Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 6451–6455, May 1999
Microbiology

Toward functional genomics in bacteria: Analysis of gene expression in *Escherichia coli* from a bacterial artificial chromosome library of *Bacillus cereus*

MICHELLE R. RONDON, SANDRA J. RAFFEL, ROBERT M. GOODMAN*, AND JO HANDELSMAN

Department of Plant Pathology, University of Wisconsin-Madison, 1630 Linden Drive, Russell Laboratories, Madison, WI 53706

Communicated by Donald R. Helinski, University of California at San Diego, La Jolla, CA, March 30, 1999 (received for review November 10, 1998)

ABSTRACT **As the study of microbes moves into the era of functional genomics, there is an increasing need for molecular tools for analysis of a wide diversity of microorganisms. Currently, biological study of many prokaryotes of agricultural, medical, and fundamental scientific interest is limited by the lack of adequate genetic tools. We report the application of the bacterial artificial chromosome (BAC) vector to prokaryotic biology as a powerful approach to address this need. We constructed a BAC library in *Escherichia coli* from genomic DNA of the Gram-positive bacterium *Bacillus cereus*. This library provides 5.75-fold coverage of the *B. cereus* genome, with an average insert size of 98 kb. To determine the extent of heterologous expression of *B. cereus* genes in the library, we screened it for expression of several *B. cereus* activities in the *E. coli* host. Clones expressing 6 of 10 activities tested were identified in the library, namely, ampicillin resistance, zwittermicin A resistance, esculin hydrolysis, hemolysis, orange pigment production, and lecithinase activity. We analyzed selected BAC clones genetically to identify rapidly specific *B. cereus* loci. These results suggest that BAC libraries will provide a powerful approach for studying gene expression from diverse prokaryotes.**

The massive accumulation of prokaryotic DNA sequences, including an increasing number of complete genome sequences, is revolutionizing the practice and potential of microbiology. Classical genetic techniques, such as those developed for *Escherichia coli*, *Salmonella typhimurium*, and *Bacillus subtilis,* are no longer the only powerful methods for the investigation of gene expression and function in bacterial systems. Because tools for genetic analysis of many prokaryotes are lacking, we are interested in the development of broadly applicable systems for the investigation of the biology of diverse prokaryotes. A promising technology emerging from genomics is the ability to investigate rapidly the biological features of any organism of choice, without prior development of a specific genetic system for that organism. Here we describe the application of a powerful tool in genomic technology, the bacterial artificial chromosome (BAC), to the study of *Bacillus cereus*, a bacterium for which classical genetic tools are not available.

The BAC vector, based on the *E. coli* F factor, was developed for cloning large fragments of eukaryotic DNA in *E. coli* (1). BACs replicate at a copy number of one to two per cell and are maintained very stably in the cell. BAC plasmids can be isolated easily from chromosomal DNA and purified in sufficient quantity for analysis and sequencing. These properties separate BACs from other cloning vectors such as yeast artificial chromosome and cosmids, which also may suffer from instability and chimera problems (1, 2).

BAC libraries of genomic DNA from numerous plant, animal, and fungal species have been constructed and are becoming the preferred approach in many large-scale sequencing projects (3–5). Molecular techniques developed with BAC technology include methods for introduction of reporter genes into mammalian systems, *in vivo* complementation of mutations, and *in vivo* and *in vitro* "retrofitting" protocols to add new sequence elements to BAC plasmids (6–9).

To date, BAC technology has been applied in a limited way to prokaryote genomics (10, 11). We believe it deserves much wider appreciation, because it offers significant advantages for cloning and analysis of prokaryotic genomes. BACs can be used to clone complex loci, such as biosynthetic pathways, secretion systems, or pathogenicity islands, because the average insert size of a BAC clone is usually greater than 100 kb and because the genes for many bacterial pathways are clustered in the genome. Because BAC inserts are large, a relatively small number of clones is required to provide complete coverage of a bacterial genome, minimizing the amount of work required to screen a BAC library for complex functions or to construct a minimum overlap library. Most significantly, and in contrast to eukaryotic BAC libraries, gene expression from bacterial BAC libraries can be detected in the host strain, thus providing a surrogate system for the analysis of complex pathways from poorly studied, difficult to manipulate, or even uncultured prokaryotes from environmental samples. Therefore, bacterial BAC libraries can serve to archive DNA for genomics purposes and, concurrently, can be used to analyze gene expression, a first step in functional genomics analysis.

