GeneHunter, a Transposon Tool for Identification and Isolation of Cryptic Antibiotic Resistance Genes

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GeneHunter is a transposon tool designed for the experimental activation and identification of silent antibiotic resistance genes. The method permits the identification of novel resistance genes that lack previously identified homologues. Using Salmonella enterica serovar Typhimurium strain LT2 as a test organism for the in vivo version of the GeneHunter method, we were able to activate, clone, and identify two cryptic antibiotic resistance genes, the aminoglycoside acetyltransferase aac(6')-Iaa and the probable Mar-A regulon activator rma. Because the method requires being able to electroporate the host with an efficiency of at least 10^6 transformants per microgram, the in vivo method is not applicable to most microorganisms. We therefore developed an in vitro transposition method, showed that it can also recover the cryptic rma gene from S. enterica serovar Typhimurium strain LT2, and showed that it is generally applicable to a variety of microorganisms by using it to recover a cryptic metallo-β-lactamase gene from the gram-positive organism Bacillus cereus. It is anticipated that the GeneHunter method will be used to identify potential resistance genes during the development and testing of novel antibiotics, new variants of existing antibiotics, and drug inhibitor combinations.

Although hundreds of antibiotic resistance genes have been identified, one of the major challenges facing the antibiotic resistance field is to predict, in advance of their appearance, where new resistance genes will arise. This challenge is especially important when new antibiotics are developed and introduced into clinical use.

One source of new resistance genes is the mutational modification of existing genes, of which the best-studied example is the plethora of TEM β-lactamase alleles that have arisen in response to the introduction of the extended-spectrum β-lactam antibiotics. Methods for predicting the evolution of existing resistance genes in response to selection by existing or novel drugs have been developed (2, 3, 5).

The advent of genomic sequencing now allows us to detect “new” resistance genes in the genomes of sequenced organisms by virtue of their homologies to known resistance genes. That approach, however, cannot detect new classes of resistance genes; it can only detect genes that are similar to known resistance genes.

Another source of new resistance genes is silent genes that are present in the chromosomes of phenotypically antibiotic-sensitive bacteria. Such silent or “cryptic” resistance genes have been reported in the chromosomes of Bacteroides fragilis (15), Salmonella enterica (11), and Citrobacter freundii (4). Cryptic resistance genes have been detected by the deliberate (15) or accidental (11) selection of resistant mutants of sensitive strains or by deliberate attempts to clone resistance genes suspected of being present in pre-antibiotic era isolates (4).

GeneHunter utilizes EZ::TN transposase, a variant of Tn5 transposase that was engineered by Epicentre Technologies based on the work of Goryshin and Reznikoff (9). In an in vitro reaction, EZ::TN transposase combines with molecules of a GeneHunter transposon to form stable transposon-transposase complexes. These complexes may be activated in vivo by electroporation, resulting in the random integration of the transposons into the chromosomes of living cells. Alternatively, they may be activated in vitro by the addition of Mg^{2+} in the presence of target molecules, such as Fosmid libraries of genomic DNA, resulting in random insertions into the target molecules. Once incorporated into the DNA target, a powerful outward-reading promoter encoded by the transposon can activate genes adjacent to the site of insertion. Salmonella enterica serovar Typhimurium strain LT2 was
chosen as a model organism based on an earlier study that revealed the presence of at least one cryptic antibiotic resistance gene within that organism’s chromosome, the aminoglycoside acetyltransferase aac(6′)-Iaα gene (16).

