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Novel Florfenicol and Chloramphenicol Resistance Gene Discovered in Alaskan Soil by Using Functional Metagenomics▽

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Functional metagenomics was used to search for florfenicol resistance genes in libraries of cloned DNA isolated from Alaskan soil. A gene that mediated reduced susceptibility to florfenicol was identified and designated *pexA*. The predicted PexA protein showed a structure similar to that of efflux pumps of the major facilitator superfamily.

Antimicrobial resistance in various bacterial pathogens is an escalating global problem, with multidrug resistance in many pathogens becoming increasingly common (15, 32). To develop alternative treatments or to use existing therapies judiciously and efficaciously, it is important to understand the origins and ecological reservoirs of antimicrobial resistance genes and the underlying resistance mechanisms (4). Identifying the sources of the resistance genes and their association with mobile genetic elements will aid in efforts to predict their emergence and dissemination in clinically relevant pathogens (14).

The dynamics of emergence and persistence of antimicrobial resistance determinants are complex and still not fully understood. Spread of resistance genes can be caused by use of an antimicrobial agent, thereby selecting for clonal dissemination of a bacterium harboring the corresponding resistance gene or—if the gene is located on a mobile element—by horizontal transfer of the respective mobile genetic element among bacteria of the same or different species and genera (10, 18). However, there is also evidence that antimicrobial treatment at a specific site is not the sole risk factor for the development or dissemination of resistance (17, 26). Resistance genes have been found in remote, "pristine" environments far removed from human influence (2). The discovery of what Waksman and Woodruff termed "antagonistic" microorganisms (35) led to the early assumption that resistance genes have arisen as a self-defense mechanism against self-produced "war munitions" or attacks from other microbes trying to gain an advantage in the competitive environment that exists in microbial communities. It has also been suggested that resistance genes serve functions other than those based on anthropomorphic definitions (8, 36). If this assumption is correct, the occurrence of genes conferring resistance to both currently used therapeutics and those yet to be approved is likely underestimated. Understanding the frequency and diversity of these resistance genes in environmental reservoirs will aid in predicting the emergence and dissemination of antimicrobial resistance genes (26, 29).

Many antimicrobial agents are produced by soil bacteria (23), and soil bacteria are still believed to represent not only a source of novel antimicrobial agents but also a source of novel resistance genes. It has been hypothesized that only 0.5% of microbes residing in soil are culturable by current methods (34), and consequently, investigations into the diversity of resistance genes that exist in nature are biased if they focus solely on cultivable microorganisms. Metagenomics is a culture-independent method of examining the DNA present in a given sample (19, 28). DNA is extracted directly from the sample and cloned into commercial vectors. Classically, metagenomic analysis was based on random sequencing of inserted DNA or amplification by PCR of target genes (9, 33). Using a similar approach but searching for a specific function using heterologous expression in a surrogate host has been designated functional metagenomics and has already been used to identify antimicrobial resistance genes (1, 2). This approach allows exploration of genes whose function may not be obvious based on their sequence. Functional metagenomics could provide powerful insight into the genetic diversity of antimicrobial resistance not yet accounted for in complex microbial communities such as those that exist in soil.

Florfenicol is a synthetic fluorinated derivative of chloramphenicol. It is a broad-spectrum antimicrobial agent approved for the control of respiratory tract infections in cattle and swine, infectious pododermatitis in cattle, and furunculosis in salmon. It acts by binding to the ribosome, thus inhibiting protein synthesis in bacteria (24). Resistance to florfenicol has been observed in many diverse bacteria, and a variety of mechanisms have been described (5, 11, 12, 16, 17, 24). These have all been discovered by analysis of organisms that exhibited phenotypic resistance to florfenicol or elevated MICs in cases where no CLSI-approved interpretive criteria were available. In some instances, the genes responsible for florfenicol resistance have also been found to be physically linked to genes

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TABLE 1. Bacterial cloning strains, plasmids, and metagenomic clones described in this work