To test the applicability of BACs to the study of bacterial functional genomics, we constructed a BAC library in *E. coli* from genomic DNA of the Gram-positive bacterium *B. cereus.* We screened the library for characteristic *B. cereus* activities expressed in *E. coli* and report that a significant number of such activities can be detected in a small library. Our results suggest that BACs provide a useful technique for heterologous expression and functional genomics in prokaryotes.

MATERIALS AND METHODS

Bacterial Strains and Plasmids Used. *B. cereus* strain UW85 was described previously (12). *E. coli* stain DH10B, the host strain for the BAC library, and the BAC vector pBeloBAC11 were obtained from H. Shizuya (1, 3).

Preparation of High Molecular Weight DNA from *B. cereus*. High molecular weight DNA was prepared by a modification of the method described in ref. 13. *B. cereus* cells were grown for 5 h at 28°C in LB to a density of 3×10^8 cells per ml. Cells were harvested by centrifugation and washed once in 1 vol of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

Abbreviation: BAC, bacterial artificial chromosome.

*To whom reprint requests should be addressed. e-mail: RGoodman@facstaff.wisc.edu.

buffer A (50 mM Tris·HCl, pH 8.0/1 M NaCl). A sample was taken to determine cell density, and the remaining culture was stored as a cell pellet at −20°C. The pellet was resuspended to a final concentration of 2×10^9 cells per ml and mixed with an equal volume of molten 1.6% SeaPlaque GTG agarose (FMC), pipetted into plug molds, and allowed to cool. The resulting plugs were incubated for 24 h at 37°C in 2 vol of buffer B [50 mM Tris·HCl, pH 8.0/100 mM EDTA, pH 8.0/100 mM NaCl/0.2% sodium deoxycholate/0.5% 20 cetyl ether (Brij-58)/0.5% *S*-laurylsarcosine] with 5 mg/ml lysozyme added. The plugs were transferred to 2 vol of buffer C (50 mM Tris·HCl, pH 8.0/500 mM EDTA, pH 8.0/100 mM NaCl/0.5% *S*-laurylsarcosine/0.2 mg/ml proteinase K) and incubated for 24 h at 50°C. This step was repeated once. Plugs were washed extensively with TE (10 mM Tris/1 mM EDTA, pH 8.0), followed by inactivation of proteinase K with PMSF. Plugs were stored at 4°C in 10 mM Tris·HCl, pH 8.0/50 mM EDTA, pH 8.0.

Digestion of *B. cereus* DNA *in Situ* and Isolation of Sized Fragments. Partial *Hin*dIII digestion was used to prepare large fragments of DNA from the plugs. Agarose plugs first were incubated in two changes of 1 ml of TE per plug for 5 h at room temperature to remove storage buffer components. Partial digestion by limiting Mg^{2+} concentration was performed as described in ref. 14. The plugs were loaded onto a 1% SeaPlaque agarose gel, and the DNA was size-fractionated by pulsed-field gel electrophoresis. Gel slices containing DNA of the appropriate size were cut out and digested with GELase (Epicentre Technologies, Madison, WI) before ligation. Separation conditions were varied to optimize removal of DNA fragments smaller than 100 kb.

Preparation of BAC Vector, Ligation, and Transformation. Protocols for library construction were taken from the URL http://www.tree.caltech.edu with the following modifications. After the plasmid pBeloBac11 was purified with the Qiagen Plasmid Maxi Kit, it was purified further by LiCl precipitation, RNase treatment, and polyethylene glycol precipitation, as detailed in ref. 15. Finally, the plasmid was treated with PlasmidSafe DNase (Epicentre Technologies) as recommended by the manufacturer. Plasmid DNA (10 μg) was digested with *Hin*dIII and dephosphorylated with HK Phosphatase (Epicentre Technologies), followed by phenol/chloroform extraction and ethanol precipitation. Ligations and transformations were performed as described in the above-mentioned URL. One microliter of ligation mix was used to transform 50 μl of DH10B competent cells by electroporation with a Bio-Rad GenePulser instrument. Samples of 100 μl were spread on LB plates containing 12.5 μg/ml chloramphenicol, 25 μg/ml isopropyl β-D-thiogalactopyranoside, and 50 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. After 36 h at 37°C, white colonies were picked for further analysis.

