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Psychrotrophic Strain of *Janthinobacterium lividum* from a Cold Alaskan Soil Produces Prodigiosin

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We have explored the microbial community in a nonpermafrost, cold Alaskan soil using both culture-based and culture-independent approaches. In the present study, we cultured >1000 bacterial isolates from this soil and characterized the collection of isolates phylogenetically and functionally. A screen for antibiosis identified an atypical, red-pigmented strain of *Janthinobacterium lividum* (strain BR01) that produced prodigiosin when grown at cool temperatures as well as strains (e.g., strain BP01) that are more typical of *J. lividium*, which produce a purple pigment, violacein. Both purple- and red-pigmented strains exhibited high levels of resistance to β-lactam antibiotics. The prodigiosin pathway cloned from *J. lividium* BR01 was expressed in the heterologous host, *Escherichia coli*, and the responsible gene cluster differs from that of a well-studied prodigiosin producer, *Serratia* sp. *J. lividum* BR01 is the first example of a prodigiosin-producer among the β-Proteobacteria. The results show that characterization of cultured organisms from previously unexplored environments can expand the current portrait of the microbial world.

Introduction

Soil microbial communities are the most phylogenetically and functionally diverse communities on Earth. A single gram of soil may contain 10^9 to 10^{11} bacteria distributed among thousands of species, which play a significant role in biogeochemical cycling, bioremediation, and the production of antimicrobials and industrially useful small molecules and enzymes (Whitman *et al.*, 1998; Janssen, 2006). Despite estimates that fewer than 1% of soil bacteria are readily culturable (Amann *et al.*, 1990), culturing from soil continues to produce a new understanding of genetic and biochemical diversity, and more intensive efforts to culture from unexplored soils will accelerate the rate of discovery in microbiology. Combining traditional culturing with metagenomics and other culture-independent methods will be a powerful strategy to enhance our understanding of soil microbial communities.

As part of an effort to define the diversity of bacteria in undisturbed cold soils, we established the Alaskan Cold Microbial Observatory in the Bonanza Creek Long-Term Ecological Research site on an island in the Tanana River in interior Alaska where the soil temperature rarely exceeds 16°C (Viereck *et al.*, 1993). The bacteria in this habitat represent a reservoir of unsampled biodiversity. Although there has been intense interest in sampling from extreme, cold environments (Shi *et al.*, 1997; Zhou *et al.*, 1997; Priscu *et al.*, 1999; Christner *et al.*, 2001; Schadt *et al.*, 2003; Lavire *et al.*, 2006; Alekhina *et al.*, 2007; Hansen *et al.*, 2007), less attention has focused on cold, nonpermafrost soils.

Previous study of this Alaskan soil microbial community using culture-independent approaches revealed high species richness based on the sequences of 1033 16S rRNA genes from a single 0.5-g sample of soil (Schloss and Handelsman, 2006). The sequences were distributed among 19 bacterial phyla, 4 of which had no cultured representatives. The Alaskan soil contains an estimated 5000 different species per gram, using ≥97% identity in 16S rRNA gene sequence to define members of the same species (Schloss and Handelsman, 2006). Moreover, functional metagenomic analyses indicate diverse genes involved in LuxR-based quorum sensing (Williamson *et al.*, 2005a) and resistance to β-lactam antibiotics (Allen *et al.*, 2009).

The previous culture-independent analyses are complemented here by a culture-dependent analysis of bacterial isolates collected from the Alaskan Cold Microbial Observatory. Two pigmented isolates, one red and one purple, were identified as *Janthinobacterium lividum*. The characterizations of these strains are presented here, including the genetic analysis of pigment production and antibiotic resistance. We report that the red-pigmented isolate produces

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prodigiosin and the purple-pigmented isolate produces violacein, both in a temperature-dependent manner.

Methods

Culture conditions

A soil core was obtained from a site, designated BP-1, on an island in the Tanana River in the Bonanza Creek Long Term Ecological Research site approximately 30 km southwest of Fairbanks, Alaska, United States (64°40.24′ N, 148°14.46′ W) (Williamson *et al.*, 2005a; Schloss and Handelsman, 2006). The top 3 cm of the soil core was removed. Two soil horizons were visible: a silt layer that was deposited in a past flooding event topped by an organic layer resulting from years of biological activity after deposition of the silt. Each horizon was passed through a 5-mm sieve to remove large roots and organic debris. A 1-g soil sample from each horizon was suspended in 10 mL sterile deionized water and sonicated for 30 s in a bath sonicator (Model 2210 Branson, Danbury, CT). The sonicated soil suspension was serially diluted, and the dilutions were plated on 10%, 1%, and 0.1% tryptic soy agar (TSA) medium; nutrient broth gellan gum; VL55 gellan gum (Sait *et al.*, 2002); and 10% tryptic soy gellan gum (TSG). All culture plates were incubated at 6°C, 12°C, and 28°C for up to 2 months. At various intervals, colonies were picked, inoculated into the appropriate broth media, and stored in 96-well plates in 15% glycerol at −80°C.

An isolate of *J. lividum* that produced red colonies was isolated on 10% TSG at 6°C in the above collection, and this strain was designated BR01. This and other *J. lividum* isolates were cultured on 1/10th-strength TSA at temperatures no higher than 25°C, and were transferred to 4°C for several days to allow for pigment development. To improve pigment production, *J. lividum* isolates were also cultured on PGA medium (0.5% peptone, 1% glycerol, and 1.5% agar).

