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# Amdinocillin (Mecillinam) Resistance Mutations in Clinical Isolates and Laboratory-Selected Mutants of *Escherichia coli*

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**Amdinocillin (mecillinam) is a β-lactam antibiotic that is used mainly for the treatment of uncomplicated urinary tract infections. The objectives of this study were to identify mutations that confer amdinocillin resistance on laboratory-isolated mutants and clinical isolates of *Escherichia coli* and to determine why amdinocillin resistance remains rare clinically even though resistance is easily selected in the laboratory. Under laboratory selection, frequencies of mutation to amdinocillin resistance varied from $8 \times 10^{-8}$ to $2 \times 10^{-5}$ per cell, depending on the concentration of amdinocillin used during selection. Several genes have been demonstrated to give amdinocillin resistance, but here eight novel genes previously unknown to be involved in amdinocillin resistance were identified. These genes encode functions involved in the respiratory chain, the ribosome, cysteine biosynthesis, tRNA synthesis, and pyrophosphate metabolism. The clinical isolates exhibited significantly greater fitness than the laboratory-isolated mutants and a different mutation spectrum. The *cysB* gene was mutated (inactivated) in all of the clinical isolates, in contrast to the laboratory-isolated mutants, where mainly other types of more costly mutations were found. Our results suggest that the frequency of mutation to amdinocillin resistance is high because of the large mutational target (at least 38 genes). However, the majority of these resistant mutants have a low growth rate, reducing the probability that they are stably maintained in the bladder. Inactivation of the *cysB* gene and a resulting loss of cysteine biosynthesis are the major mechanism of amdinocillin resistance in clinical isolates of *E. coli*.**

Because of the rapid increase in antibiotic resistance, several first-line antibiotics have been rendered less suitable for empirical treatment. For example, in Sweden, the increasing resistance to trimethoprim has led to a shift in the empirical therapy of uncomplicated urinary tract infections (UTIs), with nitrofurantoin and pivmecillinam now being suggested as first-line therapy (1). It is also recommended as a first-line UTI treatment by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases (2). Amdinocillin (Amd; also known as mecillinam) is an extended-spectrum penicillin derivative, a 6β-amidinopenicillanic acid developed in the 1970s (3, 4). It has been widely used in the Scandinavian countries for UTI treatment since the early 1980s (5, 6). A large proportion of the drug is excreted unchanged in the urine (1, 7), leading to high concentrations in urine and a limited effect on the commensal flora (1, 8, 9). Amd binds to and inhibits the transpeptidase activity of penicillin-binding protein 2 (PBP2) (10–13). PBP2 is responsible for the elongation of rod-shaped cells, and cells treated with Amd become enlarged, nondividing spheres that ultimately lyse (11, 14).

Resistance development has remained low in countries where it is used (15–17). This finding is seemingly a paradox, since a large number of genes (Table 1) can, when mutated, confer resistance to Amd, resulting in a high frequency of mutation to resistance during laboratory selection. Resistance mutations found in mutants isolated in laboratory selections affect many different cellular functions, for instance, (i) cell elongation and division (10, 11, 18–22); (ii) cellular amino acid levels and tRNA synthetases (23–25); (iii) composition of lipopolysaccharide in combination with cya/crp (26–28); (iv) the Rcs global regulatory system that controls, for example, capsule production and cell division (29, 30); and (v) cysteine biosynthesis (31, 32). Some of these mutations are associated with an intracellular increase in the concentration of the molecule ppGpp—the signal for the stringent response. It is known that elevated cellular levels of ppGpp render the target of Amd, PBP2, nonessential (33). Extensive work has been done to elucidate how elevated ppGpp levels can cause Amd resistance (10, 21–23, 33–37), but as of now, the mechanism remains unclear. Furthermore, elevated levels of ppGpp have also been shown to upregulate virulence genes in both *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (38, 39), suggesting that pathogens could concomitantly become antibiotic resistant and more virulent because of these mutations.

In this study, we isolated and analyzed Amd$^r$ *E. coli* mutants obtained during laboratory selections and Amd$^r$ clinical isolates obtained from UTIs where *E. coli* was the causative agent. Our major objectives were (i) to identify the main genetic determinant(s) of Amd resistance in clinical isolates of *E. coli* and (ii) to determine why resistance development remains slow in clinical settings, even though Amd resistance emerges very rapidly during selection in the laboratory. Results show that in clinical isolates of