**MATERIALS AND METHODS**

**Bacterial strains.** Wild-type *S. enterica serovar Typhimurium* strain LT2 (Hall collection), *Bacillus cereus* strain ATCC 10987 (America Type Culture Collection), and *Escherichia coli* strain EC100D protA+ [F− merA Δ(mrr-hsdRMS-merBC) Δ(pirD-pirE) lacX74 recA1 endA1 araD139 Δ(ara-leu)1759Δ6 galU galK λ− rpsL, nupG trp* (DHFR)] (Epici) and EP300 [F− merA Δ(mrr-hsdRMS-merBC) Δ(pirD-pirE) lacX74 recA1 endA1 araD139 Δ(ara-leu)1759Δ6 galU galK λ− rpsL, nupG trp* (Epici)] were used in this project. Stock solutions of antibiotics were prepared in 0.1 M NaPO4 buffer (pH 7.0) (except for tetracycline and doxycycline, 500 μg/ml). All strains that carry the pir* gene, as well as a multiple cloning site (MCS) into which can be cloned any desired gene. Plasmid plasmid pMOD3-pTAC was constructed by amplifying the pTAC promoter, bp 63 to 250 of plasmid pSE380 (Invitrogen), using primers FP-1 (TGGTTCC GAATATCCGTCGAATTCGATCCACACACATCTAT) and RP-1 (GGGTACCTAGAGATCCCGGAAGCGTCGACCGGTG GAATTC). The method of Geiser et al. (8) was used to insert the ampicillin into plasmid pTAC by replacing the pTAC promoter with that of intermediate plasmid, 221 bp of the ampicillin resistance gene of that intermediate plasmid, 221 bp of the ampicillin resistance gene of EZ::TN as a standard. Electrocompetent cells were prepared by the method of Sharma and Shimke (17), except that cells were resuspended in 20% glycerol in the medium to induce transcription from the pTAC promoter. After 16 h of incubation, the zones of inhibition were measured.

**Plasmids.** Plasmid pMOD3-<R6K>ori/MCS (Epici) includes an ampicillin resistance gene and an EZ::TN transposon that contains, between the inverted repeats required for transposition, an R6K origin of replication that permits replication in *E. coli* strains that carry the pir* gene, as well as a multiple cloning site (MCS) into which can be cloned any desired gene. Plasmid pMOD3-pTAC was constructed by amplifying the pTAC promoter, bp 63 to 250 of plasmid pSE380 (Invitrogen), using primers FP-1 (TGGTTCC GAATATCCGTCGAATTCGATCCACACACATCTAT) and RP-1 (GGGTACCTAGAGATCCCGGAAGCGTCGACCGGTG GAATTC). The method of Geiser et al. (8) was used to insert the ampicillin into plasmid pMOD3-<R6K>ori/MCS (Epici) as a replacement for bp 580 to 589 of pMOD3-<R6K>ori/MCS:MCS. pMOD3-pTAC served as the backbone for constructing pGHC and pGHC.

Plasmid pGHC (GeneHunter chloramphenicol) (Fig. 1A) was constructed by amplifying the chloramphenicol resistance gene of plasmid pACYC184 (bp 3591 to 461) (6) with primers FP-2 (CCCGGGGATCCTCTAGAGTCGACCGTAA GCATATTGGCTCGAATTCATCCGCTCACAATTCCACACACATTA) and RP-1 (GGGTACCTAGAGATCCCGGAAGCGTCGACCGGTG GAATTC). The method of Geiser et al. (8) was used to insert the ampicillin into plasmid pMOD3-<R6K>ori/MCS: (Epici) as a replacement for bp 580 to 589 of pMOD3-<R6K>ori/MCS:MCS. pMOD3-pTAC served as the backbone for constructing pGHC and pGHC.

**Determination of MICs.** Ampicillin (Sigma), cefotaxime (Sigma), cefepime (Bristol-Myers Squibb), cefoxitin (Merck), cefuroxime (Sigma), cefazolin (Glaxo Wellcome), aztreonam (Bristol-Myers Squibb), imipenem (Merck), ampicillin (Sekisui Chemical Co.), ciprofloxacin (Bayer), levofloxacin (Ortho-McNeil), tetracycline (Sigma), and doxycycline (American Pharmaceutical Partners) were used in this project. Stock solutions of antibiotics were prepared in 0.1 M NaPO4 buffer (pH 7.0) (except for tetracycline and doxycycline, which were prepared using sterile water), filter sterilized, and stored at −80°C in single-use aliquots. All MICs were determined in Mueller-Hinton broth according to the method of Barlow and Hall (5). For *E. coli* cells, 1 mM isopropyl-β-D-thiogalactopyranoside was included in the medium to induce transcription from the pTAC promoter.