Plasmid Preparation of BAC DNA. DNA was isolated as described in ref. 15, with modifications. Cells from an overnight culture (1.5 ml) were centrifuged in a 1.5-ml microcentrifuge tube and resuspended in 100 μl of resuspension solution (50 mM glucose/10 mM EDTA/10 mM Tris·Cl, pH 8.0). Freshly prepared 0.2 M NaOH/1% SDS (200 μl) was added and the suspension was mixed by inversion of the tube. After a 5-min incubation at room temperature, 150 μl of 7.5 M ammonium acetate and 150 μl of chloroform were added and mixed by inversion of the tube. The samples were incubated for 10 min on ice and then centrifuged at 14,000 rpm for 10 min in an Eppendorf model 5415C microcentrifuge. The supernatant fluid was added to 200 μl of 30% polyethylene glycol 8000/1.5 M NaCl, mixed by inversion, and incubated on ice for 15 min. Precipitated BAC DNA was collected by centrifugation for 10 min, all of the remaining liquid was removed from the tube, and the pellet was resuspended in 25 μl of sterile water. One-fifth of the sample was used per restriction digest. Digests were analyzed by pulse-field gel electrophoresis on a Pharmacia GeneNavigator using hexagonal electrodes, with the following parameters: 1% SeaKem agarose gel (FMC); linear pulse time ramp from 5 to 15 s; 18-h run time; 165 V.

Activity Screens. Media for testing esculin hydrolysis, lecithinase activity, starch hydrolysis, casein hydrolysis, and lipase activity were as described (16). Ampicillin resistance was tested on LB plates containing 50 μg/ml sodium ampicillin. Hemolytic activity was determined on sheep/veal blood agar plates prepared by the Wisconsin State Hygiene Laboratory. *zmaR*-containing clones were identified by colony hybridization and confirmed to be zwittermicin A-resistant by radial streak assay (17).

λ-Tn*phoA* Mutagenesis. Mutagenesis was performed as described (18, 19). Mid-logarithmic-phase cells were infected with λ-Tn*phoA* at a ratio of 1:1, incubated for 2 h at 28°C, and then plated on LB plates with chloramphenicol (Cm, 12.5 μg/ml) and kanamycin (Km, 50 μg/ml). Colonies were pooled and plasmid DNA was prepared from the pools as described above. One microliter of plasmid DNA was used for transformation of DH10B by electroporation. Cm^R Km^R colonies were selected and then replicated to indicator medium to screen for the loss of function phenotype.

Mini-Tn*10*-Kan Mutagenesis. We transformed pLOFKm (20) by electroporation into competent cells of strains containing the BAC of interest. Cm^R Km^R transformants were selected, pooled, and processed as described above for λ-Tn*phoA* mutants.

Sequencing. BAC DNA for sequencing was prepared by using the Qiagen Plasmid Midi kit, following the protocol for BACs as specified by the manufacturer. The final pellet was suspended in 1 ml of sterile water, precipitated with 2 vol of ethanol, and resuspended in 75 μl of 10 mM Tris·Cl, pH 8.0. Sequencing reactions were performed with 2 μg of DNA and 10 pmol of primer, using BigDye reaction mix (Perkin—Elmer). Reaction products were purified with MicroSpinPreps (Pharmacia) or with CentriSep columns (Princeton Separations, Adelphia, NJ). Sequencing reactions were run on an Applied Biosystems 377 sequencer at the University of Wisconsin Biotechnology Center. Standard T7 and SP6 primers (Promega) were used to generate end sequence, and *phoA* primers were *phoA*1 (5′-AATATCGCCCTGAGCAGCCCG-3′) (21) and *phoA*4 (5′-TAGGAGGTCACATGGAAGTCA-GATC-3′) (22).