Strain, plasmid, gene, or clone	Relevant characteristic(s)[a]	Source or reference
E. coli strains and plasmids for cloning		
Epi300	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) *endA1 recA1*; high-transformation efficiency of large DNA	Epicentre, Madison, WI
DH5α	F^- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 recA endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Invitrogen, Carlsbad, CA
pCC1FOS	Chl^r; fosmid cloning vector	Epicentre, Madison, WI
pDrive	Amp^r Kan^r; TA PCR cloning vector	Qiagen, Valencia, CA
EZ-Tn*5* <KAN-2>	Kan^r; Tn*5* for transposon mutagenesis	Epicentre, Madison, WI
Metagenomic clone in pCC1FOS from Alaskan soil DNA, Ak20-3	Ffl^r Chl^r; contains a gene encoding a major facilitator superfamily drug exporter	This work
ORF of active gene from the metagenome, *pexA*	Confers resistance to florfenicol and chloramphenicol	This work

[a] Abbreviations: Amp, ampicillin; Chl, chloramphenicol; Ffl, florfenicol; Kan, kanamycin.

conferring resistance to other antimicrobial agents (5, 11, 25). This could imply that, although the drug is used strictly in animals, the use of florfenicol might select for and amplify resistances to antimicrobials that are relevant to human health (18, 22).

The present study aimed to discover florfenicol resistance genes by using metagenomic libraries constructed from DNA extracted from the soil of remote sites in Alaska. Our hypothesis is that genes that confer resistance to florfenicol exist in the environment, even in the absence of a sufficiently high selective pressure imposed by the presence of florfenicol. Identifying novel resistance genes, particularly those that might be found in noncultivable microbes, can help to predict the emergence of resistance. Identifying the genes that are linked to a resistance gene will aid in our understanding of coselection and persistence of resistance genes.

Construction and screening of metagenomic libraries. The metagenomic libraries screened in this study were reported previously (2). Briefly, they were constructed using soil samples collected from an island in the Tanana River in the National Science Foundation's Long-Term Ecological Research site at Bonanza Creek Experimental Forest near Fairbanks, AK. The samples were transported at 4°C, stored at −20°C, and thawed at room temperature just before use. Cells were lysed either directly in the sample or after being separated from the sample matrices. DNA from the cells was ligated into pCC1BAC (Epicentre, Madison, WI) or pCC1FOS (CopyControl fosmid library production kit; Epicentre). *Escherichia coli* Epi300 (Epicentre) was used as the host for these vectors. Recombinant clones were scraped from Luria-Bertani (LB) agar supplemented with chloramphenicol (12.5 μg/ml) into selective LB broth plus 20% glycerol. The libraries were stored in pools at −80°C.

The metagenomic libraries were inoculated into 3 ml LB broth plus chloramphenicol (12.5 μg/ml) and incubated for 2 to 3 h at 37°C with shaking. Cultures were plated onto LB agar with florfenicol (16 μg/ml). Although breakpoints for resistance to florfenicol exist for only a few animal pathogens (7), we previously reported that a common genetic basis for florfenicol resistance in *E. coli* resulted in a MIC of ≥16 μg/ml (27). Half of the plates were incubated at 37°C, and half were incubated at room temperature. Clones growing on these selective plates were evaluated by restriction endonuclease analysis and retransformation into chemically competent *E. coli* DH5α to confirm the phenotype (Table 1).

A total of 13,201 Mb of DNA from Alaskan soil was screened. A single fosmid clone, Ak20-3, grew on LB plates supplemented with florfenicol (16 μg/ml) when incubated at room temperature. Restriction endonuclease analysis of this clone with NotI and XhoI showed an insert of approximately 40 kb.

Identification and analysis of active gene. The clone Ak20-3 was subjected to *in vitro* transposon mutagenesis using the commercially available EZ-Tn*5* <KAN-2> insertion kit (Epicentre). Mutants exhibiting susceptibility to florfenicol were sequenced using the manufacturer's primers to identify the inactivated gene. Other insertion mutants were randomly chosen and used to sequence the remaining inserted DNA by using the manufacturer's primers. The sequence was assembled using Sequencher (Genecodes, Ann Arbor, MI) and SeqMan (Lasergene software; DNAStar, Madison, WI) programs. Finishing was done by primer walking. The Artemis program (21) served to identify putative open reading frames (ORFs), which were annotated using BLAST (Basic Local Alignment Search Tool) (3). Predicted ORFs within the DNA insert are listed in Table 2.