**Disk diffusion assays.** Overnight cultures were spread onto Mueller-Hinton medium agar plates, and BBL antibiotic disks containing aminoglycosides (30 μg of amikacin, 10 μg of tobramycin, 10 μg of gentamicin, 30 μg of kanamycin), β-lactams (10 μg of ampicillin, 30 μg of cefotaxime, 30 μg of cefepime, 30 μg of cefoxitin, 30 μg of cefuroxime, 30 μg of ceftazidime, 30 μg of aztreonam, 10 μg of imipenem), or fluoroquinolones (5 μg of ciprofloxacin, 5 μg of levofloxacin) and tetracyclines (30 μg of tetracycline, 30 μg of doxycycline) were applied to the plates. For *E. coli* cells, 1 mM isopropyl-β-D-thiogalactopyranoside was included in the medium to induce transcription from the pTAC promoter. After 16 h of incubation, the zones of inhibition were measured.

**Electroporation.** The GeneHunter method depends upon high electroporation efficiencies, i.e., efficiencies of >1010 transformants per μg using plasmid pUC19 as a standard. Electrocompetent cells were prepared by the method of Sharma and Shimke (17), except that cells were resuspended in 20% glycerol in the final step and that 50-μl single-use aliquots were stored at −80°C. All steps were carried out at 4°C, and centrifugation times were reduced to 5 min. Electroporation was done in a cold room using prechilled cuvettes with a 1-mM gap. Fifty
microliters of electrocompetent cells were combined with up to 4 μl of DNA in H2O or low-salt buffer and subjected to a 1.6-kV pulse at a capacitance of 25 μF and a resistance of 200 Ω. Instantly following the pulse, 1 μl of SOC broth at 30°C was added to the cuvette and well mixed with the cells, and the cuvette was held at 30°C. Within a few minutes the electrocompetent cell suspension was added to a sterile 18-by-150-mm tube containing an additional 1 ml of SOC broth, and the suspension was placed onto a rotating drum at 37°C for 90 min to permit expression of the drug resistance gene.

In vivo library construction. To avoid restriction degradation of a GeneHunter transposon electroporated into Salmonella, the plasmid pHGIC was prepared from S. enterica serovar Typhimurium strain LT2. Approximately 10 μg of Salmonella-modified pHGIC was digested with the endonuclease PshAI (New England Biolabs) and purified using the MinElute PCR purification kit (Qiagen), and the digested DNA was diluted to a concentration of 474 ng/μl. One of the two resulting fragments is the GeneHunter<cam> transposon. One microgram of digested plasmid was combined with 2 μl of EZ-TN transposesase (Epipcr) and 1 μl of sterile 100% glycerol, in that order. The reaction was incubated at room temperature for 30 min, and the entire volume was then electroporated into S. enterica serovar Typhimurium. After expression, 8 ml of L broth was added to the culture, and serial dilutions of the culture were plated onto L-chloramphenicol agar plates to determine the library size. The remainder of the transformed population was transferred to 200 ml of L-chloramphenicol and incubated overnight in a shaker culture. Ten milliliters of the overnight culture was pelleted, resuspended in 3 ml of P buffer and 300 μl of dimethyl sulfoxide, and diluted to sterile microtube tubes in 100-μl single-use aliquots. Those aliquots were stored at −80°C until needed.

Formation of self-replicating plasmids from genomic fragments containing a GeneHunter transposon. Genomic DNA was purified from transposon-bearing isolates using the MasterPure DNA purification kit (Epipcr). Ten micrograms of DNA was combined with 750 μl of Tris-EDTA (pH 8.0) containing 10% glycerol and sheared in a Nebulizer (Invitrogen) for 45 s at 9.5 kV/cm² to produce fragments with an average length of approximately 5 kb. The sheared DNA was purified by ethanol precipitation and resuspended in 70 μl of sterile water. Five micrograms of purified DNA was blunt ended and 5'-phosphorylated using the End-It DNA end repair kit (Epipcr), and 500 ng of the repaired DNA was self ligated at room temperature overnight with T4 DNA Ligase (Gibco) in a volume of 20 μl. The ligation was purified with the MinElute PCR purification kit (Qiagen) and transformed into electrocompetent E. coli EC100D pir−. Because GeneHunter transposons encode an Rok expression origin of replication, circularized DNA elements containing a transposon are sustained as low-copy-number (one to two per cell) plasmids in an E. coli strain expressing the pir gene product.