16S rRNA gene sequencing and analysis

Frozen stocks of 273 strains isolated on 1/10th-strength TSA were streaked for isolation and used to inoculate 1/10th-strength tryptic soy broth; broth cultures were grown until turbid. The cultures were lysed by freezing at −20°C followed by thawing. Genomic DNA from the lysed cells was used as the template for PCR with the primers 27f (5′-AGRGTTTGATYMTGGCTCAG) and 1492r (5′-GGYTACC TTGTTACGACTT) (Integrated DNA Technologies, Coralville, IA). Cycle conditions were an initial 5-min denaturation at 95°C, followed by 30 cycles of 45 s at 94°C, 45 s at 55°C, 90 s at 72°C, and a final extension of 10 min at 72°C. PCR products were purified using AmpPure (Agencourt Bioscience, Beverly, MA) and sequenced using the 27f primer and BigDye version 3.1 (Applied Biosystems, Foster City, CA). BLASTN (Altschul *et al.*, 1990, 1997) was used to determine the phylum-level designation of each isolate.

Genomic DNA was purified from cultures of *J. lividum* according to the manufacturer's instructions with the GenElute Bacterial Genomic Kit (Sigma-Aldrich, St. Louis, MO), and the 16S rRNA gene was amplified, purified, and sequenced as described above. Full-length sequences were obtained for the *J. lividum* 16S rRNA genes using primers 27f, 1492r, 787r (5′-CTACCAGGGTATCTAAT), and 787f (5′-ATTAGATA CCCYGGTAG) (Integrated DNA Technologies).

Screen for antibiotic production

To identify putative antibiotic-producing isolates, the Alaskan soil culture collection was replica printed from storage onto three types of media: 1/10th-strength TSA, VL55, and 1/10th-strength TSG. Duplicates were made of each plate to test the isolates for inhibition of *Bacillus subtilis* 168 and *Pseudomonas putida* F117. The plates were incubated at 6°C, 12°C, or 28°C until visible colonies formed. Colonies were fixed by spraying 0.5% water agar in a thin layer across the plate. A 100-μL overnight culture of each tester strain, *B. subtilis* 168, and *P. putida* F117, was mixed with 5 mL of soft Luria-Bertaini agar (LBA) (0.8% agar) and poured over each plate. After overnight incubation at 28°C, isolates that produced a zone of inhibition were then identified by 16S rRNA gene analysis as described above. *J. lividum* BR01 and BP01 were tested in the same manner for antibiosis against additional tester strains: *Salmonella enterica*, *Streptomyces griseus*, *Bacillus cereus* UW85, *Bacillus mycoides*, *E. coli* DH5α, *Erwinia herbicola*, and *Pseudomonas aeruginosa*.

In vivo *transposon mutagenesis*

The EZ-Tn5 <DHFR-1> transposome (Epicentre, Madison, WI) was introduced into electrocompetent *J. lividum* BR01 and BP01 according to the manufacturer's instructions. The mutant phenotype was confirmed and genomic DNA was isolated from each nonpigmented mutant (GenElute™ Bacterial Genomic DNA Kit; Sigma-Aldrich) and cloned into the *Pst*I site of pGEM-3zf(+) (Promega, Madison, WI). Ligation reaction products were electroporated into *E. coli* DH5α and plated onto LBA supplemented with 15 μg/mL of trimethoprim (to select for the transposon) and 100 μg/mL ampicillin (to select for the vector). Thousands of colonies from each mutagenesis reaction were screened. Plasmids were isolated from the resulting clones according to manufacturer's instructions with the ExpressMatrix (Bio101; Morgan, Irvine, CA). The manufacturer-supplied transposon-based sequencing primers were used to determine the sequence of the insertion site.

J. lividum *fosmid library construction and analysis*

Genomic DNA was isolated from *J. lividum* BR01 and BP01 (Table 1) using the GenElute Bacterial Genomic Kit (Sigma-Aldrich). A Hamilton syringe was used to shear the genomic DNA to fragments of 40 kb, which served as inserts for fosmid libraries using the CopyControl Fosmid Library Production Kit (Epicentre) (Table 1). Among 1000 colonies, clones harboring the pigmentation genes (pBRred and pBPpur) (Table 1) were identified by growing the two libraries for 40 h at room temperature and identifying red or purple colonies. Clones harboring genes conferring resistance to β-lactam antibiotics (pBRbeta and pBPbeta) (Table 1) were identified by growing the two libraries overnight at 37°C on LBA containing ampicillin (100 μg/mL).

Fosmid DNA was isolated from induced overnight cultures of pBRred, pBPpur, pBRbeta, and pBPbeta by alkaline lysis with buffers P1, P2, and N3 (Qiagen, Valencia, CA) followed by ethanol precipitation (Sambrook and Russell, 2001). To identify the genes responsible for the respective phenotypes, fosmid DNA was mutagenized with a transposon via the GPS-1 Genome Priming System (New England