Clone Grouping. Denatured miniprep DNA (2 μl) of each BAC clone was spotted to a nylon membrane (Magnagraph; Micron Separations) previously wet with water and then with 2× SSC (1.3 M sodium chloride/30 mM sodium citrate, pH 7). Membranes were kept wet while spotting the DNA by placing them on filter paper soaked with 2× SSC. DNA was crosslinked to the membrane by using a Stratalinker. The 700- and 900-kb fragments were generated by *Not*I digestion of *B. cereus* chromosomal DNA and separated by pulse-field gel electrophoresis using 0.5× TBE buffer modified to contain only 0.1× EDTA. Run parameters were 11°C for 15 h at 70-s pulse time and then 11 h at 120-s pulse time. The gel was stained briefly with ethidium bromide, and gel slices were cut out. After dialysis in sterile, deionized water to remove excess ethidium bromide, the DNA was labeled *in situ* by using the Genius Kit (Boehringer Mannheim), according to the protocol for in-gel labeling from the FMC catalog. Hybridizations were done according to the protocol for Magnagraph membranes.

RESULTS

Construction of a *B. cereus* BAC Library. The BAC library consists of 323 clones, containing approximately 30 Mb of *B. cereus* DNA. We estimated the size of the UW85 genome at 5.5 Mb, based on *Not*I and *Sfi*I digests (data not shown); on this basis, our library represents a 5.75-fold coverage of the UW85

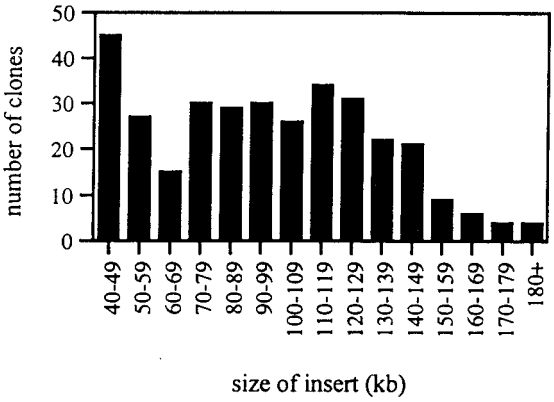

FIG. 1. Size distribution of BAC clones. Insert size was estimated by digestion of the plasmids with *Not*I, followed by analysis of the digestion products by pulse-field gel electrophoresis.

genome. The range of insert sizes is from 40 to greater than 175 kb (Fig. 1). The average insert size was 98 kb. Greater than half (216 clones) of the BAC clones contain insert DNA greater than 80 kb in size. We estimate that the probability of the library containing any particular 1-kb gene is 99.7% (23).

Numerous molecular techniques, such as contig building, end-sequence analysis, hybridization, and clone pooling, have been applied successfully to BAC libraries for rapid identification of a clone of interest, physical mapping, and comparative genomics (24, 25). We identified a set of BAC clones all hybridizing to a single genomic *Not*I fragment from *B. cereus*. Using a 700-kb *Not*I fragment as a probe, we identified 27 clones that hybridized to this fragment (Fig. 2). These clones contain approximately 3,060 kb of DNA, representing 4.4-fold coverage of the probe fragment. The fragment represents 12.7% of the genome, and the clones identified contain 10.4% of the DNA in the library, indicating that the genomic DNA in this fragment is represented proportionately in the library. We also probed by using a 900-kb fragment and found 19 clones that hybridized to this fragment (data not shown). This represents a 2.7-fold coverage of the fragment. These clones contain 8% of the DNA in the library, although the fragment represents 16% of the genome. This section of the genome therefore may be underrepresented in the library.

Potential of BACs as Surrogate Expression Vectors for Analysis of Prokaryotic Genes. We estimated the frequency of gene expression in the library by testing it for easily detectable *B. cereus* activities. The BAC library was replicated by using 48-prong replicators to various media to test for the expression of *B. cereus* activities in *E. coli*. Of nine activities tested by the use of specific indicator media, six were found in the library (Table 1). Additionally, zwittermicin A-resistant clones were identified by hybridization to a *zmaR*-containing probe and confirmed to be resistant by radial streak assay (E. A. Stohl and J.H., unpublished results). In all cases the BAC clones were isolated from putative positive colonies and retransformed into DH10B, and the activity was found to be BAC-associated. This represents a useful frequency of detection, considering that a number of the activities tested for represent extracellular functions, which may be less well expressed in a Gram-negative organism, and because the gene expression and protein export machinery of *B. cereus* and *E. coli* are likely to have numerous differences.