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**TABLE 1** Known Amd resistance-encoding genes and the functions of their products[a]

| Gene(s) (alias[es]) | [ppGpp] dependence | Reference(s) | Function |
|---|---|---|---|
| *mrdA* (*pbpA*) | Unknown | 10, 11, 19 | Cell division and elongation |
| *mrdB* (*rodA*) | Unknown | 10, 20 | |
| *mreB* (*envB*) | Unknown | 18 | |
| *mreC* | Unknown | 18 | |
| *ftsQ* | No | 21, 22 | |
| *ftsA* | No | 21, 22 | |
| *ftsZ* | No | 21, 22 | |
| *rpoB* | Yes | 35 | RNA synthesis |
| *cysB* | Partly | 31, 32 | Cysteine biosynthesis |
| *cysE* | Partly | 31, 32 | |
| *argS* (*lov*) | Yes | 23 | tRNA synthetases |
| *alaS* | Yes | 23 | |
| *slt* | No | 32 | Transglycosylation |
| *lon* | Unknown | 29 | Rcs regulatory system |
| *rcsB* | Unknown | 29, 30 | |
| *rcsC* | Unknown | 29, 30 | |
| *yrfF* (*mucM*, *igaA*) | Unknown | 29 | |
| *cyaA* | Unknown | 26, 27 | Global regulation |
| *crp* | Unknown | 26, 27 | |
| *spoT*[b] | Yes | 28 | ppGpp degradation and synthesis |
| *rfa*, *rfb*, *rfc*[b] | | 28 | Lipopolysaccharide |
| *galE*[b] | Yes | 28 | |
| *aroK* | Yes | 34 | Shikimate kinase |

[a] Elevated cellular levels of ppGpp (known as the stringent response) give increased Amd resistance. Some of the known resistance-encoding genes have been shown to confer resistance or not to confer resistance, depending on the cell's capacity to produce ppGpp.
[b] In combination with each other and *cysB* and *cysE* (28, 32).

Amd$^r$ *E. coli*, inactivation of the *cysB* gene was the major cause of resistance. In contrast, in laboratory-isolated mutants, many other types of Amd resistance mutations were found. However, many of these mutants showed large reductions in fitness, suggesting that in spite of the high frequency of mutation to resistance *in vitro*, resistance development remains low in clinical settings because most of these mutants grow too slowly to be stably maintained.

## MATERIALS AND METHODS

**Bacterial strains and media.** All of the laboratory strains used in this study are derivatives of *E. coli* MG1655. All of the clinical strains are *E. coli* UTI isolates kindly provided by Gunnar Kahlmeter, Växjö, Sweden. Clinical strains whose whole genomes have been sequenced were isolated from Swedish patients in 2005 to 2007, and additional strains came from other European countries (United Kingdom, Germany, Finland, and Greece). All of the strains used in this study are listed in Table S1 in the supplemental material. When not specified otherwise, bacteria were grown in Mueller-Hinton (MH) broth (Becton, Dickinson and Company) and plated on MH agar. When more suitable, Luria-Bertani (LB) medium (Sigma-Aldrich) was used instead. Some experiments were performed with urine; morning urine (from E. Thulin) was sterile filtered, samples from several days were pooled, and the urine was sterile filtered again and then frozen in aliquots.

When appropriate, the medium was supplemented with the antibiotics Amd (4 to 32 mg/liter), tetracycline (Tet; 12.5 to 30 mg/liter), chloramphenicol (Cam; 12.5 mg/liter), kanamycin (Kan; 100 mg/liter), and ampicillin (Amp; 100 mg/liter), all from Sigma-Aldrich. Strains were saved by freezing overnight cultures in 10% dimethyl sulfoxide at −80°C.

To screen for cysteine auxotrophy, strains were grown for 48 h on minimal medium, M9 glucose (0.4%) agar plates, and M9 glucose plates supplemented with 0.3 mM cysteine (from Sigma-Aldrich).

**MIC assays and cross-resistance testing.** Amd MICs were determined by Etest (AB bioMérieux, Solna Sweden). Bacteria were grown overnight and then diluted 500-fold in phosphate-buffered saline (PBS; 13 mM phosphate, 137 mM NaCl, pH 7.4) before being spread evenly on MH agar plates. An Etest strip was placed on the agar, and the results were analyzed after ~24 h. When appropriate, the MH agar plates were supplemented with 0.3 or 0.75 mM cysteine. The Amd MIC for *E. coli* MG1655 was also tested on M9 glucose agar plates.

Potential cross-resistance to 11 antibiotics (Tet, meropenem, cefotaxime, Amp, ciprofloxacin, fosfomycin, trimethoprim, trimethoprim-sulfamethoxazole, erythromycin, Cam, and nalidixic acid) in the reconstructed laboratory-selected mutants was analyzed by Etest as described above.

**Isolation of mutants.** Thirty independent overnight cultures in MH medium of *E. coli* MG1655 were diluted in PBS, and then approximately $10^7$ CFU were spread evenly on MH agar plates with four different concentrations of Amd, 4, 8, 16, and 32 mg/liter, corresponding to 32, 64, 128, and 256 times the MIC for *E. coli* MG1655, respectively. Colonies were picked after one and two overnight incubations. Single colonies of isolated mutants were restreaked on MH agar plates with the same concentration of Amd as that on which they were isolated. The mutation frequency was calculated as the median number of colonies per plate of each selection concentration divided by the total number of CFU (40).