In vitro library construction. Forsmid libraries of the genomes of Salmonella enterica serovar Typhimurium LT2 and of B. cereus ATCC 10987 were prepared using the Copy-control forsmid construction kit (Epipcr) according to the manufacturer’s instructions. The Salmonella library contained 3,500 clones, and the B. cereus library contained 9,100 clones. Since each clone carries an average of 45 forsmid, the genome DNA, the Salmonella library provided 20-fold coverage, and the B. cereus library provided 75-fold coverage of the respective genomes.

The GeneHunter<kan> transposon was prepared from E. coli K2-grown pHGK, pGhK was digested with NdeI (New England Biolabs) to linearize the plasmid, and the reaction was purified with the QiaQuick PCR cleanup kit (Qiagen). The GeneHunter<kan> transposon was amplified from 620 pg of the digested plasmid in a 100-μl PPU Turbo (Stratagene) PCR using the primers FP4 (ATTCCAGGTCGCGCAACTGT) and RP4 (GTGACTGGAGCGGGAAGCGGAAG). The PCR product was digested with DpnI (to eliminate the template plasmid) and with PshAI (New England Biolabs), and the digest was purified using a MinElute PCR purification kit.

One hundred fourteen nanograms of transposon was combined with 1.6 μg of forsmid, 1 μl of EZ-TN transposesase buffer, and 1 μl of EZ-TN transposesase in a total volume of 10 μl. The reaction was incubated overnight at 37°C using a PCR Sprint thermal cycler (Thermo Hybaid) with the hot lid set to 94°C and terminated by adding 1 μl of EZ-TN Stop solution (Epipcr) and heating the reaction for 10 min at 70°C. The reaction was combined with 3 μl of 3 M sodium acetate (pH 4.6) and 1 μl of 20-mg/ml glycohe (Boehringer) and ethanol precipitated, and the DNA pellet was resuspended in 10 μl of buffer EB (Qiagen).

The entire volume of the purified reaction was added to 100 μl of electrocoment EP300 (Epipcr), and the cells were electrooporated in two equal aliquots and expressed in 4 ml of SOC broth. After expression, the transformed cells were transferred to 200 ml of L broth containing 12.5 μg of chloramphenicol/ml (to select for the forsmid-borne chloramphenicol resistance gene), and 10-fold serial dilutions of the culture were plated onto L-agar plates containing 12.5 μg of chloramphenicol/ml to determine the library size and onto L-agar plates containing 75 μg of kanamycin/ml to determine the fraction of the library that contained a GeneHunter transposon. The remainder of the culture was grown overnight at 37°C, and the following day, single-use aliquots were frozen at −80°C.

**RESULTS**

In vivo GeneHunter transposon. In several experiments, 15% of the chloramphenicol-resistant transformants carried intact plasmids and were therefore not the result of transposition. All attempts to decrease the fraction of plasmid-bearing transformants by digesting the plasmid with other enzymes in addition to PshAI failed; apparently, there is a small fraction of plasmid that is indigestible but is fully transformable. Accounting for the proportion of cells that contained undigested plasmid, a library of 5.1 × 105 individual GeneHunter transposon insertions was produced in S. enterica serovar Typhimurium LT2; i.e., an average of an insertion event every 9 bp.

The GeneHunter library was selected for increased resistance to 16 commonly used antibiotics from the β-lactam, tetracycline, fluoroquinolone, and aminoglycoside classes by plating aliquots equivalent to seven times the library size onto L agar containing 2× serial dilutions of the drug, starting at a concentration equal to that of clinical resistance to that antibiotic (14) and decreasing in concentration to the MIC for S. enterica serovar Typhimurium strain LT2 or E. coli strain EC100D. A similar number of wild-type cells were plated on the same series of antibiotic plates as a control. Resistance phenotypes could be distinguished on the basis of the sizes of colonies that grew at higher concentrations than did the wild-type controls. Approximately 12 colonies of each resistant phenotype were patched onto L-chloramphenicol plates for further study.