Genetic Analysis of BAC Clones: Identification of Loci Involved in Hemolysis, Esculin Hydrolysis, and Orange Pigment Production. Once a BAC clone expressing a particular activity is isolated, methods for rapid identification of the locus responsible for the activity will contribute to functional and physical analysis. We tested the possibility that transposon mutagenesis, combined with direct sequencing of BACs, could provide the appropriate information rapidly. Transposon mutants were isolated for a hemolysin-producing clone (BACB61; 40-kb insert), an esculin-hydrolyzing clone (BACB94; 80-kb insert), and an orange pigment-producing clone (BACB142; 125-kb insert). These mutants were generated by infecting with lambda phage containing the Tn*phoA* marker or by electroporation with a suicide plasmid containing a mini-Tn*10*-Km transposon. These methods for plasmid mutagenesis were successful in all cases. The locus containing the transposon was identified by sequencing from the transposon into the flanking DNA by using primers specific for the ends of the transposon (Table 2).

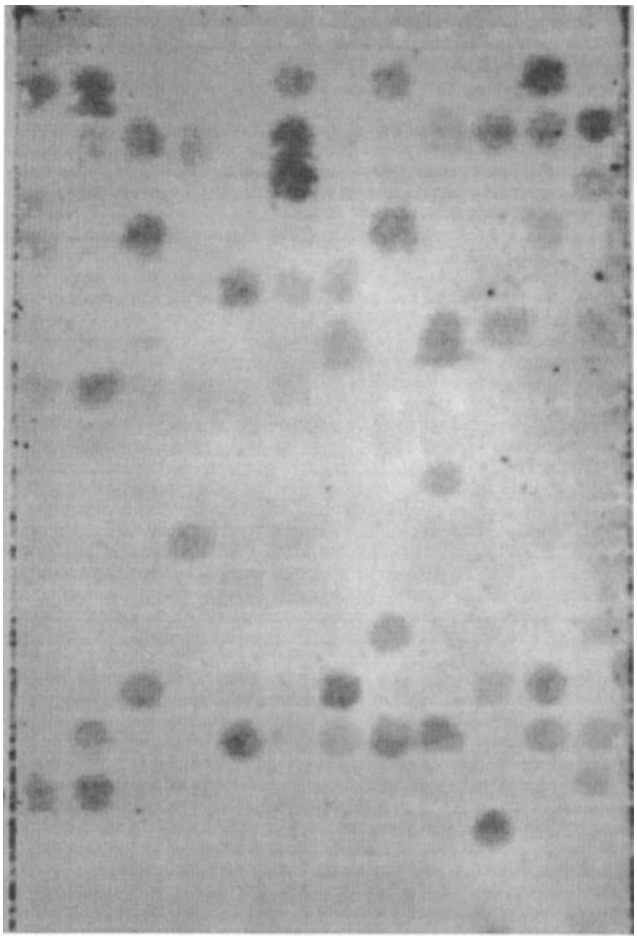

FIG. 2. Hybridization of BAC clones with a genomic *Not*I fragment. A 700-kb genomic *Not*I fragment from strain UW85 was used to probe a blot containing DNA from all BAC clones with inserts greater than 80 kb in size. These clones (total, 216) provide 4-fold coverage of the UW85 genome and can be screened on one blot.

The hemolytic activity of BACB61 likely is due to production of hemolysin II (26), because mutations that abolish

Table 1. Expression of *B. cereus* activities in *E. coli*

Activity tested	No. of clones detected
Starch hydrolysis	0
Casein hydrolysis	0
Hemolysis	2
Esculin hydrolysis	2
Orange pigment	2
Ampicillin resistance	1
Zwittermicin A resistance	2
Lecithinase	2
Chitinase	0
Lipase	0

Table 2. Identification of *B. cereus* loci

Clone number	Activity	Transposon	Homology, %
BACB61	Hemolysis	Tn*phoA*	YqiI (54% over 79 aa)
		Mini-Tn*10*-Km	HlyII (95% over 103 aa)
			Eag (52% over 51 aa)
BACB94	Esculin hydrolysis	Tn*phoA*	LevR (58% over 144 aa)
			CelR (47% over 127 aa)
BACB142	Orange pigment	Tn*phoA*	CatA (72% over 51 aa)
			CatV (40% over 62 aa)

Accession numbers for the above-mentioned loci: YqiI, Z99116; HlyII, U94743; Eag, X99724; LevR, M60105; CelR, U07818; CatA, M75944; CatV, M80796. References are cited in the text. Homology values indicate percent identify.

