APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2008, p. 6803–6805 Vol. 74, No. 21
0099-2240/08/$08.00+0 doi:10.1128/AEM.00873-08

Metagenomic Screening for Bleomycin Resistance Genes▽†

Toshio Mori,[1] Shiori Mizuta,[1] Hikaru Suenaga,[1] and Kentaro Miyazaki[1,2]*

Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan,[1] and Department of Medical Genome Sciences, Graduate School of Frontier Sciences, University of Tokyo, Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan[2]

Received 16 April 2008/Accepted 5 September 2008

A metagenomic library of activated sludge was screened for bleomycin resistance genes. Two genes were identified that differed greatly from each other, from the genes of bleomycin-producing actinomycetes, and from those of clinical isolates. Therefore, the nonclinical environment is a rich reservoir of new resistance elements, and metagenomics can be used to sample the resistome rapidly.

The antibiotic bleomycin (Bm) was first discovered in the culture broth of "*Streptomyces verticillus*" (14, 17), an antitumor agent used in clinical settings. Bm-producing actinomycetes have resistance genes (*ble*) that are often clustered in biosynthetic operons (3, 12, 15). However, resistance has spread to bacteria that do not produce Bm, and the genes that confer this resistance have been identified in clinical isolates, Tn*5* of gram-negative bacteria (4), and pUB110 of gram-positive bacteria (11). Nonetheless, the resistance mechanism is conserved; the acidic Bm resistance protein (BRP) sequesters the basic Bm to prevent DNA cleavage by Bm (17).

The natural, or nonclinical, environment is also a reservoir for various antibiotic resistance genes (1, 2, 8, 10). Because *ble* genes have been reported only from clinical isolates and actinomycetes, we explored nonclinical environments for these genes to better understand the origin and distribution of antibiotic resistance. Our aims were to determine the following: (i) whether *ble* could be retrieved from a nonclinical environment, (ii) the evolutionary position of environmental BRPs, and (iii) the resistance mechanism of environmental BRPs. We produced a metagenomic library (5) using activated sludge as a DNA source (13). Activated sludge, which is used to treat industrial wastewater polluted with phenolic compounds, contains a dense microbial community. To survive in this niche, each bacterium must develop resistance to myriad natural antibiotics, mostly those produced by actinomycetes (1). The library contained 3.2 Gb of DNA comprising 96,000 clones of *Escherichia coli* EPI300-T1^R, each carrying a fosmid with an ~33-kb insert (13). One hundred clones were mixed in each well of a 96-well plate, and a total of 10 plates were screened in 100 μl of LB medium containing 12.5 μg/ml chloramphenicol and 50 μg/ml phleomycin, in which the host *E. coli* alone could not grow. After 14 h at 37°C, three resistant clones were identified, 4H7, 7A10, and 8D4. For each fosmid, a shotgun library was produced; each library comprised 388 clones, each with an ~2- to 3-kb insert in pUC118. The libraries were used for DNA sequencing and functional screening.

The 4H7 fosmid contained a 37.4-kb insert (see Table S1 in the supplemental material). Putative *ble* (open reading frame 17 [ORF17]) was identified from among 30 ORFs. For the 7A10 and 8D4 fosmids, sequences were not assembled into a long contig. However, the read sequences from these two fosmids were identical. Therefore, we assumed that they shared the same region of environmental DNA and combined them to generate a single contig. The 31.07-kb fragment contained 26 ORFs (see Table S2 in the supplemental material), including putative *ble* (ORF24) and mobile genetic elements. The G+C content of both fosmids was much lower (62.9 mol% for 4H7 and 57.5 mol% for 7A10) than that of actinomycetes (>70 mol%), and the genes involved in Bm biosynthesis were missing, suggesting that metagenomic *ble* is not part of the actinomycete self-resistance gene group. Functional screening of the shotgun libraries at 500 μg/ml phleomycin identified ORF17 (for 4H7) and ORF24 (for 7A10) as the determinants, as expected. The predicted pI values of these proteins were 4.57 and 4.51, respectively, suggesting their ability to sequester Bm.

The amino acid sequences of 4H7 and 7A10 showed only weak similarity (32% and 25% identity, respectively) to known BRPs (encoded by the *ble* gene from *Streptoalloteichus hindustanus* [Sh*ble*] or carried by pUB110 and Tn*5*) and 30% similarity to each other. Using these BRPs as queries, we performed BLAST searches of several public databases, including all nonredundant GenBank CDS translations, and collected sequences with scores of >90 (multiple alignment shown in Fig. S1 in the supplemental material). This resulted in approximately 120 amino acids with proline near the N terminus, which is critical for dimer formation in known BRPs (6), and an acidic pI of <5, indicating their abilities to sequester Bm.