Table 1. Strains and Plasmids Used in This Study

Strain or plasmid	*Relevant characteristics*[a]	*Reference*
Strains		
Janthinobacterium lividum strain BR01	Isolated from Alaskan soil; produces red pigment (prodigiosin) when grown at low temperatures	This study
J. lividum strain BP01	Isolated from Alaskan soil; produces purple pigment (violacein) when grown at low temperatures	This study
Escherichia coli strain EPI300	DH10B derivative with inducible promoter in front of *trfA*; induction increases copy number of pCC1FOS from *oriV*	Epicentre, Madison, WI
E. coli strain DH5α	General purpose lab strain	Hanahan (1983)
Plasmids		
pCC1FOS	Chl^R; fosmid cloning vector with inducible copy number when propagated in *E. coli* EPI300	Epicentre
pGEM-3zf(+)	Amp^R; standard cloning vector	Promega, Madison, WI
pBRred	Chl^R; subclone of *J. lividum* BR01 in pCC1FOS that turns *E. coli* EPI300 pink at cool temperatures	This study
pBPpur	Chl^R; subclone of *J. lividum* BP01 in pCC1FOS that turns *E. coli* EPI300 purple at cool temperatures	This study
pBRbeta	Chl^R; amp^R; subclone of *J. lividum* BR01 in pCC1FOS that confers resistance to β-lactam antibiotics on *E. coli* EPI300	This study
pBPbeta	Chl^R; amp^R; subclone of *J. lividum* BP01 in pCC1FOS that confers resistance to β-lactam antibiotics on *E. coli* EPI300	This study

[a]Antibiotic resistance phenotypes are abbreviated as follows: Chl^R, chloramphenicol resistant; Amp^R, ampicillin resistant.

Biolabs, Ipswich, MA). Each mutagenesis reaction was transformed into *E. coli* EPI300 and plated on LBA supplemented with antibiotics to select for the fosmid (20 μg/mL chloramphenicol) and transposon (20 μg/mL kanamycin). DNA was isolated from mutants that had lost the respective phenotype, and the transposon-based sequencing primers were used to sequence the genes of interest using BigDye version 3.1 (Applied Biosystems). Additional random insertion mutants were prepared to sequence the entire fosmid insert to at least threefold coverage. The region of pBRred containing *pigJK* was sequenced to ~15-fold coverage, and both the *pigBC* and *pigJK* junctions were PCR amplified and sequenced to confirm the annotation. Sequences were assembled using the STADEN software package (v. 1.4.0; http://staden.sourceforge.net/) and annotated using open reading frame (ORF) Finder (www.ncbi.nih.gov/gorf/gorf.html). ORF maps were drawn in SeqBuilder (Lasergene; DNASTAR, Madison, WI).

Antibiotic susceptibility

Minimum inhibitory concentration (MIC) assays were performed according to Clinical Laboratory Standards Institute guidelines (NCCLS, 2004). Briefly, serial dilutions of antibiotics were made in a 96-well plate from 512 to 0.5 μg/mL, with no antibiotics in the last well. The final volume in each well was 90 μL in Mueller–Hinton broth (Becton, Dickinson and Company, Sparks, MD). Ten microliters containing $\sim 1 \times 10^5$ cfu of *J. lividum* BR01, *J. lividum* BP01, *E. coli* pBPpur, *E. coli* pBRbeta, and *E. coli* pBPbeta were added to the appropriate wells. MICs were tested with the following β-lactam antibiotics: ampicillin (Research Products International Corp., Mt. Prospect, IL), carbenicillin (Fisher Scientific, Fair Lawn, NJ), amoxicillin, penicillin G, piperacillin, cephalexin, cefamandole, and ceftazidime (Sigma, St. Louis, MO). Assays were performed in duplicate in three separate experiments using independent cultures with *E. coli* EPI300 carrying the pCC1FOS vector as the reference strain (Epicentre). Growth was evaluated within 24 h.

Purification of prodigiosin from J. lividum *BR01*

A 500 μL overnight culture of *J. lividum* BR01 grown in LB medium at 30°C was used to inoculate 500 mL of nutrient broth containing 1% glycerol in a 4 L Erlenmeyer flask, which was then incubated at 10°C with shaking at 250 rpm for 11 days, by which time the culture had become red-orange. Cells were removed by centrifugation, and the cell pellet, which retained most of the red color, was extracted three times by resuspending the cells in 50 mL of methanol, incubating on a rocker overnight, and centrifuging at 10,000 rpm for 15 min at 4°C. The final methanol extract was dried, and 486 mg of material was recovered. This extract was purified by column chromatography with a silica gel using a step gradient of dichloromethane and methanol. The active fractions were further purified by high performance liquid chromatography (HPLC) on a C-18 column using a water–methanol mixture acidified with 0.05% trifluoroacetic acid. The HPLC elution started with 4 min at 75% methanol and then increased to 100% methanol over 10 min followed by 5 min at 100% methanol (prodigiosin: $tR = 11.3$ min; heptylprodigiosin: $tR = 15.7$ min).

Purification of violacein from J. lividum *BP01*

Eleven 500 mL Erlenmeyer flasks each containing 50 mL of 0.5% (w/v) Bacto peptone and 1% (v/v) glycerol were inoculated with 50 μL of an overnight culture of *J. lividum*

BP01. Cultures were incubated at room temperature on a platform shaker at 200 rpm. After 13 days the cultures were dark purple. The cultures were combined, frozen, and lyophilized. Approximately 150 mL of methanol was added to the lyophilized culture and this mixture was incubated at 4°C for approximately 1 h on a rocker. The mixture was centrifuged and the methanol extract was decanted. This extraction process was repeated twice except that the incubation at 4°C was extended to 24 h. The methanol extracts were combined and dried to yield approximately 7.5 g of oil, which was purified according to the protocol outlined in Brady *et al.* (2001). Briefly, the oil was dissolved in 800 mL of 80% methanol and extracted three times with 800 mL of hexanes. The aqueous portion was diluted to 50% methanol and extracted twice with 1000 mL of chloroform followed by two more extractions with 500 mL of chloroform. The chloroform extract yielded 143 mg of purple oil. This extract was subjected to normal-phase column chromatography on a silica gel. Fractions were eluted from the column using a hexanes:ethyl acetate gradient acidified with 0.01% trifluoroacetic acid (TFA). The purple fractions from the silica column were combined and further purified using reverse-phase HPLC on a C-18 column (isocratic: 40% acetonitrile: 60% water acidified with 0.05% TFA; violacein: $tR = 9.2$ min).