The gene encoding the decreased susceptibility to florfenicol was identified by transposon mutagenesis. A single transposon insertion at bp 24262 resulted in the clone being unable to grow on LB agar plates supplemented with florfenicol (16 μg/ml). This insertion site was located within an ORF at bp 24055 to 25302 that was tentatively designated *pexA* (phenicol exporter A) and coded for a protein of 415 amino acids (aa).

Amino acid alignment was done using MEGA4 (30). The new resistance protein was aligned with known phenicol exporter proteins of the major facilitator superfamily (MFS) using the ClustalW method. The resulting alignment was used to construct a phylogenetic tree using the minimum evolution method. The resulting tree was optimized using 1,000 bootstrap replicates and a random seed.

The PexA amino acid sequence showed only low similarity with other protein sequences deposited in the GenBank protein database. All similar sequences were part of the major facilitator superfamily of secondary transporters. The

TABLE 2. Predicted genes encoded by the metagenomic clone Ak20-3

ORF no.	ORF start	ORF stop	Predicted function of closest match	Accession no. of closest match (% identity)
1	726	1	Translation elongation factor Tu	ACU90852 (78)
2	2150	1398	rRNA methylase	EEUO3523 (36)
3	3403	2147	Hypothetical protein	No matches
4	4751	3366	Two-component, sigma54-specific, transcriptional regulator, Fis family	ABB31018 (52)
5	7010	4764	PAS/PAC sensor signal transduction histidine kinase	ABA89583 (37)
6	7699	7007	Hypothetical protein	No matches
7	7821	8717	Integrase/recombinase XerD/RipX family	EEI76895 (27)
8	8701	9171	Nucleoside deaminase	EAQ78406 (54)
9	11575	9146	Acyl coenzyme A dehydrogenase	ABC44659 (48)
10	12510	11662	Hypothetical protein	No matches
11	13390	12572	tRNA/rRNA methyltransferase	ABQ05252 (41)
12	13876	13394	Hypothetical protein	No matches
13	14256	13873	Hypothetical protein	No matches
14	15545	14451	Chaperone protein DnaJ	EFA67184 (50)
15	18316	15575	Excinuclease ABC, A subunit	ACU05744 (39)
16	18381	18830	Hypothetical protein	No matches
17	18972	20777	GTP-binding protein LepA	CAN93996 (62)
18	20725	21231	Methyltransferase	EDS77032 (53)
19	21260	22489	Sulfite dehydrogenase subunit SorA	EEO97828 (50)
20	22467	22847	Hypothetical protein	No matches
21	22875	23780	Universal stress protein	CAE79992 (31)
22	25302	24055	*pexA*[a]; drug resistance transporter	ACN95065 (33)
23	25586	26110	Hypothetical protein	No matches
24	27709	26126	ATP-dependent RNA helicase	EDL56381 (49)
25	28854	27946	Ribosomal protein S6 modification protein	ACA98689 (67)
26	29351	28854	Conserved hypothetical protein	EDL56110 (52)
27	29561	29673	5S rRNA	CP001661 (85)
28	29750	32672	23S rRNA	CP001089 (80)
29	32838	34362	16S rRNA	CP001629 (83)
30	34847	37603	Hypothetical protein	No matches
31	37616	38896	UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase	BAH38593 (50)
32	40406	38973	Glutamyl-tRNA synthetase	ACY17153 (53)
33	41537	40455	*N*-Acetylglucosaminyltransferase	BAC13049 (44)
34	42378	41530	Oligopeptide ABC transporter, ATP-binding protein	AAD35151 (56)

[a] *pexA* is the designation given in this work.

closest similarities, of 33% amino acid identity, were observed between PexA and drug resistance transporters from *Wolbachia* spp. (accession no. YP_002726856, YP_198189, and NP_966057). Phylogenetic analysis of the amino acid sequence of PexA shows very low identity with any of the known florfenicol/chloramphenicol exporters (Fig. 1). The Tmpred program (http://www.ch.embnet.org/software/TMPRED_form.html) was used to detect possible transmembrane helices in the PexA structure. The results predicted that the PexA protein has 11 transmembrane helices.