hemolytic activity are located in an ORF with homology to *hlyII* of another *B. cereus* strain (GenBank accession no. U94743). Before this analysis, strain UW85 was not known to contain this gene. Interestingly, mutations in the BAC clone that confer reduced but detectable levels of hemolytic activity also were isolated. Insertions producing this activity were in one of two ORFs: an ORF homologous to *B. subtilis* cell wall amidases (27) or an ORF encoding an S layer homology motif (28, 29). Restriction enzyme digestion analysis (not shown) indicated that the two loci were linked closely on BACB61, suggesting a possible functional or transcriptional linkage. Further analysis of this locus is required to understand its structure and contribution to hemolytic activity.

Two independently isolated mutations of BACB94, which confers the ability to hydrolyze esculin on *E. coli*, were found to be in an ORF with homology to the *B. subtilis* protein LevR. LevR is a transcription factor regulating expression of the levanase operon (30). It is likely that there is a β-glucosidase utilization operon present on BACB94 that is regulated by the LevR homolog, conferring esculin hydrolysis activity. Other genes similar to *levR* are postulated to regulate genes involved in cellobiose utilization [*celR* from *B. stearothermophilus* (31)] or β-glucoside utilization [*arbG* of *Erwinia chrysanthemi* (32)]. An alternative explanation is that the LevR homolog fortuitously activates the cryptic *bgl* operon in *E. coli*, because LevR has homology to BglG, the antiterminator protein that regulates *bgl* expression (33). These hypotheses now can be tested experimentally.

Two independently isolated transposon mutants were identified that disrupted pigment production in BACB142, the orange pigment-producing BAC clone. Both of these transposons disrupted a single ORF with homology to bacterial catalase enzymes (34, 35). These proteins contain a heme cofactor, which may be responsible for the orange color of the colonies carrying BACB142. Alternatively, the presence of genes on this BAC could disrupt or modify *E. coli* heme metabolism. Consistent with the latter idea, there appears to be an ORF directly upstream of the catalase ORF that has homology to bacterial ferrochelatases (data not shown), suggesting there may be alterations in heme metabolism in this strain. Overexpression of a heme biosynthetic enzyme from *B. stearothermophilus* in *E. coli* resulted in the colonies having a reddish color (36). Alternatively, there could be a gene downstream from the catalase gene on BACB142 that is responsible for the orange color and whose expression is altered by the presence of the transposon, although sequence analysis of the downstream region did not reveal any homology to known genes.

Genomic Comparisons by Using BAC Plasmids. Despite their large size and low copy number, sequencing directly from BACs is practical, as seen above. We generated sequence information at the ends of the insert DNA from the three characterized BACs, BACB61, BACB94, and BACB142, by using standard primers directed to vector sequences. This resulted in four of six cases in the identification of homologous sequences from *B. subtilis* (data not shown), reflecting the high information density of bacterial genomes. These data could be used to align a BAC to a sequenced genome from a related bacterium, if possible, or to select clones for complete sequencing. Construction, phenotypic analysis, and sequencing of insert ends with vector-directed primers of BAC libraries from several *Bacillus* species could provide a genomic overview of this group of organisms, which contain both industrially important and pathogenic species, and would provide a valuable resource for further genomic analysis.

DISCUSSION

Our approach to functional genomics combines the utility of BAC libraries to access large, contiguous segments of DNA with the small genome size of prokaryotes and heterologous expression in *E. coli*. This merger results in a broadly applicable approach to the study of prokaryotic biology. Clearly, not every prokaryotic species is a candidate for whole genome sequencing, nor can we afford to develop genetic tools for each species of interest. BAC libraries offer a universal method to perform genetic, physical, and functional analyses of a prokaryotic genome without the need for an extensive investment in sequencing or specific methods development.

We predict that BAC libraries of bacterial genomes will yield new insights into prokaryotic biology, especially libraries of those species that, thus far, are poorly understood. Bacteria that are of significant biological interest for antibiotic production or ecological behavior, such as *B. cereus*, may be recalcitrant to the usual bacterial genetic and molecular techniques but can be studied via a BAC library approach.