The BRPs and homologs appear to have evolved from a common ancestral protein (Fig. 1). Because the primary role of BRPs is to sequester Bm via electrostatic interactions, sequences can be very diverse as long as their sequestering abilities are preserved. Numerous hits were obtained that were homologous to 7A10 BRP and affiliated with *Proteobacteria*. As noted above, in 7A10, the *ble* gene neighbored genes that are typically found as part of mobile elements. The ORF4 product

* Corresponding author. Mailing address: Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan. Phone: 81-29-861-6060. Fax: 81-29-861-6413. E-mail: miyazaki-kentaro@aist.go.jp.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 12 September 2008.

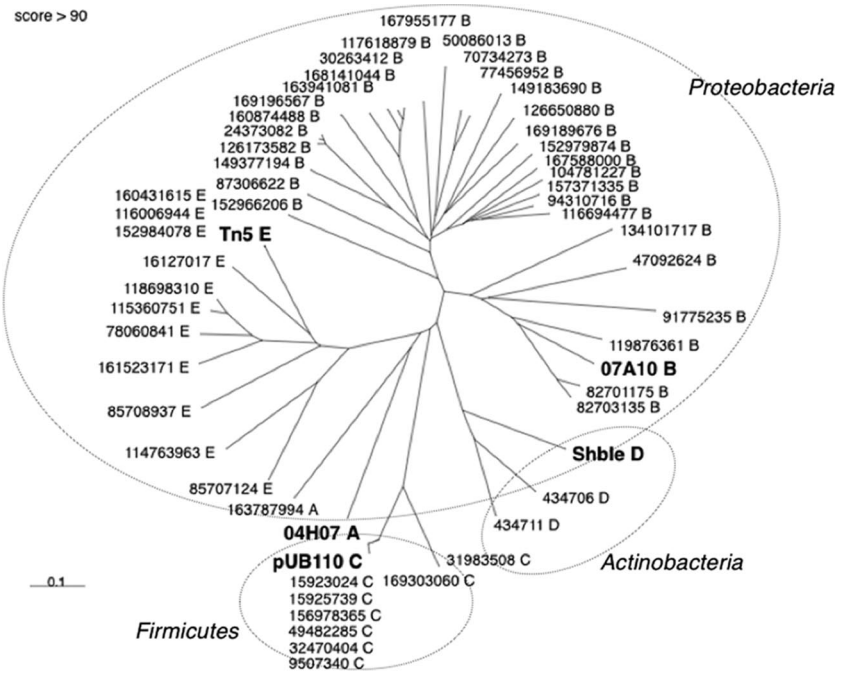

FIG. 1. Phylogenetic tree of BRPs. Using metagenomic BRPs (4H7 and 7A10) and known BRPs (Sh*ble*, pUB110, and Tn*5*) as queries, a BLAST search of the GenBank database was carried out. Fifty-six proteins with matching scores of >90 were selected. Multiple alignment was carried out using ClustalX software (16), using the default parameters (see Fig. S1 in the supplemental material). The program TreeView (9) was used to present the results graphically. Proteins that have been shown experimentally to exhibit Bm resistance are denoted in bold. Sequences are in GenInfo numbers with suffixes A to E, depending on the query sequence. A, 4H7; B, 7A10; C, pUB110, D, Sh*ble*; and E, Tn*5*.

was similar to resolvase from ISXc4 belonging to the Tn*3* family of *Xanthomonas* (7). In addition, there were mercuric resistance proteins (ORF25 and ORF26) just downstream from the BRP (ORF24), suggesting that this type of BRP is likely harbored by some mobile elements (e.g., transposons, insertion elements, etc.) and is distributed in *Proteobacteria*. A large number of hits were also obtained when Tn*5* BRP was used as the query. Therefore, *ble* seems to be widespread in the environment, and this is mediated by mobile elements. When 4H7 BRP was used as the query, no closely related (BLAST score of >90) homologous sequences were identified, giving it a distinct position in the phylogenetic tree. The closest homologue (gi163787994 A) was included in Fig. 1 (a BLAST score of 88) but was on a relatively deep branch relative to 4H7.