Susceptibility testing. MICs were determined by broth microdilution according to the recommendations given in the document M31-A3 of the Clinical and Laboratory Standards Institute (CLSI) (7). Florfenicol MICs were determined by using a microtiter plate according to CLSI guidelines with a range of 2 to 64 μg/ml of florfenicol. The clone was tested for susceptibility to the antibiotics amikacin, amoxicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim-sulfamethoxazole with a commercially available panel (CMV1AGNF, Sensititre Gram-negative NARMS plate; Trek Diagnostic Systems, Westlake, OH). For each strain, both tests were performed at 30°C and at 37°C and read manually after 24 h and 48 h. Based on the growth in the wells of the microtiter plates, the MICs of 17 different antimicrobial agents were determined. The commercially available *E. coli* ATCC 25922 served to ensure the quality of the plates, and *E. coli* DH5α and *E. coli* DH5α carrying the empty cloning vector were used for comparative reasons.

To evaluate the activity of the gene against chloramphenicol, it was necessary to move the gene into a plasmid that did not have a chloramphenicol resistance gene. PCR was conducted with primers (20) that bind 248 bp upstream and 177 bp downstream of the reading frame coding for florfenicol resistance. These primers (F, 5′-TTCACTGCAGGGATCGTGAC-3′; R, 5′-CAACTGCAGAAAAGCGAAAAG-3′) yielded a 1,701-bp PCR amplicon that contained the 1,248-bp coding sequence of interest. The amplicon was cloned into the pDrive cloning vector (Qiagen PCR cloning kit) according to kit instructions.

MICs were recorded after 48 h of incubation (Table 3). Clones with the insert had florfenicol and chloramphenicol MICs of 16 μg/ml, but only on the microtiter plates grown at 30°C. At 37°C, clones with and without the insert showed MICs of florfenicol and chloramphenicol of 2 μg/ml each. The Ak20-3 mutant with the transposon insertion within the *pexA* ORF had a florfenicol MIC of 2 μg/ml at 30°C and 37°C. The clones with the insert did not differ in their susceptibility to any

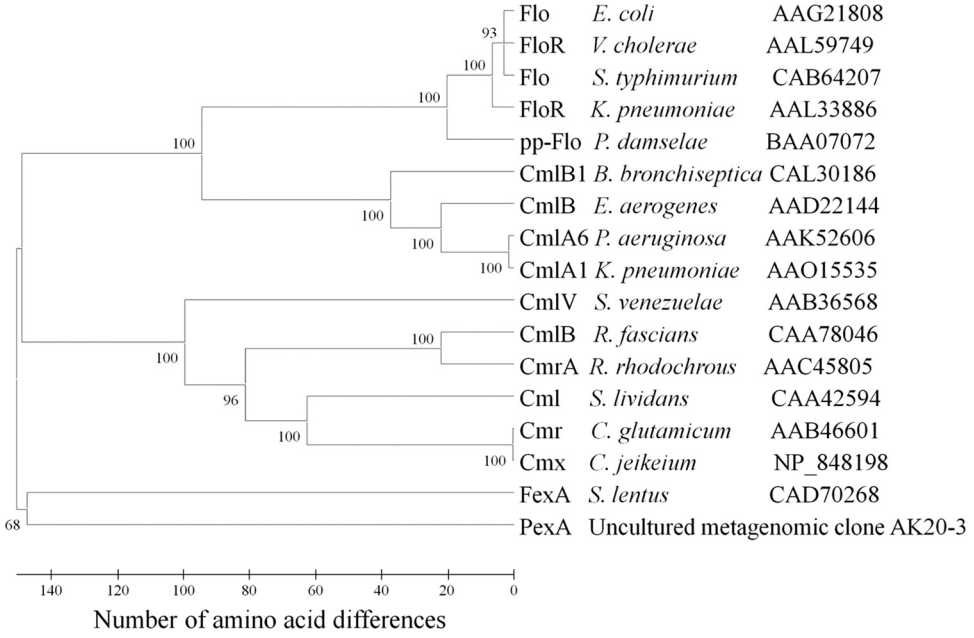

FIG. 1. Phylogenetic tree of the known chloramphenicol/florfenicol exporter proteins. For the different exporter proteins, information on bacterial hosts, database accession numbers, and gene designations (as given in the database entries) is provided. Numbers above each node show the percentage of tree configurations that occurred during the 1,000 bootstrap trials. The tree was constructed using MEGA4 (30).

of the antimicrobials, aside from chloramphenicol and florfenicol, from those without the insert at 30°C or 37°C (data not shown). This suggests that *pexA* mediates resistance to phenicols and not to any other antimicrobial agents.