BAC libraries of eukaryotic genomes typically contain thousands of clones (3). This number is required for sufficient coverage of large eukaryotic genomes. In contrast, prokaryotic genomes, such as that of *B. cereus*, require only a few hundred clones for equivalent coverage. For example, the BAC library of *Mycobacterium tuberculosis* required 68 BACs for a minimal overlap library of the 4.4-Mb genome, with one 150-kb gap (10). Yet, the same powerful techniques developed for eukaryotic BACs can be applied to prokaryotic BAC libraries, further increasing the utility of these libraries.

One of the advantages of BACs is that they appear to maintain heterologous DNA more stably than other cloning systems. This would be an advantage in cloning DNA from diverse microorganisms and might be especially relevant when gene expression from the clones is desired. The *M. tuberculosis* library has an average insert size of 70 kb, and inserts larger than 110 kb were not obtained (10), indicating that there might be species-specific limitations to some applications of BAC technology. We did not observe these problems with our *B. cereus* library, because inserts in our library were as large as 260 kb.

Given that six of ten of the activities screened for were detected in our library, we envision that diverse genes from bacteria can be cloned and analyzed successfully in BACs. We have not yet determined whether this frequency of detection of gene expression is an average level for BAC libraries of

prokaryotic genomes. An answer to that will come from the construction and screening of more BAC libraries. Xu *et al*. (11) used pBeloBac11 to clone *Enterococcus faecalis* genes in *E. coli*, although in this library inserts were only 30–45 kb in size. Adequate levels of expression would be especially important when the activity of interest is hard to screen for or requires a large amount of genetic information for expression.

Though not broadly screened for in our library, BACs are the ideal tool for cloning and analysis of entire bacterial pathways, such as antibiotic biosynthesis pathways, biodegradative operons, or pathogenicity islands. Before our genetic analysis, it was unknown whether some activities such as hemolysis or orange pigment production were due to one or two genes or whether they represented biological activities of small molecules requiring a suite of genes for their production. Although we have applied BAC technology to *B. cereus*, a readily cultured organism, this approach will be even more powerful for analysis of the genomes of bacterial species in the environment, which may be accessed via BAC cloning, even if the cells themselves cannot be cultured at the present time (37, 38).

We thank Brett Tyler for introducing us to BACs and for a critical discussion that led to this work. We thank Amy Klimowicz and Daniel Cook for phenotypic screening of parts of the library, Elizabeth Stohl for screening the library for *zmaR*-containing clones, Brad Borlee for isolating the BACB94 Tn*phoA* mutants, the W. S. Reznikoff laboratory for the plasmid DNA isolation method, and Alan Bettermann for help with minipreps. Carlos Rios provided the λ-Tn*phoA* materials. pLOF-Km was provided by Sang-jin Suh. M.R.R. gratefully acknowledges support for a part of this research from a postdoctoral research fellowship (1 F32 GM18871-O1A1) from the National Institutes of Health. This research was supported by the University of Wisconsin University–Industry Research program, The McKnight Foundation, Ariad Pharmaceuticals, and the National Institutes of Health (R03 AI42786-02.

