

## Genes Involved in Intrinsic Antibiotic Resistance of *Acinetobacter baylyi*<sup>∇†</sup>

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Received 10 May 2006/Returned for modification 13 July 2006/Accepted 10 August 2006

**Bacterial genes defining intrinsic resistance to antibiotics encode proteins that can be targeted by antibiotic potentiators. To find such genes, a transposon insertion library of *Acinetobacter baylyi* was screened with subinhibitory concentrations of various antibiotics to find supersusceptible mutants. A DNA microarray printer was used to replica plate 10,000 individual library clones to select mutants unable to grow at 1/10 the MICs of 12 different antibiotics. Transposon insertions in 11 genes were found to cause an eightfold or higher hypersusceptibility to at least one antibiotic. Most of the mutants identified exhibited hypersusceptibility to  $\beta$ -lactam antibiotics. These included mutants with disruptions of genes encoding proteins involved in efflux (*acrB* and *oprM*) as well as genes pertaining to peptidoglycan synthesis and modification (*ampD*, *mpl*, and *pbpG*). However, disruptions of genes encoding proteins with seemingly unrelated functions (*gph*, *argH*, *hisF*, and *ACIAD0795*) can also render cells hypersusceptible to  $\beta$ -lactam antibiotics. A knockout of *gshA*, involved in glutathione biosynthesis, enhanced the susceptibility to metronidazole, while a knockout of *recD*, involved in recombination and repair, made the bacteria hypersusceptible to ciprofloxacin. Disruption of *acrB* in *Escherichia coli* rendered the cells hypersusceptible to several antibiotics. However, knockout mutants of other homologous genes in *E. coli* showed no significant changes in antibiotic MICs, indicating that the intrinsic resistance genes are species specific.**

The growing problem of antibiotic resistance among bacterial pathogens and the escalating difficulty in finding new antibiotics drive the search for new approaches to antibacterial chemotherapy. One such approach is the development of antibiotic potentiators that can enhance antibiotic efficiency when the antibiotics and the potentiators are used in combination, as well as reduce the chances of the emergence of antibiotic resistance.

Currently, potentiators are available for only one type of antibiotics,  $\beta$ -lactams. The  $\beta$ -lactamase inhibitors currently used in clinics, clavulanate, sulbactam, and tazobactam, have dramatically enhanced the efficacies of important  $\beta$ -lactam antibiotics (6). Other potentiators are being developed, for example, those that target the multidrug resistance efflux pumps (14). However, to date, there are no such potentiators in clinical use.

A number of nonessential bacterial proteins may potentially contribute to the intrinsic antibiotic resistance. The susceptibilities of bacteria to antibiotics depend on many factors, including the structure and the composition of the cell envelope, the presence of inactivating enzymes, and the availability of efflux pumps. The corresponding genes may be present in the genome either because they were selected in the course of evolution to help the organism tolerate antibiotics that it may encounter in the environment or because the encoded enzymes, which have specialized cellular functions, may fortuitously contribute to antibiotic resistance. Conceivably,

inactivation of such enzymes by inhibitors may increase the potencies of the antibiotics currently in medical use.

In the present study, we used a genetic approach to identify the putative targets of such antibiotic potentiators. We generated a random transposon gene-knockout library of *Acinetobacter baylyi*, a close relative of the opportunistic pathogen *Acinetobacter baumannii*, and selected mutants for the inability to grow at a subinhibitory antibiotic concentration. To identify the *A. baylyi* genes contributing to the intrinsic resistance to antibiotics, 10,000 bacterial clones carrying random transposon insertions were replica plated in the presence of subinhibitory concentrations of 12 different antibiotics. This otherwise laborious task was facilitated by the use of a microarray-printing robot to “print” the clones for replica plating. Hypersusceptible mutants were identified, and the genes whose disruption increases cell susceptibility to antibiotics were determined by direct genomic DNA sequencing. If a disruption of a certain gene leads to antibiotic hypersusceptibility, inhibition of the encoded protein product is likely to have the same effect. Therefore, an inhibitor can potentially be designed that, when used in combination with the corresponding antibiotic, will enhance its efficacy.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Acinetobacter baylyi* strain ADP1 was from the American Type Culture Collection (ATCC 33305). *Escherichia coli* strain BW25113 gene deletion mutants were obtained from H. Mori, Nara Institute of Science and Technology, Japan (1d). Both *A. baylyi* and *E. coli* were grown either in liquid cultures in Luria-Bertani (LB) medium or on LB agar plates at 37°C. When appropriate, overnight cultures were supplemented with kanamycin (KAN; 12.5  $\mu$ g/ml), spectinomycin (50  $\mu$ g/ml), and streptomycin (10  $\mu$ g/ml) for *A. baylyi* or with KAN (30  $\mu$ g/ml) for *E. coli*.

**Antibiotics and reagents.** Chloramphenicol, gentamicin, KAN, metronidazole, piperacillin, rifampin, streptomycin, spectinomycin, and vancomycin were from Sigma; ampicillin and tetracycline were from Fisher; azithromycin was from Pfizer; ceftazidime and imipenem (with cilastatin) were from GlaxoWellcome;

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† This paper is dedicated to the memory of Alexander A. Neyfakh, deceased 20 April 2006.