GenBank accession numbers

pBRred and pBPpur sequences have been deposited in GenBank under accession numbers EF063590 and EF063591, and pBRbeta and pBPbeta sequences have been deposited under accession numbers EF063589 and EF063592, respectively. The 16S rRNA gene sequences for *J. lividum* BR01 and BP01 have been deposited in GenBank under accession numbers EU330448 and EU330449, respectively. GenBank accession numbers of each unique 16S rRNA gene sequence of the bacteria that were isolated on 10% TSA have been deposited under numbers HM113566-HM113684. These sequences are associated with the Alaskan soil metagenome project (NCBI GenomeProject database ID 28853).

Results

Biodiversity of Alaskan soil includes a red-pigmented J. lividum *strain*

To culture diverse bacteria, multiple media types, temperatures, and incubation lengths were employed. Dilute nutrients, such as those found in VL55 media, and long incubation times have been shown to increase the diversity of culturable isolates from soil (Janssen *et al.*, 2002; Sait *et al.*, 2002). Multiple cool temperatures were chosen for bacterial cultivation to capture a range of temperatures experienced in the Alaska soil habitat.

To assess the biodiversity of the Alaskan soil culture collection, the 16S rRNA genes of a subset of isolates were sequenced. Partial 16S rRNA sequences were obtained from 273 bacteria that were isolated on 10% TSA; the sequences represented 26 different genera distributed among the Proteobacteria (37.7%), Bacteroidetes (48%), Actinobacteria (12.8%), and Firmicutes (1.5%) (Fig. 1). The 16S rRNA gene

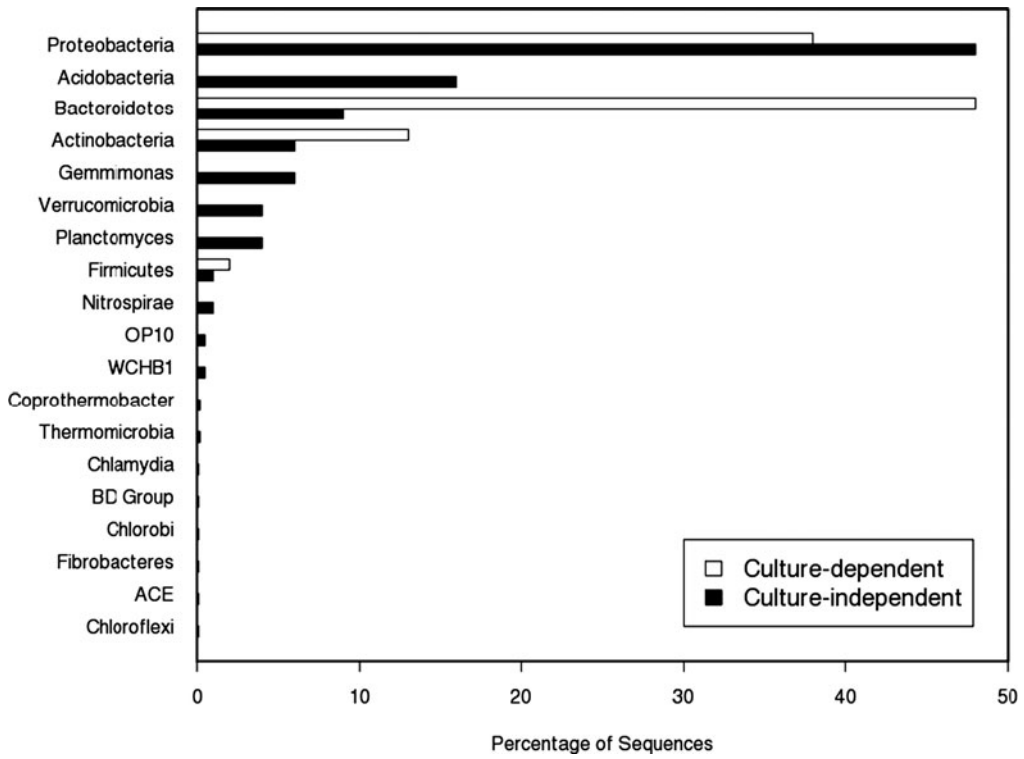

FIG. 1. The relative abundance of each phylum in the Alaskan soil culture collection isolated on 1/10th-tryptic soy agar ($n = 273$ 16S rRNA gene sequences) and in a culture-independent, PCR-derived clone library ($n = 1033$ 16S rRNA gene sequences) (Schloss and Handelsman, 2006). White bars represent sequences identified by culture-dependent methods, and black bars represent sequences identified by culture-independent methods. Candidate phyla OP10, WCHB1, BD group, and ACE have no sequenced representatives.