1. Shizuya, H., Birren, B., Kim, U.-J., Mancino, V., Slepak, T., Tachiri, Y. & Simon, M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8794–8797.
2. Monaco, A. P. & Larin, Z. (1994) *Trends Biotechnol.* **12,** 280–286.
3. Kim, U.-J., Birren, B. B., Slepak, T., Mancino, V., Boysen, C., Kang, H.-L., Simon, M. I. & Shizuya, H. (1996) *Genomics* **34,** 213–218.
4. Woo, S.-S., Jiang, J., Gill, B. S., Paterson, A. H. & Wing, R. A. (1994) *Nucleic Acids Res.* **22,** 4922–4931.
5. Venter, J. C., Smith, H. O. & Hood, L. (1996) *Nature (London)* **381,** 364–366.
6. Mejía, J. E. & Monaco, A. P. (1997) *Genome Res.* **7,** 179–186.
7. Yang, X. W., Model, P. & Heintz, N. (1997) *Nat. Biotechnol.* **15,** 859–865.
8. Antoch, M. P., Song, E.-J., Chang, A.-M., Vitaterna, M. H., Zhao, Y., Wilsbacher, L. D., Sangoram, A. M., King, D. P., Pinto, L. H. & Takahashi, J. S. (1997) *Cell* **89,** 655–667.
9. Kim, S. Y., Horrigan, S. K., Altenhofen, J. L., Arbieva, Z. H., Hoffman, R. & Westbrook, C. A. (1998) *Genome Res.* **8,** 404–412.
10. Brosch, R., Gordon, S. V., Billaut, A., Garnier, T., Eiglmeier, K., Soravito, C., Barrell, B. G. & Cole, S. T. (1998) *Infect. Immunol.* **66,** 2221–2229.
11. Xu, Y., Jiang, L., Murray, B. E. & Weinstock, G. M. (1997) *Infect. Immunol.* **65,** 4207–4215.
12. Handelsman, J., Raffel, S., Mester, E. H., Wunderlich, L. & Grau, C. R. (1990) *Appl. Environ. Microbiol.* **56,** 713–718.
13. Harrell, L. J., Andersen, G. L. & Wilson, K. H. (1995) *J. Clin. Microbiol.* **33,** 1847–1859.
14. Birren, B. & Lai, E. (1993) *Pulsed Field Gel Electrophoresis* (Academic, San Diego).
15. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
16. Gerhardt, P. (1994) *Methods for General and Molecular Bacteriology* (Am. Soc. Microbiol., Washington, DC).
17. Milner, J. L., Stohl, E. A. & Handelsman, J. (1996) *J. Bacteriol.* **178,** 4266–4272.
18. Manoil, C. & Beckwith, J. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 8129–8133.
19. Kaufman, M. R. & Taylor, R. K. (1994) *Methods Enzymol.* **235,** 426–448.
20. Herrero, M., de Lorenzo, V. & Timmis, K. (1990) *J. Bacteriol.* **172,** 6557–6567.
21. Taylor, R. K., Manoil, C. & Mekalanos, J. J. (1989) *J. Bacteriol.* **171,** 1870–1878.
22. Leclerc, G. J., Tartera, C. & Metcalf, E. S. (1998) *Infect. Immunol.* **66,** 682–691.
23. Clarke, L. & Carbon, J. (1976) *Cell* **9,** 91–99.
24. Zhu, H., Choi, S., Johnston, A. K., Wing, R. A. & Dean, R. A. (1997) *Fungal Genet. Biol.* **21,** 337–347.
25. Boysen, C., Simon, M. I. & Hood, L. (1997) *Genome Res.* **7,** 330–338.
26. Sinev, M. A., Budarina, Z. I., Gavrilenko, I. V., Tomashevskii, A. Y. & Kuz'min, N. V. (1993) *Mol. Biol.* **27,** 753–760.
27. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., *et al.* (1997) *Nature (London)* **390,** 249–256.
28. Mesnage, S., Tosi-Couture, E., Mock, M., Gounon, P. & Fouet, A. (1997) *Mol. Microbiol.* **23,** 1147–1155.
29. Lupas, A., Engelhardt, H., Peters, J., Santarius, U., Volker, S. & Baumeister, W. (1994) *J. Bacteriol.* **176,** 1224–1233.
30. Débarbouillé, M., Martin-Verstraete, I., Klier, A. & Rapoport, G. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2212–2216.
31. Lai, X. & Ingram, L. O. (1993) *J. Bacteriol.* **175,** 6441–6450.
32. El Hassouni, M., Henrissat, B., Chippaux, M. & Barras, F. (1992) *J. Bacteriol.* **174,** 765–777.
33. Amster-Choder, O. & Wright, A. (1993) *J. Cell. Biochem.* **51,** 83–90.
34. Bol, D. K. & Yasbin, R. E. (1991) *Gene* **109,** 31–37.
35. Haas, A., Brehm, K., Kreft, J. & Goebel, W. (1991) *J. Bacteriol.* **173,** 5159–5167.
36. Chou, P.-L., Ohtsuka, M., Minowa, T., Yamasoto, K., Sakano, Y., Matsuzama, H., Ohta, T. & Sakai, H. (1995) *Biosci. Biotech. Biochem.* **59,** 1817–1824.
37. Stein, J. L., Marsh, T. L., Wu, K. Y., Shizuya, H. & DeLong, E. F. (1996) *J. Bacteriol.* **178,** 591–599.
38. Handelsman, J., Rondon, M. R., Brady, S. F., Clardy, J. & Goodman, R. M. (1998) *Chem. Biol.* **5,** R245–R249.