<sup>∇</sup> Published ahead of print on 28 August 2006.

and ciprofloxacin was from Bayer. All enzymes except DpnI were from Fermentas; DpnI was from Promega.

**Determination of MICs.** Logarithmically growing cells were diluted in LB medium to an optical density at 600 nm of 0.002, and 100  $\mu$ l was placed in the wells of 96-well plates. Antibiotics were added in twofold dilutions, and the bacteria were grown overnight. The MIC was determined as the lowest antibiotic concentration at which no visible growth occurred.

**Construction of the transposome insertion library.** A transposome  $\Omega$  cassette (22) was used to create a random transposon insertion library of *A. baylyi*, as described previously (11). About 30,000 colonies were obtained on spectinomycin-streptomycin agar plates. A total of 10,000 colonies were picked, individually inoculated into 96-well plates, grown overnight, and stored at  $-80^{\circ}\text{C}$  with 15% glycerol.

**Selection of hypersusceptible clones of *A. baylyi*.** Cells were inoculated into 100  $\mu$ l of LB medium in 96-well plates and grown overnight. Cultures were diluted 10-fold into fresh LB medium and grown for 4 h with shaking at 700 rpm by using a Brinkmann TiterMix100 microtiter plate shaker. The cells were then diluted 20-fold in 50  $\mu$ l LB broth supplemented with 20% sucrose to prevent drying of the bacterial culture spots during printing. Submicroliter volumes of the cultures were then spotted (in duplicate) on the surfaces of 13 nylon membranes (GE Osmonics) (768 spots per 25-mm by 75-mm membrane with a printing area of 20 mm by 60 mm and a 1-mm distance between spots). Spotting was done by using a LabNext DNA microarray printer. Four pins of the microarray printer, each 0.2 mm in diameter, were dipped into the bacterial cultures; and extra fluid was removed by prespotting onto the first nylon membrane. Replicas of 384 clones were then printed in duplicate on 13 nylon membranes. Printer pins were sterilized by two rounds of dipping into 40% ethanol, followed by air drying. When the printing job was complete, one membrane (a control) was layered on top of an agar plate without antibiotic; the rest of the membranes were placed atop agar plates containing subinhibitory concentrations (1/10 the wild-type MIC) of one of the antibiotics: azithromycin, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, imipenem, metronidazole, piperacillin, rifampin, or tetracycline. The membranes were also placed on LB agar plates containing ampicillin (3  $\mu\text{g}/\text{ml}$ ) or vancomycin (10  $\mu\text{g}/\text{ml}$ ). After 16 to 18 h of incubation at  $37^{\circ}\text{C}$ , the colonies on the filters were analyzed, and clones that grew on the control plate but failed to grow on plates containing antibiotics at subinhibitory concentrations were identified. For such clones, the MICs of the antibiotics were then determined in liquid cultures.

In order to determine the site of the transposon insertion in antibiotic-hypersusceptible clones, genomic DNA was isolated from 3-ml overnight cultures by using a genomic DNA isolation kit (Sigma). Sequencing of the genomic DNA segment adjacent to the site of the transposon insertion was performed by using an outwards-directed transposon-specific primer (5' AGAGTCGACCTGCAG GCATGC) and the Promega *f*mol DNA cycle sequencing system. The disrupted gene was identified by using the genome sequence of *A. baylyi* strain ADP1 (NCBI accession number CR543861) (2).

To validate the causative relation between the transposon insertion and the hypersusceptible phenotype, total DNA was prepared from the hypersusceptible mutants that were identified and was used to directly transform logarithmically growing wild-type *A. baylyi* (20). Transformants were selected on a spectinomycin-streptomycin agar plate. Individual colonies were picked, the location of the transposon insertion was verified by PCR, and the antibiotic MICs were determined.

**Targeted inactivation of *A. baylyi* genes.** Selected *A. baylyi* genes were disrupted by using the method of chromosomal gene replacement (17). A PCR product containing a kanamycin resistance marker, Tn903, which was amplified from the KAN-2 transposon (Epicenter), flanked by the 600-bp-long upstream and downstream regions of the gene of interest was prepared by consecutive rounds of PCR. First, the kanamycin resistance marker and 300- to 500-bp-long regions flanking the gene of interest were amplified by PCR. During PCR, the NotI and SdaI restriction sites were introduced at the appropriate ends of the upstream and downstream gene-flanking regions of the PCR-amplified kanamycin resistance gene. The PCR products were cut with the restriction enzymes NotI and SdaI, ligated together, treated with DpnI, and then used as the template for the second round of PCR with primers specific to the distal ends of the gene-flanking regions. The PCR products were purified by using a Wizard SV Gel and PCR cleanup system (Promega). In some cases, the second round of PCR produced several bands. In this case, the correct product was extracted from the gel and purified by using a QIAquick gel extraction kit (QIAGEN) or a Wizard SV Gel and PCR cleanup system (Promega). The resulting PCR product was used to transform exponentially growing *A. baylyi* cells, and transformants were selected on LB medium-KAN plates. The disruption of the appropriate gene in the selected transformants was verified by PCR. The MICs for the

generated mutants were determined and compared with the MICs for the original transposon-disrupted clone and wild type.