Table 2. Minimum Inhibitory Concentration (μg/mL) of Eight β-Lactam Antibiotics on *Janthinobacterium lividum* and Subclones After 24 h of Growth

	Minimum inhibitory concentration of strain or construct[a]				
β-Lactam antibiotic	J. lividum *BR01*	J. lividum *BP01*	E. coli *pBRbeta*	E. coli *pBPbeta*	E. coli *pCC1FOS (empty vector)*
Amoxicillin	256	128	>512	>512	4
Ampicillin	256	512	512	>512	8
Carbenicillin	128	256	128	512	4
Penicillin G	128	64	>512	>512	32
Piperacillin	>512	>512	>512	>512	4
Cephalexin	256	256	256	256	8
Cefamandole	256	256	256	512	0.5
Ceftazidime	8	16	8	4	0.5

[a]Names are defined in Table 1.

sequences of a different subset of 138 randomly selected isolates, representing the various media used in the initial isolation, were sequenced and revealed greater abundance of Proteobacteria (52.2%), Actinobacteria (20.3%), and Firmicutes (2.8%), and fewer Bacteriodetes (24.6%) (data not shown) than were detected among the 16S rRNA gene sequences from 10% TSA isolates alone. Overall, far fewer phyla were isolated by culturing than by a culture-independent analysis of the same soil (Fig. 1).

A screen of the Alaskan culture collection for antimicrobial activity identified six putative antibiotic producers. All inhibited the growth of *B. subtilis* 168 but not of *P. putida* F117. One isolate that produced a red pigment was further characterized.

The sequence of the 16S rRNA gene of the red antibiotic producer revealed that it was 100% identical to *J. lividum* (GenBank accession DQ640007). An additional 24 *J. lividum* isolates in the culture collection were identified with similar 16S rRNA gene sequences. Although the definition of *J. lividum* specifies that it produces a purple pigment (*Janthinobacterium* = purple bacterium, *lividum* = bluish), 9 of 24 *J. lividum* Alaskan soil isolates produced a red pigment (e.g., strain BR01) and the remaining 15 isolates produced the purple pigment typical of *J. lividum* (e.g., strain BP01) (Table 1). The full-length 16S rRNA gene sequences of a representative purple and red strain, *J. lividum* BP01 and *J. lividum* BR01, differed by two nucleotides over 1455 base pairs. Neither the red nor the purple pigments were visible in cultures grown above 20°C, although growth rate was not affected by temperature.

Given the difference in pigment production despite nearly identical 16S rRNA sequences, we examined other phenotypes of *J. lividum* BR01 and BP01. Except for the unique red pigmentation of nine *J. lividum* isolates and the temperature dependency of pigment production, the phenotypes of the Alaskan soil *J. lividum* isolates were similar to those of previously reported *J. lividum* isolates (De Ley *et al.*, 1978). Phenotypes tested include motility, proteolysis, lactose fermentation (data not shown), antibiosis, and antibiotic resistance. Pigmented *J. lividum* BR01 and BP01 inhibited the growth of *S. enterica*, *S. griseus*, *B. subtilis* 168, *B. cereus* UW85, and *B. mycoides*, but not *E. coli* DH5α, *E. herbicola*, or *P. aeruginosa*. No growth inhibition was observed for any tester strain when the *J. lividum* strains were grown at temperatures that prevented the appearance of pigmentation. Both strains of *J. lividum* (BR01 and BP01) exhibited high levels of resistance to seven of eight β-lactam antibiotics tested (Table 2).

Genetic characterization of β-lactam resistance in J. lividum *BR01 and BP01*

The genetic basis for β-lactam antibiotic resistance in *J. lividum* BR01 and BP01 was explored by selecting a fosmid clone library of each strain on ampicillin, followed by transposon mutagenesis and sequence analysis of the clones that conferred resistance on *E. coli.* We identified five clones from the *J. lividum* BR01 library and three from the BP01 library, and a restriction digest revealed overlap between ampicillin-resistant clones from the same library (data not shown). Therefore, a representative clone from each library was characterized and sequenced (clones designated pBRbeta and pBPbeta). Both pBRbeta and pBPbeta conferred high levels of β-lactam antibiotic resistance on *E. coli*, and in some cases (when tested on amoxicillin and penicillin G, e.g.) exhibited greater resistance than did the *J. lividum* strain from which the genes were isolated (Table 2). Sequencing revealed much congruency between pBRbeta and pBPbeta; the former has a 33.1-kb insert containing 32 ORFs, the latter has a 38.9-kb insert containing 33 ORFs, and they contain 22 ORFs in common (Fig. 2). Within the overlapping region, pBPbeta and pBRbeta are syntenous except for one additional ORF in the pBPbeta sequence that is not found in pBRbeta (Fig. 2). The 22 overlapping ORFs are, on average, 95.2% (SD = 3.9) identical between the two fosmids. Both encode a putative β-lactamase repressor protein, a putative carboxypeptidase, and a putative metallo-β-lactamase, and the amino acid sequences of the pairs of homologs have 99.2%, 95.4%, and 93.3% identity, respectively. The predicted protein sequence encoded by the two metallo-β-lactamase genes share 81% identity with a previously published metallo-β-lactamase (THIN-B) from *J. lividum* (Rossolini *et al.*, 2001). The genes encoding the metallo-β-lactamases from *J. lividum* BR01 and BP01 are designated *bla*LRA-20 and *bla*LRA-21, respectively (*bla*, β-lactamase; LRA, β-lactam resistance from Alaskan soil).