Many of the known MFS proteins from clinically relevant bacteria involved in the active efflux of antimicrobial agents are inducibly expressed. Sequence analysis of the region upstream of *pexA* revealed a structure similar to that of translational attenuators previously reported to be located upstream of staphylococcal *cat* genes for chloramphenicol resistance or the *fexA* gene for chloramphenicol/florfenicol resistance (11). Upstream of *pexA*, a small reading frame for a 10-aa peptide which contained a potential ribosome stall sequence (5′-GUC UGGACUGCU-3′) similar to previously described ones in regulatory regions of other inducibly expressed phenicol resistance genes was detected (Fig. 2). A pair of two imperfect inverted repeated sequences of 11 and 14 bp, the latter of which contained the *pexA*-associated ribosome binding site, was also detected in the *pexA* upstream region. Calculation of the stability of the mRNA secondary structure formed by these inverted repeats showed a distinctly lower stability of $\Delta G = -24.3$ kJ/mol than that calculated for the mRNA secondary structure in the *fexA* upstream region ($\Delta G = -74.7$ kJ/mol) (9).

To check whether the newly identified phenicol resistance gene was induced by low concentrations of either florfenicol or chloramphenicol, *E. coli* DH5α strains containing the pCC1FOS vector with and without the Ak20-3 insert were passed three times on nonsupplemented LB agar plates at 24-h intervals. Colonies from the antibiotic-free LB plate were then

TABLE 3. MICs of control strains, fosmid clone, and subclone from the Alaskan soil metagenome for florfenicol and chloramphenicol at 30°C after 48 h of incubation

Strain (vector)	Clone (mutation)	MIC (μg/ml)[a]	
		Chl	Ffl
DH5α(pCC1FOS[b])	Ak20-3	64	16
DH5α(pCC1FOS)	Ak20-3 (EZ-Tn*5* <KAN-2>)	64	2
DH5α(pCC1FOS)		64	2
DH5α(pDrive)	*pexA*	16	16
DH5α		4	2
DH5α(pDrive)		4	2

[a] Abbreviations: Chl, chloramphenicol; Ffl, florfenicol.
[b] pCC1FOS contains a *cat* gene for chloramphenicol resistance.

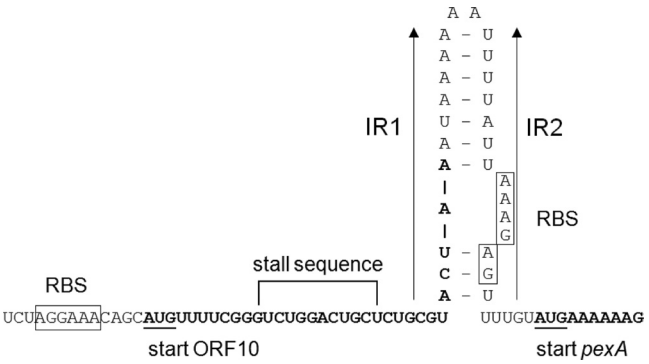

FIG. 2. Presentation of the predicted *pexA* regulatory region. The predicted regulatory region- and *pexA*-associated ribosome binding sites (RBS) are boxed. The start codons of the ORF for the regulatory peptide and the *pexA* gene are underlined, and the corresponding coding sequences are displayed in bold letters. The inverted repeated (IR) sequences IR1 and IR2 are marked by arrows, and an mRNA secondary structure formed by these IR sequences is shown. Calculation of the stability of this stem-loop structure followed the specifications given by Tinoco et al. (31).

used to inoculate 3-ml aliquots of cation-adjusted Mueller-Hinton broth containing florfenicol or chloramphenicol (0.5 μg/ml); these aliquots were incubated with shaking at 30°C for 3 h. Cells were pelleted at 8,000 rpm for 3 min and then resuspended in sterile water, diluted in Mueller-Hinton broth, and used to inoculate the aforementioned microtiter plates. MIC determination followed CLSI standards (7). No differences in MICs of any of the antimicrobial agents tested, including chloramphenicol and florfenicol, were observed between the preincubated and nonpreincubated strains when grown at 30°C or 37°C.