**Identification of *E. coli* genes homologous to *A. baylyi* genes.** Eleven *A. baylyi* genes were used as queries in a BLAST search of the *E. coli* MG1655 genome (NCBI accession number U00096) (4). The top hits were then used as queries in a BLAST search of the *A. baylyi* ADP1 genome (NCBI accession number CR543861) (2). The antibiotic MICs for *E. coli* strains from the Keio collection (1a) were determined as described above for *A. baylyi*. The MICs of *E. coli* wild-type strain BW25113 were as follows: ampicillin, 100  $\mu\text{g}/\text{ml}$ ; azithromycin, 12.5  $\mu\text{g}/\text{ml}$ ; ceftazidime, 0.3  $\mu\text{g}/\text{ml}$ ; chloramphenicol, 8  $\mu\text{g}/\text{ml}$ ; ciprofloxacin, 0.06  $\mu\text{g}/\text{ml}$ ; gentamicin, 10  $\mu\text{g}/\text{ml}$ ; imipenem, 1.3  $\mu\text{g}/\text{ml}$ ; metronidazole, 4,000  $\mu\text{g}/\text{ml}$ ; piperacillin, 2.5  $\mu\text{g}/\text{ml}$ ; rifampin, 1  $\mu\text{g}/\text{ml}$ ; tetracycline, 4  $\mu\text{g}/\text{ml}$ ; and vancomycin, 325  $\mu\text{g}/\text{ml}$ .

## RESULTS

**Selection and validation of antibiotic-hypersusceptible mutants of *A. baylyi*.** Previously we described an approach that allows the selection of antibiotic-hypersusceptible gene-knockout mutants of *A. baylyi* (11), based on the release of DNA from the cells lysed in the presence of subinhibitory concentrations of antibiotics. Although this method identified several genes of interest, its intrinsic limitations left open the possibility that many interesting mutations have not been detected. A more straightforward approach for identification of the genes contributing to the intrinsic resistance of bacteria to antibiotics is the selection of hypersusceptible mutants in a gene-knockout library by direct replica plating of individual mutants on plates containing subinhibitory antibiotic concentrations. In order to accomplish this inherently laborious task, especially when thousands of mutants are to be screened against a number of antibiotics, we applied a variant of the clone microarray printing technique (26). This technique allowed the replication of arrays of clones of the *A. baylyi* ADP1 transposon library for the simultaneous screening for mutants susceptible to low concentrations of various antibiotics (11). The drugs that were used in our screen included the cell wall synthesis inhibitors ampicillin, imipenem, piperacillin, ceftazidime, and vancomycin; the protein synthesis inhibitors azithromycin, chloramphenicol, gentamicin, and tetracycline; the transcription inhibitor rifampin; and the DNA synthesis inhibitor metronidazole.

After the initial screening of 10,000 clones, we identified 29 clones that exhibited diminished or no growth on plates with low concentrations (1/10 the MICs) of ampicillin, ceftazidime, piperacillin, vancomycin, ciprofloxacin, imipenem, rifampin, and metronidazole. These clones showed at least an eightfold reduction in MICs for at least one antibiotic compared to that for the wild type. (We were unable to find mutants with MIC reductions of eightfold or greater for the translation inhibitors azithromycin, chloramphenicol, gentamicin, and tetracycline). Sequencing of the regions flanking the transposon insertions, performed directly with the genomic DNA prepared from these clones, revealed the identities of the disrupted genes. Independent transpositions were found in 22 distinct genes, with some clones having disruptions within the same gene.

Since *A. baylyi* has a very high natural competence for transformation and recombination (2), we were able to easily transfer transposon-mediated gene disruptions into the fresh wild-type bacteria to verify whether the hypersusceptibility phenotype was the direct result of the disruption of the iden-

TABLE 1. Antibiotic susceptibilities of the gene disruption mutants of *Acinetobacter baylyi* ADP1 mutants