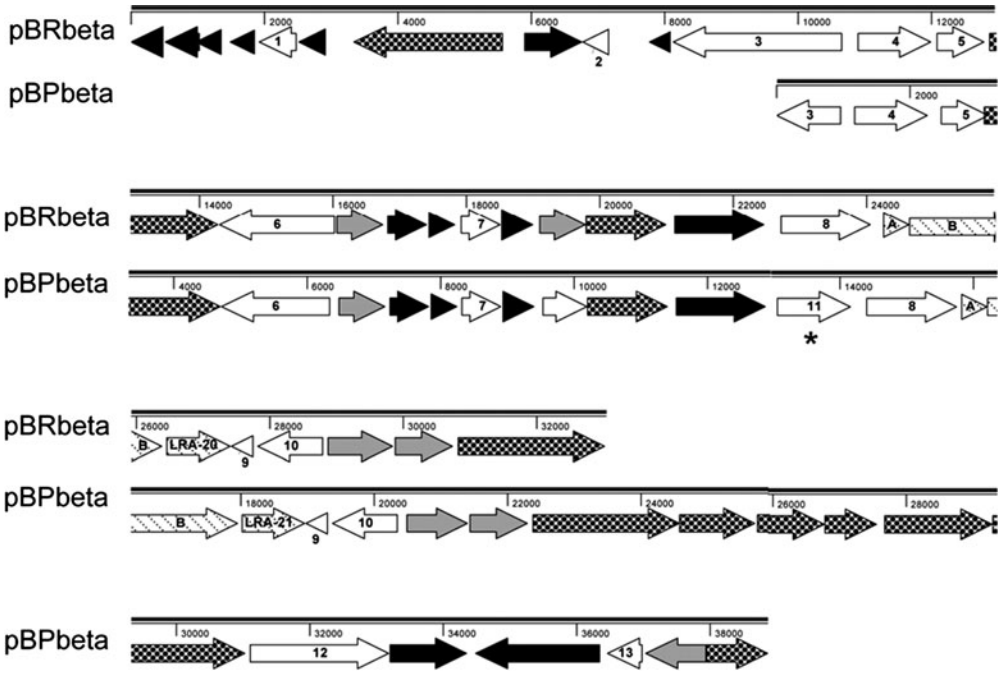

FIG. 2. Maps of open reading frames (ORFs) from pBRbeta (33,133 bp) and pBPbeta (38,875 bp), aligned across homologous regions. The ORFs with dotted diagonal lines in each map encode putative β-lactamase repressor proteins (A), carboxypeptidases (B), and metallo-β-lactamases (LRA-20 and LRA-21). The asterisk below the map of pBPbeta designates the ORF that is missing in pBRbeta. ORFs filled with black, gray, and black dots have homology to hypothetical ORFs, regulatory proteins, and transport proteins, respectively. Those ORFs lacking a color have homology to a methyltransferase (1), hemerythrin-like protein (2), oxidoreductase (3), branched-chain amino acid aminotransferase (4), ribonuclease I (5), AMP-dependent synthetase and ligase (6), electron chain protein (7), adventurous gliding motility protein (8, 11), TPR repeat protein (9), alcohol dehydrogenase (10), ferrichrome-iron receptor (12), and isochorismate hydrolase (13).

Isolation and characterization of J. lividum *BR01 pigment-producing gene cluster*

The red compound produced by *J. lividum* BR01 was purified, and a nuclear magnetic resonance (NMR) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis indicated that it was a mixture of prodigiosin and heptylprodigiosin (Fig. 3A, B). Transposon mutagenesis identified two genes, *smpB* and *pigE*, that were required for pigmentation and antibiosis. SmpB forms a complex with SsrA RNA that targets aberrant proteins and mRNAs for degradation (Keiler, 2008). This complex is ubiquitous among bacteria and is required for viability, response to stresses, and pathogenesis in many bacteria (Keiler, 2008). The other gene implicated in pigmentation, *pigE*, is part of the prodigiosin pathway in *Serratia* sp. (Williamson *et al.*, 2005b).

To obtain the entire complement of genes required for pigment production, the *J. lividum* BR01 fosmid library was screened in *E. coli* for red-pigmented clones. One pink clone,

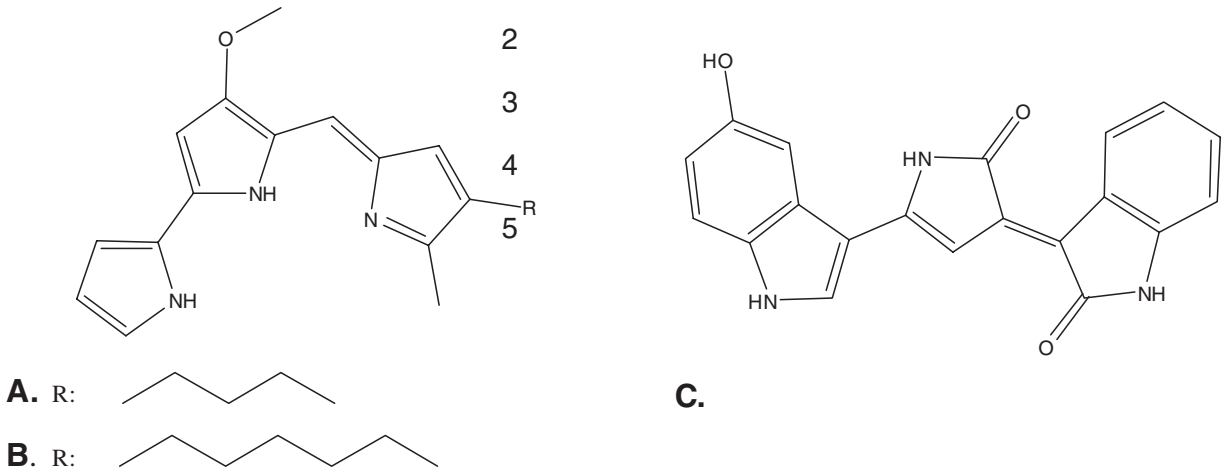

FIG. 3. Structures of compounds isolated in this study. Prodigiosin (**A**) and heptylprodigiosin (**B**) were purified from a culture of *Janthinobacterium lividum* BR01, and violacein (**C**) was purified from a culture of *J. lividum* BP01.