Crude genomic DNA was prepared by boiling the antibiotic-resistant colonies of interest. The presence of a GeneHunter transposon in those isolates was detected by analytical PCR with primers FP5 (CCCCGGGATCCTCTAGATGTCGCCG TAAGTTGGCAGCAGCATCACCAGCAGC) and RP5 (TA AAAGCTTTTAAAGCTTGGCCTAGGTTTTGAGA GAAGC) to amplify the chloramphenicol resistance determinant carried on pHGIC transposons. The presence of the GeneHunter plasmid was detected by repeating the PCRs with primers FP6 (GGTCTAGAGATTTCAACATTCGC TTGTCG) and RP6 (CCGAGCTCTTGTCGTGACCGT TACCAGC) to amplify the ampicillin resistance gene carried on that vector. Isolates found to yield amplicon with primers FP6 and RP6 were discarded, as were isolates that did not amplify with either primer pair. Colonies found to amplify with primers FP5 and RP5 only (i.e., those that contained the GeneHunter transposon but lacked the GeneHunter plasmid) were saved for further study.

To qualitatively distinguish resistance phenotypes, the resistance levels for isolates of interest were evaluated by the disk diffusion method.

Three classes of mutants were distinguished, two of which exhibited increased resistance to aminoglycosides and one of which exhibited increased resistance to fluoroquinolones and tetracyclines. One isolate of each class was chosen for identification of the gene responsible for the increased resistance.

To be sure that the resistance phenotype was the result of activating a cryptic gene and not the result of, for instance,
disrupting a porin gene, it is necessary to transfer the candidate antibiotic resistance gene into a separate strain, where it can then be assessed for an ability to confer resistance. To facilitate that process, the GeneHunter transposon encodes an R6Kγ origin of replication that allows a circularized DNA element containing the transposon to be sustained as a low-copy-number plasmid in an organism expressing the pir gene product. Mobilizing the putative resistance gene in this fashion serves both to demonstrate the potential for horizontal spreading of the gene and to facilitate sequencing of the gene.

Identification of activated genes. Self-replicating plasmid containing the GeneHunter transposon and approximately 3.3 kb of flanking sequence was prepared from each of the three isolates as described in Materials and Methods and transformed into strain EC100D pir'**. Four isolates that grew at the highest concentration of the relevant antibiotic (tobramycin for GHC1 and GHC2, tetracycline for GHC3) were chosen for further analysis.

Plasmid purified from each of those isolates was PCR amplified from both ends of the transposon with primers FP7 (GAGCCATATGGCGGAACACCCCGAGAA) and RP7 (GCTAAGCTACTCAGCTAGCACC). The shortest amplicon was sequenced using the ABI Prism BigDye Terminator cycle sequencing ready reaction kit (PE Applied Biosystems) with primers FP7 and RP7. The location of the insertion site within Salmonella's chromosome was determined by using the edited sequences as queries in a BLAST (1) search of the completed S. enterica serovar Typhimurium LT2 genome.

When self-ligated genomic DNA from the resistant LT2 isolates was transformed into pir** E. coli, the resistance phenotypes of the transformed E. coli were similar to the phenotypes of the LT2 strains from which the plasmids had been derived (Table 1). Plasmids pLT2GHCl and pLT2GHCl contained a GeneHunter transposon integrated upstream from gene aac(6')-Iaa and oriented in the direction of gene transcription (Table 2). The transposon present in pLT2GHCl was about 200 bp from the gene's start codon; however, the insertion present in pLT2GHCl2 was located more than 2 kb upstream from aac(6')-Iaa. In this case, there appears to be an inverse relationship between the amount of aminoglycoside resistance conferred and the distance from the GeneHunter-encoded promoter to the transcriptional start of aac(6')-Iaa, a phenomenon which may result from different levels of gene expression (Table 1). The phenotypes conferred by pLT2GHCl resulted from an insertion that activated the gene rma, a probable MarA regulon activator that was reported only several months prior to our experiments (Table 2) (18).