Gene product	Gene	Gene identifier <sup>a</sup>	MIC (μg/ml) <sup>b</sup>											
			AMP	PIP	CAZ	IPM	VAN	AZM	CHL	GEN	TET	RIF	CIP	MTZ
	Wild type		128.0	10.0	5.0	0.20	160.0	0.3	4.0	1.3	1.0	0.6	0.1	2000
Acriflavin resistance protein B	<i>acrB::kan</i>	2879610	<b>8.0</b>	<b>0.3</b>	<b>0.3</b>	0.10	160.0	0.2	1.0	1.3	<b>0.1</b>	0.3	<b>0.006</b>	2000
Outer membrane protein OprM	<i>oprM::kan</i>	2879678	<b>8.0</b>	<b>0.6</b>	<b>0.6</b>	0.05	160.0	0.2	2.0	1.3	0.3	0.3	<b>0.013</b>	2000
Anhydro- <i>N</i> -acetylmuramyl tripeptide amidase	<i>ampD::kan</i>	2880355	<b>4.0</b>	<b>0.6</b>	<b>0.3</b>	<b>0.01</b>	80.0	0.3	4.0	0.7	1.0	0.3	0.025	2000
UDP-MurNAc-L-Ala-D-Glu- <i>meso</i> -diaminopimelate ligase	<i>mpl::kan</i>	2878498	<b>8.0</b>	<b>1.3</b>	<b>0.6</b>	0.05	160.0	0.3	4.0	1.3	1.0	0.6	0.100	2000
D-Alanyl-D-alanine endopeptidase	<i>pbpG::kan</i>	2879431	<b>8.0</b>	<b>0.6</b>	<b>0.6</b>	0.10	40.0	0.1	2.0	0.7	0.5	<b>0.08</b>	0.050	1000
Argininosuccinate lyase	<i>argH::kan</i>	2877980	<b>16.0</b>	2.5	2.5	0.05	160.0	0.2	4.0	0.7	1.0	0.2	0.100	2000
Imidazole glycerol phosphate synthase	<i>hisF::kan</i>	2878927	<b>16.0</b>	<b>1.3</b>	1.3	<b>0.03</b>	80.0	0.2	4.0	0.7	1.0	0.6	0.025	2000
Unknown	ACIAD0795:: <i>kan</i>	2877853	<b>8.0</b>	<b>0.6</b>	2.5	0.20	160.0	0.2	4.0	1.3	1.0	0.3	0.100	2000
Phosphoglycolate phosphatase	<i>gph::kan</i>	2881034	<b>8.0</b>	2.5	1.3	<b>0.03</b>	160.0	0.3	4.0	0.7	1.0	0.3	0.050	2000
Glutamate cysteine ligase	<i>gshA::kan</i>	2879245	128.0	10.0	5.0	0.20	80.0	0.3	4.0	1.3	1.0	0.3	0.100	<b>250</b>
Exodeoxyribonuclease V α chain	<i>recD::kan</i>	2879479	64.0	10.0	5.0	0.10	80.0	0.3	2.0	1.3	1.0	0.6	<b>0.013</b>	1000

<sup>a</sup> Gene identifiers are from the complete *Acinetobacter baylyi* genome (NCBI accession number CR543861).

<sup>b</sup> MICs with eightfold or higher decreases are shown in boldface.

tified gene. From the 22 gene disruptions, 11 withstood this test: *acrB*, *oprM*, *ampD*, *mpl*, *pbpG*, *argH*, *hisF*, *gph*, ACIAD0795, *gshA*, and *recD* (Table 1). We further constructed 11 strains in which these genes were completely deleted and replaced by a kanamycin resistance marker (see Materials and Methods). MIC determination for the deletion mutants showed levels of antibiotic hypersusceptibility similar to those obtained with the transposon disruption mutants. In order to exclude possible polar effects of chromosomal gene replacement, we performed deletions of the genes located immediately downstream from several of the identified genes (Fig. 1). We used the gene replacement technique in an attempt to inactivate the genes ACIAD0042, *dsbB*, ACIAD2946, ACIAD3665, ACIAD0064, and ACIAD0282, located downstream from *gph*, *gshA*, *oprM*, *mpl*, *ampD*, and *argH*, respectively (the genes *recD*, *pbpG*, and ACIAD0795 had no downstream protein genes belonging to the same operon). While we were not able to obtain the knockout mutants for ACIAD0064 and ACIAD0282, the *A. baylyi* mutants lacking ACIAD0042, *dsbB*, ACIAD2946, and ACIAD3665 showed no changes in susceptibility compared to that of the wild type. This result indicated that the hypersusceptibilities of the initial mutants were likely the direct result of the disruption of the corresponding genes rather than a polar effect of the transposon insertion. Altogether, these experiments led us to conclude that inactivation of the 11 genes identified in *A. baylyi* ADP1 (*acrB*, *oprM*, *ampD*, *mpl*, *pbpG*, *argH*, *hisF*, *gph*, ACIAD0795, *gshA*, and *recD*) led to the antibiotic hypersusceptibility phenotypes.