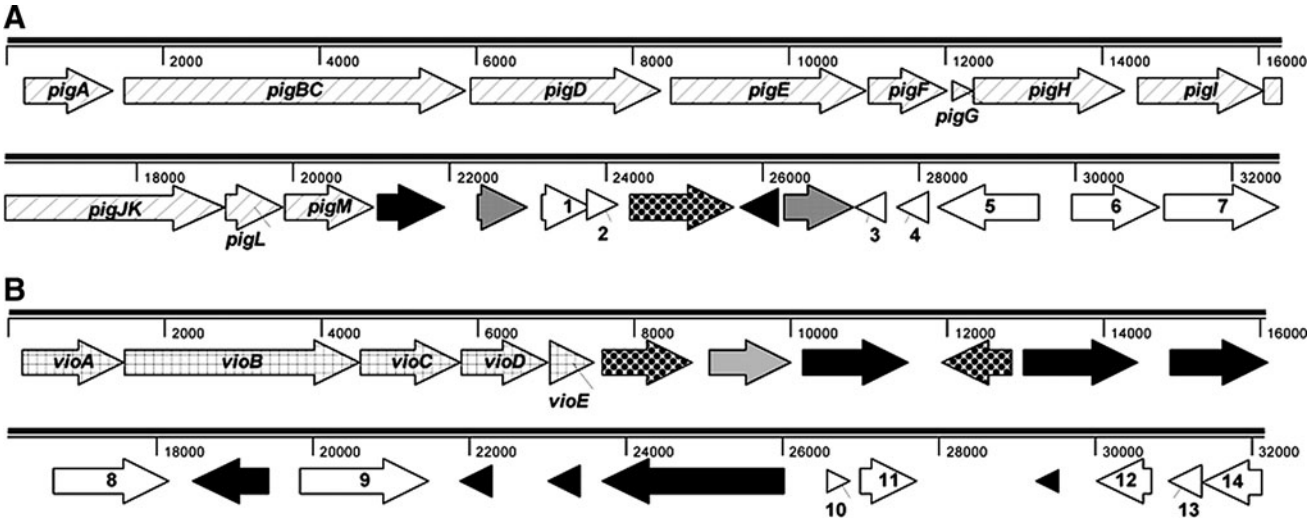

FIG. 4. Maps of ORFs from pBRred (32,611 bp) (**A**) and pBPpur (32,163 bp) (**B**). The ORFs with diagonal lines correspond to homologues of the *pig* pathway, which encodes prodigiosin biosynthetic genes, and the ORFs with grid lines correspond to homologues of the *vio* pathway, which encodes violacein biosynthetic genes. ORFs filled with black, gray, and black dots have homology to hypothetical ORFs, regulatory proteins, and transport proteins, respectively. Those ORFs lacking fill have homology to a D-aspartate O-methyltransferase (1), rhodanase (2), glyoxalase (3), osmotically inducible protein OsmC (4), L-sorbosone dehydrogenase (5), NAD(+) kinase (6), RecN (7), class C β-lactamase (8), α-amylase (9), transposase (10, 11), DNA polymerase III ε subunit (12), ribonuclease H (13), and methyltransferase (14).

pBRred, was identified and its insert (32.6 kb) sequenced, revealing a cluster of 11 genes that transposon mutagenesis indicated are required for prodigiosin biosynthesis (20.8 kb) (Fig. 4). The mean G+C content of the *pig* genes (61.7%) is consistent with the mean G+C content of the remaining ORFs on pBRred (62.8%) and the ORFs on the pBRbeta clone (63.4%). All of the deduced Pig proteins from *J. lividum* BR01 exhibit higher sequence identity to the proteins from the marine γ-Proteobacterium *Hahella chejuensis* (40–76%) (Kim *et al.*, 2006) than to the proteins from either *Serratia* sp. ATCC 39006 (32%–60%) or *S. marsescens* ATCC 274 (34%–60%) (Supplemental Table 1, available online at www.liebertonline.com).

Growth of the clone at 28°C or 37°C resulted in reduced pigment production, and optimal pigment production was observed when clones were grown at room temperature. It is possible that the reduced pigmentation of pBRred (pink) compared with *J. lividum* BR01 (red) was due to limitations of heterologous expression of the pathway.

Isolation and characterization of J. lividum *BP01 pigment-producing gene cluster*

The proton nuclear magnetic resonance (^{1}H-NMR) spectrum and LC-MS/MS analysis of the purple compound produced by *J. lividum* BP01 matched that obtained with purified violacein (Fig. 3C). Random mutagenesis indicated that interruption of the *smpB* gene resulted in the loss of pigmentation, but no biosynthetic genes were identified by mutagenesis. When the fosmid library of *J. lividum* BP01 genome was then screened in *E. coli*, one clone (pBPpur) was identified that was dark purple when grown at room temperature but not when grown at 28 or 37°C. The complete sequence of the pBPpur fosmid clone (32.2 kb; Fig. 4) revealed the prototypical violacein biosynthetic operon (7.3 kb), which consists of the five genes *vioABCDE* (Sanchez *et al.*, 2006) and was originally identified in *Chromobacterium violaceum*. *In vitro* transposon mutagenesis of pBPpur resulted in nonpigmented mutants, and these insertions mapped to each of the genes (*vioABCDE*) in the violacein pathway. PCR amplification of the violacein cluster in *J. lividum* BR01 and of the prodigiosin cluster in *J. lividum* BP01 was negative (data not shown). Additionally, annotation of the DNA flanking the violacein gene cluster revealed a putative β-lactamase (Fig. 4). pBPpur was therefore tested for resistance to eight β-lactam antibiotics in an MIC assay, but the clone did not confer resistance on *E. coli* (data not shown).