To characterize the metagenomic BRPs and Sh*ble*, each *ble* gene was cloned into pET-28a(+), and N-terminal His_6-tagged proteins were produced in *E. coli* BL21(DE3) or BL21(DE3) pLysS. Isopropyl-β-D-thiogalactopyranoside was added (0.01 to 1 mM) during the mid-log phase. Proteins were extracted using BugBuster (Novagen, Carlsbad, CA) and purified to homogeneity by successively passing them through Ni-nitrilotriacetic acid (Qiagen, Hilden, Germany) and anion-exchange Resource Q (GE Healthcare, Piscataway, NJ) columns. The purified proteins were dissolved in 0.1 M Tris-HCl (pH 7.0), and the concentrations were determined using a Pierce bicinchoninic acid protein assay kit (Rockford, IL) with bovine serum albumin as the standard. As judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2a), all of the BRPs were indistinguishable in terms of mass (~15 kDa; the proteins contained an ~2.3-kDa His_6 tag), which agreed with predictions from the sequences (13,478 Da for 4H7, 13,543 Da for 7A10, and 13,796 Da for Sh*ble*). The binding of the BRPs to Bm was confirmed by the gel retardation assay (6). BRP (20 μg) was incubated with 20 μg Bm (~10-fold molar excess of Bm over BRP) in 30 μl of 10 mM Tris-HCl (pH 7.5) at 16°C. After 3 h, 6 μl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) was added, and 10 μl of the mixture was separated by nondenaturing polyacrylamide gel (15%) electrophoresis at 4°C. In all cases, the proteins migrated more slowly in the presence of Bm, indicating the formation of complexes (Fig. 2b). A DNA protection assay was then performed (3). BRP (0 or 10 μM) was mixed with 5 μM Bm in 10 mM Tris-HCl (pH 7.5). After incubation at 25°C for 5 min, 0.1 μg of EcoRI-linearized pUC19, 0.1 mM $FeSO_4$, and 1 mM dithiothreitol were added for a total volume of 20 μl. The mixture was incubated at 25°C; after 10 min, 40 mM EDTA was added. The solution (8 μl) was separated by agarose (2%) gel electrophoresis. The excess BRP prevented cleavage of the Bm DNA (Fig. 2c).

All of the above physical and biochemical characterizations imply functional similarity between metagenomic *ble* and Sh*ble*, regardless of the large sequence diversity. In conclusion, we identified two novel *ble* genes from a nonclinical environment. One gene (4H7) is likely coded on a chromosome, and the other (7A10) is likely coded on a transposon. We also found numerous putative *ble* genes in public databases, suggesting a wide distribution in the natural environment. All of the functionally characterized BRPs contained approximately 120 amino acids with a proline residue near the N terminus (see Fig. S1 in the supplemental material) and an acidic pI of