#### Effects of gene disruptions in *E. coli* on antibiotic sensitivity.

In order to test whether the intrinsic resistance genes discovered in *A. baylyi* play similar roles in other gram-negative bacteria, we investigated the antibiotic sensitivities of *E. coli* strains in which the homologs of these genes were deleted. The *E. coli* homologs of the *A. baylyi* genes were identified by a reciprocal BLAST search (24). First, the highest-scoring *E. coli* homolog was identified for each of the 11 *A. baylyi* intrinsic

resistance genes. Then, each of the highest-scoring *E. coli* genes was used as a query in the BLAST search of the *A. baylyi* genome. For 10 of the 11 genes (with the exception of ACIAD0795), the reciprocal BLAST search consistently revealed the homologous genes in *A. baylyi* and *E. coli*. The similarity scores for the homologous genes were 46% or higher for the aligned sections of the *E. coli* and *A. baylyi* proteins (Table 2). Of these 10 genes, all but 1 (*oprM*) showed the highest similarity to the expected functional homologs in the *E. coli* genome. *E. coli* lacks a homolog of the *oprM* gene (which encodes a component of the tripartite efflux pump in *Pseudomonas aeruginosa* and several other bacteria) (13). The putative *E. coli* functional equivalent of *oprM* is *tolC*, which encodes a component of the *acrAB-tolC* tripartite efflux pump whose inactivation is known to lead to antibiotic hypersusceptibility (reviewed in reference 12). However, *E. coli tolC* shows only limited similarity to *A. baylyi oprM*. The *E. coli* gene showing the highest degree of similarity (55%) to the *A. baylyi oprM* gene was *cusC*, which encodes an outer membrane component of the copper efflux system (7). Therefore, *E. coli cusC* was targeted for disruption. Of the 11 genes whose disruption makes *A. baylyi* hypersensitive to antibiotics, only 1, ACIAD0795, did not have a clear homolog in *E. coli*. A 48-amino-acid segment of this putative 167-amino-acid *A. baylyi* protein showed homology to the conserved domains of the YbiS and ErfK proteins of *E. coli*, whose functions are unknown but which may be related to the outer membrane porins and the stress response, respectively (3, 5). However, the reciprocal BLAST search with either YbiS or ErfK against the *A. baylyi* genome showed that these proteins have the highest homology to another *A. baylyi* gene, ACIAD2475. Nevertheless, we did investigate the effects of *ybiS* and *erfK* deletions on the sensitivity of *E. coli* to antibiotics.

The *E. coli* deletion mutants (*acrB*, *cusC*, *mpl*, *ampD*, *pbpG*, *argH*, *hisF*, *gph*, *gshA*, *recD*, *ybiS*, and *erfK*) from the Keio collection (1a), which is composed of *E. coli* clones in which all nonessential genes are replaced by a kanamycin