The activation and identification of aac(6')-Iaa from two individual GeneHunter transpositions and the additional activation of rma demonstrates that the GeneHunter system can activate and identify cryptic resistance genes.

In vitro results. The method described above is of limited practical use because high transformation efficiencies are required to produce libraries sufficiently large to ensure that the entire genome has been screened. We have been unable to obtain sufficiently high transformation efficiencies outside of E. coli and Salmonella. Practical application of GeneHunter as a tool for identifying novel resistance genes requires being able to screen a wide variety of pathogens and nonpathogens for the presence of resistance genes that might eventually be transferred to pathogens of interest. We therefore developed an in vitro transposition method that is applicable to any microorganism whose genes can be translated in E. coli.

The in vitro approach requires first constructing a BAC or fosmid library from the genome of interest and then transposing GeneHunter in vitro into that library to permit expression of resistance genes. Because BAC and fosmid vectors all carry a chloramphenicol resistance gene, it was necessary to construct a kanamycin variant of the GeneHunter transposon (Fig. 1B).

A fosmid library was constructed that provided 28-fold coverage of the Salmonella genome, and GeneHunter<kan> was inserted into the fosmid library to give an insertion library of 4.8 × 10⁶ inserts, sufficient to average an insertion every 11 bp. Because GeneHunter<kan> carries an aminoglycoside resistance gene, it was not possible to screen for activation of

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**TABLE 1. Minimum inhibitory concentrations for in vivo transposition mutants**

<table>
<thead>
<tr>
<th>Drug</th>
<th>S. enterica serovar Typhimurium LT2</th>
<th>LT2::GHC1</th>
<th>LT2::GHC2</th>
<th>E. coli EC100D pir**</th>
<th>pLT2GHCl</th>
<th>pLT2GHCl2</th>
<th>pLT2GHCl3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>32 4</td>
<td>64 8</td>
<td>8</td>
<td>256 16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tobramycin</td>
<td>8 4</td>
<td>64 16</td>
<td>2</td>
<td>128 64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kanamycin</td>
<td>25 4</td>
<td>32 8</td>
<td>8</td>
<td>128 32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamycin</td>
<td>8 2</td>
<td>4 1</td>
<td>2</td>
<td>2 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>4 0.016</td>
<td>0.031 0.00195</td>
<td>0.016</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>8 0.031</td>
<td>0.0625 0.0039</td>
<td>0.031</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>16 1</td>
<td>4 0.5</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td>16 2</td>
<td>4 0.5</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Clinical resistance as specified by the NCCLS (13). MICs greater than the clinical resistance level are indicated in boldface.

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**TABLE 2. Sites of in vivo GeneHunter insertions into S. enterica serovar Typhimurium LT2 genome**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Site of insertion</th>
<th>Gene activated</th>
<th>Gene coding sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT2::GHC1</td>
<td>1707244*</td>
<td>aac(6')-Iaa</td>
<td>1707358–1707795</td>
</tr>
<tr>
<td>LT2::GHC2</td>
<td>1705497</td>
<td>aac(6')-Iaa</td>
<td>1707358–1707795</td>
</tr>
<tr>
<td>LT2::GHC3</td>
<td>638964</td>
<td>rma</td>
<td>639016–639357</td>
</tr>
</tbody>
</table>

* Bases in Salmonella genome numbered according to reference 12.
DISCUSSION

The cryptic gene \textit{rmA} was originally identified in an antibiotic-resistant strain of \textit{S. enterica} serovar Paratyphi B where that gene had been activated (7). The cryptic aminoglycoside acetyltransferase \textit{aac(6')-Iy} from \textit{S. enterica}, a gene homologous to \textit{aac(6')-Iaa}, was identified in a similar fashion (11). In this project, we were able to experimentally activate and identify both \textit{aac(6')-Iaa} and \textit{rmA} in an antibiotic-sensitive strain of \textit{Salmonella} and the \textit{\beta}-lactamase II gene of \textit{B. cereus} without relying on chance to produce antibiotic-resistant mutants. Use of the GeneHunter system has allowed us to artificially activate cryptic genes in a tightly controlled environment where they can be readily characterized and sequenced.