The sequence upstream of *pexA* shows some homology to the translational attenuators upstream of other known antimicrobial resistance genes (11). Temperature could explain the inability to observe a difference in expression after preincubation with a low concentration of florfenicol or chloramphenicol. Differences in the translational components between the natural host of *pexA* and the surrogate *E. coli* host could have also played a role in the apparent noninducibility of *pexA* in the *E. coli* host despite the presence of what appears to be a translational attenuator upstream of *pexA*.

An rRNA operon was predicted using RNAmmer 1.2 (13) within the DNA insert that carries *pexA*. A BLAST (3) query to the GenBank database, excluding all uncultured bacteria, indicated that the predicted 1,525-bp 16S rRNA segment shows highest similarity to the genera *Geobacter* (89% query coverage, 95% identity) and *Desulfomicrobium* (100% query coverage, 83% identity), both members of the *Deltaproteobacteria*/*Epsilonproteobacteria*. Both of these microbes have been isolated from environmental sources.

Although the ultimate source of this gene is unknown, this finding supports the idea that resistance genes exist independently of exposure to therapeutic concentrations of antimicrobial agents and may serve unknown functions in their natural environment. Due to the proximity of *pexA* to an rRNA operon, it seems unlikely that it was inserted there or would be excised in a lateral transfer event because structures resembling mobile genetic elements, such as insertion sequences or transposons, have not been detected. In the present case, *pexA* confers an elevated MIC of chloramphenicol, which is produced by a soil bacterium. In its natural host, *pexA* could provide protection against chloramphenicol excreted by *Streptomyces* spp. However, genes which are anthropomorphically defined as "resistance genes" could also have functions other than survival in the presence of antimicrobial agents. It has been known for some time that at low concentrations antimicrobial agents have multiple effects on bacterial cells, including changes in gene expression, increased mRNA stability, increased rates of mutation, and increased genetic transfer (6, 8). Reactions due to subinhibitory concentrations of antimicrobials probably represent the true function of resistance genes in nature, and it is important to recognize that these genes are selected for under conditions other than treatment with antimicrobials. Understanding these origins could provide clues to novel interventions against resistant organisms or discovery of new antimicrobial compounds.

The results of this functional metagenomic analysis are contingent upon a given gene's ability to be expressed in *E. coli.* It is probable that most of the genes in these libraries are not expressed in this surrogate host, and therefore, we likely underestimate the frequency of resistance determinants in environmental samples. The gene described in this work is active at 30°C or lower temperatures, and—according to the results of broth microdilution—does not provide protection of the *E. coli* host against concentrations equal to or greater than 16 μg/ml of either florfenicol or chloramphenicol. This could be due to problems with heterologous expression in *E. coli*. Activity and stability of a given gene are likely dependent on the living conditions of the natural host. The environment from which the DNA was extracted was Alaskan soil. The microbe that originally harbored the gene was probably acclimated to life at temperatures much lower than 30°C. Perhaps *pexA* would have increased activity at such temperatures, but due to the limitations of using *E. coli* as a surrogate host, assessment of such activity is not possible.

Despite limitations, functional metagenomics has been shown to be effective in discovering diverse resistance mechanisms. It has been shown that human pathogens have likely acquired antimicrobial resistance determinants through horizontal gene transfer from other microbes within their community. Functional metagenomics could be used as a tool to screen microbial communities as a whole in order to fully assess the potential emergence and dissemination of antimicrobial resistance genes. This could be a powerful tool for the approval process of new antimicrobial compounds. Databases of existing metagenomic libraries could be constructed. Target libraries could be screened, using antimicrobial agents being considered for use, in order to evaluate whether a resistance mechanism already exists, and if so, resistance genes could be identified and attempts could be made to predict their rate of dissemination based on the genes to which the novel resistance genes are physically linked.

ID and nucleotide sequence accession numbers. The Alaskan soil metagenome project has been registered with the NCBI (National Center for Biotechnology Information) Genome Project database (identification [ID], 28853). The GenBank accession number for metagenomic clone AK20-3 is HM537013.

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