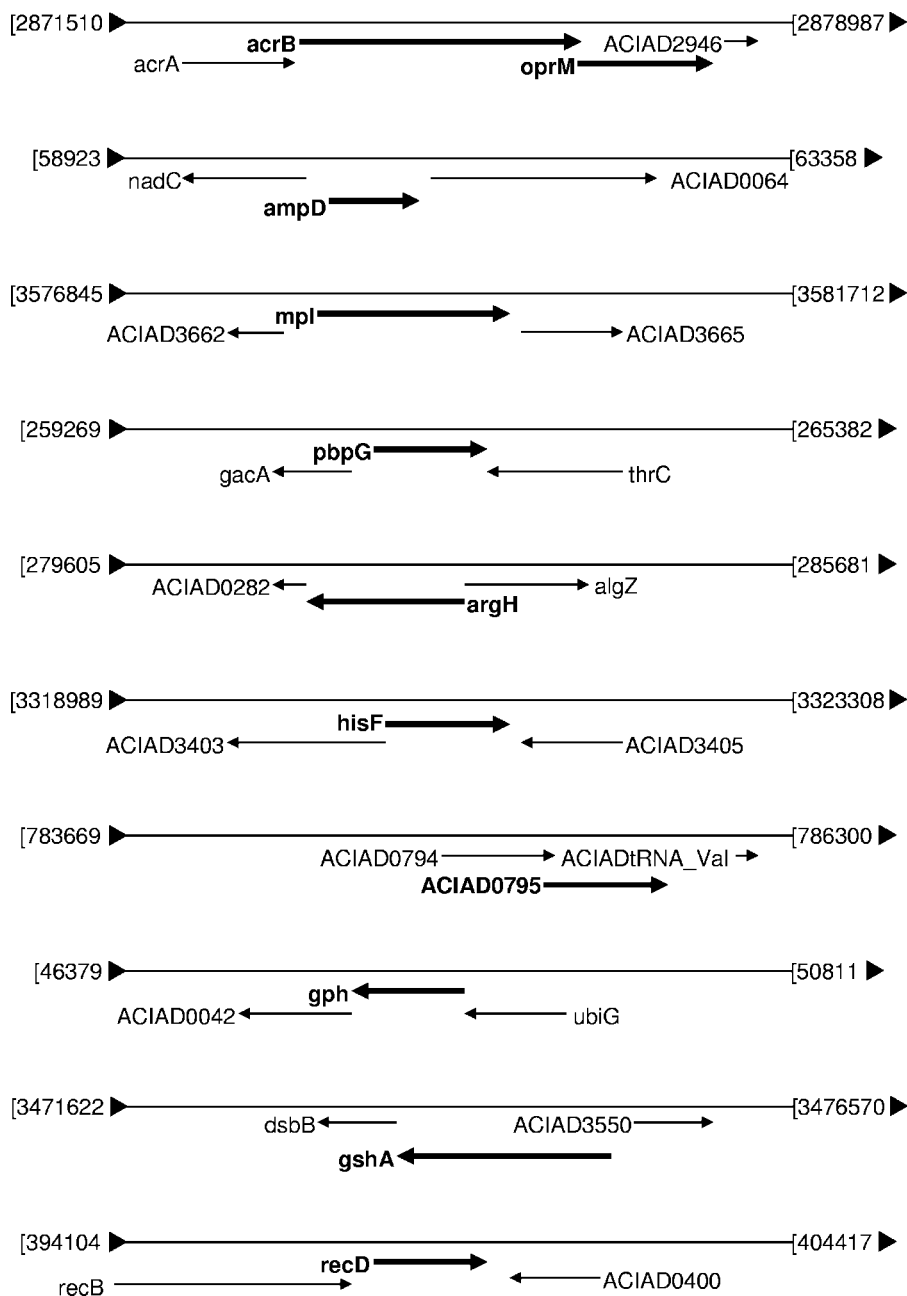


FIG. 1. Gene maps of the *Acinetobacter* ADP1 chromosome in the vicinity of genes whose inactivation renders cells hypersusceptible to antibiotics. Numbers refer to positions in the complete sequence of the *A. baylyi* ADP1 genome, and ACIAD numbers correspond to the genome annotation (NCBI accession number CR543861) (2).

resistance marker, were used for antibiotic sensitivity testing. The disruption of the *acrB* gene in *E. coli* rendered the cells hypersusceptible to several antibiotics: ampicillin (MICs, 12.5 µg/ml versus 100 µg/ml for the wild type), piperacillin (MICs, 0.1 µg/ml versus 2.5 µg/ml for the wild type), chloramphenicol (MICs, 1 µg/ml versus 8 µg/ml for the wild type), and azithromycin (MICs, 0.8 µg/ml versus 12.5 µg/ml for the wild type). Surprisingly however, no significant changes in the MICs for any of the other strains tested were observed, indicating that the newly identified potentiator targets may be specific to *A. baylyi*.

### DISCUSSION

Our goal in this work was to develop a strategy for identifying new targets for antibiotic potentiators. Using a straightforward replica-plating approach, we succeeded in identifying genes whose disruption renders *A. baylyi* hypersusceptible to several clinical antibiotics. The corresponding gene products could be considered putative targets for antibiotic potentiators. In these studies, we used *A. baylyi* ADP1 as a model organism. The very high degree of competence of *A. baylyi* greatly facilitates the transfer of the identified transposon insertion muta-



TABLE 2. Homology between homologous portions of the proteins in *A. baylyi* and *E. coli* as identified by BLAST search

<i>A. baylyi</i> (length [no. of amino acids])	<i>E. coli</i> (length [no. of amino acids])	% Similarity	Length (amino acids) of the homologous region <sup>a</sup>
AcrB (1,059)	AcrB (1,049)	72	1,062
OprM (484)	CusC (457)	55	434
AmpD (196)	AmpD (183)	66	181
Mpl (453)	Mpl (457)	68	452
PbpG (352)	PbpG (310)	63	248
ArgH (477)	ArgH (457)	61	455
HisF (269)	HisF (258)	61	257
ACIAD0795 (167)	YbiS (306)	65	38
ACIAD0795 (167)	ErfK (310)	58	48
Gph (235)	Gph (252)	46	232
GshA (530)	GshA (518)	57	476
RecD (592)	RecD (608)	50	526

<sup>a</sup> The total length of the homologous region, including gaps introduced for the optimal alignment.

tions into wild-type cells and the subsequent preparation of strains with chromosomal replacement of the identified genes. In general, however, our approach can be applied to any microorganism for which a gene-knockout library is available. The task of identifying the intrinsic antibiotic resistance genes was significantly aided by the use of a DNA microarray printer, which allowed the fast and easy replica plating of 10,000 individual strains on plates with 12 different antibiotics.

Eleven genes in *A. baylyi* whose disruption leads to antibiotic hypersusceptibility were identified. Among these genes was the homolog of *acrB*, which encodes a component of the multidrug resistance pump in *E. coli* (19). Disruption of such pumps in other bacteria is known to confer multidrug resistance (12). Some of the previous studies also indicated that mutations in the *rec* genes may increase cell sensitivity to quinolones (18, 25). This result validated our approach and demonstrated that the colony-printing technique can be used effectively to detect mutants hypersusceptible to antibiotics. To the best of our knowledge, the other genes that we identified were not previously described as direct contributors to the intrinsic antibiotic resistance and thus were not previously considered targets for putative antibiotic potentiators.