Discussion

This is the first report of a red-pigmented *J. lividum* strain, revealed by the construction and analysis of a collection of bacteria cultured from nonpermafrost Alaskan soil. This study extends the known diversity of *J. lividum*, which is a member of the Burkholderiales in the β-Proteobacteria and has been found primarily in aquatic environments (Saeger and Hale, 1993) and soils (Gleave *et al.*, 1995; Rossolini *et al.*, 2001; Du *et al.*, 2007; Kawakami *et al.*, 2007). *J. lividum* has been reported to control amphibian fungal pathogens (Harris *et al.*, 2009), and can be toxic to other bacteria, viruses, and protozoa (Lichstein and Van De Sand, 1946; Andrighetti-Fröhner *et al.*, 2003; Matz *et al.*, 2004). Our results demonstrate the importance of culturing from novel environments and of challenging common assumptions; *J. lividum* BR01 and BP01 were nearly ignored because of the familiarity of the red and purple pigments. Our original predictions were that the purple isolate was *C. violaceum* and that the red isolate was *S. marcescens*. However, neither prediction was correct as 16S rRNA gene sequencing indicated that both isolates are *J. lividum*. The 16S rRNA gene sequences of BR01 and BP01 are virtually identical, both strains contain

metallo-β-lactamases, and the genes surrounding the β-lactamase genes are highly similar, yet the strains diverged in the acquisition of pathways for the production of different small molecules (presumably after the evolution of the β-lactamase genes). This highlights the point that neither colony morphology nor 16S rRNA gene sequence alone indicates the taxa present in a community or their characteristics (Jaspers and Overmann, 2004).

Pigment production in *J. lividum* BR01 and BP01 requires growth at temperatures below 20°C. *Serratia* sp. produce prodigiosin at temperatures below 30°C but not at 37°C (Williams *et al.*, 1971). *J. lividum* BP01 is the first example of cold-dependent pigment production among violacein producers. The temperature-dependent pigment and antibiosis by *J. lividum* BR01 and BP01 raise interesting questions about the ecological role of the pigment in these strains. The Alaskan soil rarely reaches temperatures above 16°C (Viereck *et al.*, 1993), so this habitat may have selected for *J. lividum* strains that produce the pigments at cool temperatures. Further studies are required to investigate the role of cold-activated pigment production and antibiosis in *J. lividum* survival *in situ*. The cloned pathways maintain the cold regulation in *E. coli* that they had in the native organisms, and the clones may therefore provide the basis for studying the mechanism of cold-specific gene regulation.

J. lividum BR01 is the first reported β-Proteobacterium that produces prodigiosin. Prodigiosins are of clinical interest because they are attractive immunosuppressive and anticancer therapeutics (Williamson *et al.*, 2006). Other prodigiosin-producing bacteria include *Streptomyces coelicolor*, *Hahella chejuensis*, *Pseudomonas magnesiorubra*, *Vibrio psychroerythreus*, and *Serratia* sp., which represent the Actinobacteria and γ-Proteobacteria (Williamson *et al.*, 2006). Prodigiosin is produced by the condensation of 2-methyl-3-amyl-pyrrole (MAP) and 4-methoxy-2,2′-bipyrrole-5-carbaldehyde (MBC) (Williamson *et al.*, 2005b). The gene clusters for prodigiosin production in *Serratia* sp. (Harris *et al.*, 2004; Williamson *et al.*, 2005b) and *H. chejuensis* (Kim *et al.*, 2006) differ from the *J. lividum* BR01 cluster in several ways. First, the discrete *pigB* and *pigC* genes of *Serratia* sp., whose products sequentially produce MAP and execute the MAP-MBC condensation reaction (Williamson *et al.*, 2005b), exist as a fused gene in pBRred. Further, the *pigJ* and *pigK* genes, which are also separate genes in *Serratia* sp., are a fused gene in pBRred. Both PigJ and PigK are involved in the biosynthesis of MBC, although the role of PigK is uncertain. The third difference is that *pigN* is absent from the *J. lividum* BR01 prodigiosin cluster. In *Serratia* sp., *pigN* and *pigF* are thought to work together to direct the final step in the synthesis of MBC (a methylation reaction), and *pigN* mutants produce norprodigiosin as well as prodigiosin (Harris *et al.*, 2004; Williamson *et al.*, 2005b). A BLASTN analysis for *pigN* in the pBRred cluster yielded no regions of identity, so it remains unclear how the final step in synthesis of MBC is accomplished in strain BR01. Further studies are required to determine the effects of the genetic differences on the biochemical functions of the prodigiosin pathway in *J. lividum* BR01. Comparison of expression of the prodigiosin genes in *J. lividum* BR01 and *Serratia* sp. may aid in understanding how the gene cluster contributes to the ecophysiology of these organisms. The discovery of a prodigiosin-producing *J. lividum* strain expands the diversity of pathways for the biosynthesis of prodigiosin.

The goal of this study was to explore the possibility that previously unexplored Alaskan soil harbors novel biodiversity. The red-pigmented *J. lividum* strain indicates potential for discovery in the Alaskan soil culture collection. In this study of a cold soil, we found known organisms and natural products in new configurations and combinations. When examined in new ways, the culturable soil community may continue to be a fruitful source of biochemical and genetic diversity.

Disclosure Statement

No competing financial interests exist.

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