The in vivo GeneHunter method has the advantage that it activates cryptic antibiotic resistance genes in situ, permitting a direct comparison between the wild-type strain and the mutant in which a single copy of the gene has been activated. On a practical basis, however, we will be most interested in resistance genes that can be horizontally transferred to other organisms. The presence of the \textit{R6K\gamma} origin of replication within the GeneHunter transposon facilitates evaluating the consequence of expression in another genetic background. The effectiveness of the method depends upon generating a sufficiently large library of insertions to be confident that all resident genes have been activated. We assume that an insertion within 50 bp upstream from a gene will usually be sufficient to promote transcription. Because half of the insertions will be such that the promoter will transcribe away from a target gene, that means that twice as many insertions, or an average of an insertion every 25 bp, are required. Given a typical genome size of about five million base pairs, a library of about 2 $\times$ 10\textsuperscript{5} insertions is required. Achieving libraries of that size requires transformation efficiencies of >10\textsuperscript{10} transformants per \(\mu\)g, an efficiency that we have been unable to achieve outside of the family \textit{Enterobacteriaceae}.

The in vitro method has the enormous advantage of being able to identify cryptic antibiotic resistance genes in both gram-positive and gram-negative organisms. Because the activated genes are detected on the basis of their expression in \textit{E. coli}, only those genes that can confer resistance in members of the family \textit{Enterobacteriaceae} are detected. Because the target genes are expressed under the control of the powerful \textit{pTAC} promoter, the only genes that should escape detection are those that require translational signals or posttranslational modifications that are not available in \textit{E. coli} or whose expression is significantly deleterious to \textit{E. coli}.

The \textit{R6K\gamma ori} in the GeneHunter transposon can facilitate sequencing if direct sequencing from a fosmid proves difficult. Digestion of the fosmid by one of several restriction endonucleases, followed by self-ligation and transformation into \textit{E. coli} EC100D \textit{pir}\textsuperscript{+}, permits selection for both the transposon-borne kanamycin resistance gene and resistance to the drug of interest. The resulting plasmid will confer resistance to the drug of interest and to kanamycin but not to chloramphenicol. Transferring the plasmid to \textit{E. coli} strain EC100D \textit{pir}-116 (Epicentre), a strain that permits the \textit{R6K\gamma ori} to replicate at up to 250 copies per cell, facilitates preparation of sufficient quantities of plasmid for sequencing.

All techniques have limitations, and the GeneHunter tech-

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**TABLE 3. Minimum inhibitory concentrations for in vitro transposition mutants**

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC ((\mu)g/ml)</th>
<th>Clinical resistance\textsuperscript{a}</th>
<th>S. typhimurium fosmid library</th>
<th>fSaltyGHK1</th>
<th>fSaltyGHK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>4</td>
<td>0.01563</td>
<td>0.03125</td>
<td>0.03125</td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>8</td>
<td>0.03125</td>
<td>0.0625</td>
<td>0.0625</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>16</td>
<td>2</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Dosycycline</td>
<td>16</td>
<td>1</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>32</td>
<td>4</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>32</td>
<td>0.125</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>64</td>
<td>0.125</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cefturoxime</td>
<td>32</td>
<td>16</td>
<td>64</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>32</td>
<td>16</td>
<td>128</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Cefepime</td>
<td>32</td>
<td>0.125</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Clinical resistance as defined by the NCCLS (13). MICs greater than the clinical resistance level are indicated in boldface.