Several of the genes identified provide intrinsic resistance to one or more  $\beta$ -lactam antibiotics (Table 1). In three cases, *ampD*, *mpl*, and *pbpG*, this conclusion is in generally good agreement with the known functions of the corresponding gene products, since they are involved in biosynthesis or recycling of peptidoglycan and thus belong to the same biochemical pathway that is targeted by  $\beta$ -lactam antibiotics. AmpD is involved in the breakdown of the anhydromuropeptides produced upon degradation of old peptidoglycan (9, 10), whereas Mpl links the tripeptide L-alanyl- $\gamma$ -D-glutamyl-meso-diaminopimelate, released by AmpD, to one of the main precursors of peptidoglycan synthesis, UDP-N-acetylmuramate (16). Gene *pbpG* encodes two low-molecular-weight penicillin-binding proteins (PBPs), PBPs 7 and 8, in *E. coli*, which play a role in the remodeling of peptidoglycan (8, 23). However, beyond the general observation that the protein products identified are involved in the biochemical pathway affected by the antibiotic, it is hard to explain why disruptions of these particular genes

and not of multiple others involved in the same pathway render cells hypersusceptible to the drugs. This uncertainty underscores the importance of experimental approaches, similar to the one described in this paper, for identification of putative targets for antibiotic potentiators.

Four other genes, *argH*, *hisF*, *gph*, and ACIAD0795, whose knockout increases the susceptibility of *A. baylyi* to  $\beta$ -lactam antibiotics, encode proteins with functions seemingly unrelated to the biosynthesis of the bacterial cell wall. ArgH and HisF are involved in the biosynthesis of arginine and histidine, respectively, while *gph* encodes the housekeeping enzyme 2-phosphoglycolate phosphatase, which is induced during oxidative stress (21). The presumed function of Gph is to metabolize the 2-phosphoglycolate produced in the repair of DNA lesions (21). A hypothetical 167-amino-acid protein is encoded by an *A. baylyi* gene, ACIAD0795, that contains a domain that exhibits similarity to the conserved domain of the *erfK* family of proteins in *E. coli*, whose function is obscure. At the moment, it is unclear why disruption of any of these four genes in *A. baylyi* causes hypersusceptibility to  $\beta$ -lactam antibiotics.

Finally, genetic knockouts of *gshA* and *recD* increased the sensitivity of *A. baylyi* to metronidazole and ciprofloxacin, respectively. Metronidazole is a prodrug which, upon activation, forms highly active radical species that cause DNA damage (15). Therefore, it makes sense that the disruption of *gshA*, the gene whose product is involved in the biosynthesis of the reducing agent glutathione, increased the sensitivity of *A. baylyi* to metronidazole. Similarly, it is not surprising that the genetic knockout of *recD*, a component of the *recBCD* complex that plays a major role in DNA repair and recombination (1), increases bacterial sensitivity to the DNA-damaging drug ciprofloxacin. Nevertheless, as mentioned earlier, without the experimental data obtained in this study, it would be difficult to "handpick" these particular genes as targets for antibiotics potentiators.

Although in our experiments a number of genes were found to contribute to the intrinsic antibiotic resistance of *A. baylyi*, we cannot accurately estimate how exhaustive our screening was and whether we identified all or even the majority of the *A. baylyi* intrinsic resistance genes. On the one hand, our screen revealed five independent insertions into *recD*. This result may indicate that either mutagenesis was close to saturation or that *recD* represents a transposon insertion hot spot. On the other hand, the random nature of colony picking and the testing of a fairly limited number of clones (which was of the same order as the number of genes in *A. baylyi*) could have left some mutants with the hypersusceptible phenotype untested.

One of the unexpected findings of this work was that disruption of many genes which contribute to the intrinsic resistance of *A. baylyi* to antibiotics had very little or no effect on the susceptibility of *E. coli* to these drugs. Although this result might be viewed as discouraging for the development of broad-range antibiotic potentiators, it opens the possibility of expansion of the spectrum of available drugs to specific classes of pathogens as well as the development of narrow-spectrum potentiators fine-tuned to combat particular infections.

#### ACKNOWLEDGMENTS

We are grateful to Alexander Mankin for his help with preparing the manuscript and to Dennis Kaznadzey (LabNext) for help in modifying

the microarray printing routine. Special thanks are extended to H. Mori (Nara Institute, Japan) and his Japanese and American collaborators for unparalleled generosity in providing the Keio collection of *E. coli* gene knockout clones.

The work was supported by NIH grants AI49214 and AI56575.