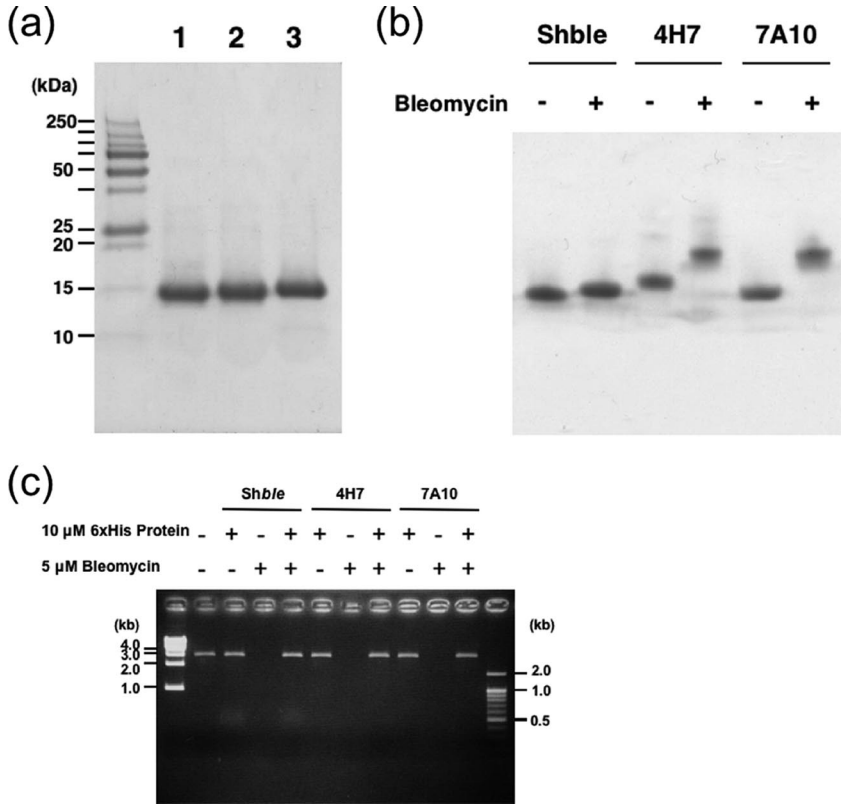

FIG. 2. Characterization of recombinant BRPs. (a) Sodium dodecyl sulfate-polyacrylamide gel (15%) electrophoresis. Lane 1, Sh*ble*; lane 2, 4H7; and lane 3, 7A10. (b) Protein retardation assay (6). Twenty micrograms each of BRP and Bm was mixed in 30 μl of 10 mM Tris-HCl (pH 7.5) and incubated at 16°C. After 3 h, 6 μl of loading dye was added, and 10 μl of the mixture was separated by nondenaturing polyacrylamide gel (15%) electrophoresis at 4°C. (c) DNA protection assay. BRP (0 or 10 μM) was mixed with 5 μM Bm in 10 mM Tris-HCl (pH 7.5). After incubation at 25°C for 5 min, 0.1 μg of EcoRI-linearized pUC19, 0.1 mM $FeSO_4$, and 1 mM dithiothreitol were added for a total volume of 20 μl. The mixture was incubated at 25°C; after 10 min, 40 mM EDTA was added. The solution (8 μl) was separated by agarose (2%) gel electrophoresis.

<5, which should be used as good clues to predict the functions of BRP-like sequences found in sequence databases.

Nucleotide sequence accession numbers. The nucleotide sequences reported herein were deposited in GenBank/EMBL/DDBJ under accession numbers AB300377 for 4H7 and AB331169 for 7A10.

REFERENCES

1. **D'Costa, V. M., K. M. McGrann, D. W. Hughes, and G. D. Wright.** 2006. Sampling the antibiotic resistome. Science **311:**374–377.
2. **D'Costa, V. M., E. Griffiths, and G. D. Wright.** 2007. Expanding the soil antibiotic resistome: exploring environmental diversity. Curr. Opin. Microbiol. **10:**481–489.
3. **Gatignol, A., H. Durand, and G. Tiraby.** 1988. Bleomycin resistance conferred by a drug-binding protein. FEBS Lett. **230:**171–175.
4. **Genilloud, O., M. C. Garrido, and F. Moreno.** 1984. The transposon Tn*5* carries a bleomycin-resistance determinant. Gene **32:**225–233.
5. **Handelsman, J.** 2004. Metagenomics: application of genomics to uncultured microorganisms. Microbiol. Mol. Biol. Rev. **68:**669–685.
6. **Kumagai, T., T. Nakano, M. Maruyama, H. Mochizuki, and M. Sugiyama.** 1999. Characterization of the bleomycin resistance determinant encoded on the transposon Tn*5*. FEBS Lett. **442:**34–38.
7. **Liu, C. C., H. R. Wang, H. C. Chou, W. T. Chang, and J. Tu.** 1992. Analysis of the genes and gene products of *Xanthomonas* transposable elements ISXc5 and ISXc4. Gene **120:**99–103.
8. **Nwosu, V. C.** 2001. Antibiotic resistance with particular reference to soil microorganisms. Res. Microbiol. **152:**421–430.
9. **Page, R. D. M.** 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12:**357–358.
10. **Riesenfeld, C. S., R. M. Goodman, and J. Handelsman.** 2004. Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ. Microbiol. **6:**981–989.
11. **Semon, D., N. R. Movva, T. F. Smith, M. Elalama, and J. Davies.** 1987. Plasmid-determined bleomycin resistance in *Staphylococcus aureus*. Plasmid **17**:46–53.
12. **Shen, B., L. C. Du, C. Sanchez, D. J. Edwards, M. Chen, and J. M. Murrell.** 2002. Cloning and characterization of the bleomycin biosynthetic gene cluster from *Streptomyces verticillus* ATCC15003. J. Nat. Prod. **65:**422–431.
13. **Suenaga, H., T. Ohnuki, and K. Miyazaki.** 2007. Functional screening of a metagenomic library for genes involved in microbial degradation of aromatic compounds. Environ. Microbiol. **9:**2289–2297.
14. **Suzuki, H., K. Nagai, H. Yamaki, N. Tanaka, and H. Umezawa.** 1969. On the mechanism of action of bleomycin: scission of DNA strands *in vitro* and *in vivo*. J. Antibiot. **22:**446–448.
15. **Tao, M., L. Wang, E. Wendt-Pienkowski, N. P. George, U. Galm, G. Zhang, J. M. Coughlin, and B. Shen.** 2007. The tallysomycin biosynthetic gene cluster from *Streptoalloteichus hindustanus* E465-94 ATCC 31158 unveiling new insights into the biosynthesis of the bleomycin family of antitumor antibiotics. Mol. Biosyst. **3:**60–74.
16. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
17. **Umezawa, H., K. Maeda, T. Takeuchi, and Y. Okami.** 1966. New antibiotics, bleomycin A and B. J. Antibiot. **19:**200–209.