\textit{aac(6')-Iaa}. Instead, the library was screened for activation of \textit{rmA} by plating both the original fosmid library and the insertion library onto serial dilutions of tetracycline agar. The non-insertion fosmid library is the proper control for the selection because we are interested only in genes that are being activated by the GeneHunter transposon. Variants within the insertion library were found that grew at higher tetracycline concentrations than did the original fosmid library. Two such isolates were chosen for further study. Table 3 shows that following retransformation into naïve EPI300 cells, the two variants exhibited increased resistance to 6 of the 10 drugs tested and that they exhibited clinical resistance to 2 of those drugs, cefuroxime and cefoxitin.

Sequencing of the fosmids fSaltyGHK1 and fSaltyGHK2 showed that the insertions were 75 and 118 bp upstream from \textit{rmA}, respectively.

To demonstrate that in vitro GeneHunter transposition would work with nonenterics, we constructed a fosmid library that provided 73-fold coverage of the \textit{B. cereus} genome and, from that, a GeneHunter<\textit{kan}> library of 6.7 $\times$ 10\textsuperscript{5} insertions, sufficient to average an insertion every 8 bp. Both the original fosmid library and the insertion library were plated onto serial dilutions of the carbapenem \textit{\beta}-lactam antibiotic imipenem. Seven isolates were recovered that grew at higher imipenem concentrations than did the original fosmid library. Disk diffusion tests were used to compare the imipenem resistance of the seven isolates with that of the original fosmid library. For five samples of the fosmid library, the inhibition zone was 28.8 $\pm$ 0.2 mm (mean $\pm$ standard error). For five samples of each of the seven isolates, fBacerGHK1 to fBacerGHK7, the diameters were 14.1 $\pm$ 0.6, 15.7 $\pm$ 0.2, 14.6 $\pm$ 0.2, 16 $\pm$ 0.3, 15.2 $\pm$ 0.4, 15.4 $\pm$ 0.2, and 15.6 $\pm$ 0.2 mm.

The sequence downstream from the outward-reading promoter of the GHK transposons in fosmid fBacerGHK5 was determined by sequencing with primer FP7. A BLAST search of the \textit{B. cereus} strain ATCC 14579 genome (10) showed that GeneHunter<\textit{kan}> had inserted 231 bp upstream from the metallo-\textit{\beta}-lactamase gene, designated \textit{\beta}-lactamase II (locus BC3440).
GeneHunter depends upon expression of heterologous genes in E. coli. Genes that cannot be expressed from their own promoters in E. coli and are located just downstream from transcription terminators may not be detected, because transcription from the pTAC promoter will usually be terminated before the gene is transcribed. Any gene that cannot be effectively transcribed from the pTAC promoter in E. coli will not be detected. Even when transcription is effective, translational barriers or the failure to carry out post-translational modifications may prevent a cryptic resistance gene from conferring improved resistance in E. coli. Such barriers to heterologous gene expression are likely to be most important in high-GC soil microorganisms, in particular antibiotic producers. It may be possible to develop tools similar to GeneHunter specifically for such organisms. Another limitation is that GeneHunter cannot be used to search for resistance genes for drugs, such as vancomycin, to which E. coli is naturally resistant. Because the GeneHunter<sup>Kan</sup> transposon confers resistance to some aminoglycosides, it is not suitable for screening for aminoglycoside resistance. That transposon could, however, be modified by replacing the kanamycin resistance gene with, for instance, the CMY-2 ampC gene for resistance to cephalosporins. Despite those limitations, our results suggest that GeneHunter is widely, if not universally, applicable.

GeneHunter will permit microbial genomes to be easily screened for the presence of genes that have the potential to confer resistance to an antibiotic of interest. The presence of silent resistance genes in the genomes of antibiotic-sensitive bacteria is sufficiently common that we cannot afford to ignore this source of novel resistance determinants. In addition to the previously mentioned examples, it appears that the metallo-β-lactamase gene in B. cereus ATCC10987 is cryptic. We hope that use of the GeneHunter system will become an integral component in the development and testing of novel antibiotics, new variants of existing antibiotics, and drug-inhibitor combinations. The obvious targets for screening are the pathogens against which drugs are to be used. However, we believe that soil microorganisms and commensal microorganisms may function as equally important reservoirs of antibiotic resistance and that those groups should be included in screening programs.

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REFERENCES