#### REFERENCES

- Amundsen, S. K., A. F. Taylor, A. M. Chaudhury, and G. M. Smith. 1986. *recD*: the gene for an essential third subunit of exonuclease V. Proc. Natl. Acad. Sci. USA **83**:5558–5562.
- Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, and H. Mori. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2**:msb4100050-E1-msb4100050-E11. [Online.]
- Barbe, V., D. Vallenet, N. Fonknechten, A. Kreimeyer, S. Oztas, L. Labarre, S. Cruveiller, C. Robert, S. Duprat, P. Wincker, L. N. Ornston, J. Weissenbach, P. Marliere, G. N. Cohen, and C. Medigue. 2004. Unique features revealed by the genome sequence of *Acinetobacter* sp. ADP1, a versatile and naturally transformation competent bacterium. Nucleic Acids Res. **32**:5766–5779.
- Bennik, M. H., P. J. Pomposiello, D. F. Thorne, and B. Demple. 2000. Defining a *rob* regulon in *Escherichia coli* by using transposon mutagenesis. J. Bacteriol. **182**:3794–3801.
- Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277**:1453–1474.
- Bochkareva, E. S., A. S. Girshovich, and E. Bibi. 2002. Identification and characterization of the *Escherichia coli* stress protein UP12, a putative in vivo substrate of GroEL. Eur. J. Biochem. **269**:3032–3040.
- Buynak, J. D. 2006. Understanding the longevity of the beta-lactam antibiotics and of antibiotic/beta-lactamase inhibitor combinations. Biochem. Pharmacol. **71**:930–940.
- Franke, S., G. Grass, C. Rensing, and D. H. Nies. 2003. Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J. Bacteriol. **185**:3804–3812.
- Henderson, T. A., M. Templin, and K. D. Young. 1995. Identification and cloning of the gene encoding penicillin-binding protein 7 of *Escherichia coli*. J. Bacteriol. **177**:2074–2079.
- Holtje, J. V., U. Kopp, A. Ursinus, and B. Wiedemann. 1994. The negative regulator of beta-lactamase induction AmpD is a *N*-acetyl-anhydromuramyl-L-alanine amidase. FEMS Microbiol. Lett. **122**:159–164.
- Jacobs, C., B. Joris, M. Jamin, K. Klarsov, J. Van Beuemen, D. Mengin-Lecreux, J. van Heijenoort, J. T. Park, S. Normark, and J. M. Frere. 1995. AmpD, essential for both beta-lactamase regulation and cell wall recycling, is a novel cytosolic *N*-acetylmuramyl-L-alanine amidase. Mol. Microbiol. **15**:553–559.
- Lee, H., N. Vazquez-Laslop, K. A. Klyachko, and A. A. Neyfakh. 2003. Isolation of antibiotic hypersusceptibility mutants of *Acinetobacter* spp. by selection for DNA release. Antimicrob. Agents Chemother. **47**:1267–1274.
- Li, X. Z., and H. Nikaido. 2004. Efflux-mediated drug resistance in bacteria. Drugs **64**:159–204.
- Li, X. Z., H. Nikaido, and K. Poole. 1995. Role of *mexA-mexB-oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39**:1948–1953.
- Lomovskaya, O., M. S. Warren, A. Lee, J. Galazzo, R. Fronko, M. Lee, J. Blais, D. Cho, S. Chamberland, T. Renau, R. Leger, S. Hecker, W. Watkins, K. Hoshino, H. Ishida, and V. J. Lee. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob. Agents Chemother. **45**:105–116.
- Mendez, G. L., and F. Megraud. 2002. Is the molecular basis of metronidazole resistance in microaerophilic organisms understood? Trends Microbiol. **10**:370–375.
- Mengin-Lecreux, D., J. van Heijenoort, and J. T. Park. 1996. Identification of the *mpl* gene encoding UDP-*N*-acetylmuramate: L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase in *Escherichia coli* and its role in recycling of cell wall peptidoglycan. J. Bacteriol. **178**:5347–5352.
- Metzgar, D., J. M. Bacher, V. Pezo, J. Reader, V. Doring, P. Schimmel, P. Marliere, and V. de Crecy-Lagard. 2004. *Acinetobacter* sp. ADP1: an ideal model organism for genetic analysis and genome engineering. Nucleic Acids Res. **32**:5780–5790.
- Niga, T., H. Yoshida, H. Hattori, S. Nakamura, and H. Ito. 1997. Cloning and sequencing of a novel gene (*recG*) that affects the quinolone susceptibility of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **41**:1770–1774.
- Okusu, H., D. Ma, and H. Nikaido. 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. **178**:306–308.
- Palmen, R., B. Vosman, P. Buijsman, C. K. Breck, and K. J. Hellingwerf. 1993. Physiological characterization of natural transformation in *Acinetobacter calcoaceticus*. J. Gen. Microbiol. **139**:295–305.
- Pellicer, M. T., M. F. Nunez, J. Aguilar, J. Badia, and L. Baldoma. 2003. Role of 2-phosphoglycolate phosphatase of *Escherichia coli* in metabolism of the 2-phosphoglycolate formed in DNA repair. J. Bacteriol. **185**:5815–5821.
- Prentki, P., and H. M. Krisch. 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29**:303–313.
- Romeis, T., and J. V. Holtje. 1994. Penicillin-binding protein 7/8 of *Escherichia coli* is a DD-endopeptidase. Eur. J. Biochem. **224**:597–604.
- Tatusov, R. L., E. V. Koonin, and D. J. Lipman. 1997. A genomic perspective on protein families. Science **278**:631–637.
- Urios, A., G. Herrera, V. Alexandre, and M. Blanco. 1991. Influence of *recA* mutations on *gyrA* dependent quinolone resistance. Biochimie **73**:519–521.
- Van Dyk, T. K., E. J. DeRose, and G. E. Gonye. 2001. LuxArray, a high-density, genomewide transcription analysis of *Escherichia coli* using bioluminescent reporter strains. J. Bacteriol. **183**:5496–5505.