**DNA isolation, sequencing, and bioinformatics.** Isolation of genomic DNA was performed with the Genomic Tip 100G DNA kit (Qiagen) according to the manufacturer's protocol. Nineteen *E. coli* Amd$^r$ mutants and 12 clinical Amd$^r$ isolates were sent to BGI (Beijing Genome Institute, China) for whole-genome sequencing (Illumina). The CLC Genomics Workbench software (5.5.1; CLC bio, Aarhus, Denmark) was used to assemble sequence reads and detect regions with sequence variation (quality-based variant detection) and regions with no coverage compared to *E. coli* MG1655 (NC_000913), respectively. The cutoff frequency was set to 75%.

In order to identify possible Amd resistance mutations in the clinical isolates, the sequences of 17 *E. coli* genomes (GenBank accession no. AE014075, CP000819, CP000946, CP000970, CP001164, CP001509,

CP001637, CP001665, CP001671, CP001855, CP001969, CP002516, CP002167, NC_002695, NC_004431, NC_013353, and NC_013361) were obtained from the National Center for Biotechnology Information. A comparison of amino acid variability in all of the known Amd resistance-encoding genes was done with the sequences from these genomes and the genome of MG1655 by using CLC Main Workbench 6.8.2 (CLC Bio) for translation and alignments of sequences. The alignments were used to find conserved and variable regions of the Amd resistance-encoding genes, and any amino acid change found in a Amd resistance-encoding gene in a clinical strain was considered to be a potential cause of Amd resistance only if it was found in highly conserved regions (that is, regions that were identical in all of the reference strains and *E. coli* MG1655).

**PCR and Sanger sequencing.** PCRs with either Phusion High-Fidelity DNA polymerase (Thermo Fisher) or TaqGold polymerase (Thermo Fisher) were done according to the following protocol: 95°C (TaqGold) or 98°C (Phusion) for 5 min; 29 cycles of 95°C (TaqGold) or 98°C (Phusion) for 30 s, the proper primer annealing temperature for 30 s, and 72°C for the proper length of the sequence of interest (1 min $kb^{-1}$); and finally 72°C for 7 min before cooling to 4°C. Purification of PCR products was performed with the Gene Jet PCR Purification kit (Thermo Fisher). PCR products were DNA sequenced by Eurofins MWG Operon (Ebersberg, Germany). All of the PCR primers used in this work are listed in Table S2 in the supplemental material.

**Strain constructions.** Selected mutations in the laboratory-selected resistant mutants were reconstructed, and gene knockouts were constructed in the *E. coli* MG1655 genetic background by standard genetic techniques (see Table 3; also see Table S1 in the supplemental material). In brief, a Cam or Kan resistance marker was introduced 10 to 20 kb away from the mutation of interest by temperature-controlled λ Red recombineering (41, 42) with a pSim5-Tet plasmid (43). P1 lysates of the constructed strains were then prepared and used for transduction into MG1655. From each transduction, a congenic strain pair carrying the mutation and the resistance marker or only the resistance marker was saved. The Cam or Kan resistance cassette was subsequently removed by introducing the pCP20 plasmid, which expresses Flp recombinase, which acts on Flp recombination sites flanking the resistance cassette, leaving an 85-nucleotide scar (see Table S1 in the supplemental material) (41, 44). Finally, the linear transformation was confirmed by both PCR (and gel electrophoresis) and sequencing of the scar sequence and the Amd resistance-encoding gene of interest.

To introduce the *cysB* mutations identified in the clinical isolates into the *E. coli* MG1655 background, a *cat-sacB* marker was introduced to replace the *cysB* gene in DA5438 by λ Red recombineering as described above. The *cat-sacB* marker used is part BBa_K864150 from the Registry of Standard Biological Parts, is expressed from the BBa_J23101 promoter (45), and has been assigned GenBank accession number KM018298. PCR-amplified *cysB* genes from the clinical $Amd^r$ strains were then transformed into the *cysB*::*cat-sacB* strain and recombinants that replaced the *cysB*::*cat-sacB* cassette with the PCR-amplified *cysB* gene were selected for by growth at high sucrose levels, as *sacB* is lethal in the presence of sucrose (46). The transformations were confirmed as described above.

All of the PCR primers used for amplification of the resistance cassettes and screening for insertion of the cassettes are listed in Table S3 in the supplemental material.

**Transformation of the *E. coli* MG1655 *cysB* gene into clinical isolates.** A plasmid carrying the functional *cysB* gene from MG1655 was introduced into clinical strains. The plasmid used (pEL3c15) was based on BioBrick vector pSB3T5 from the Registry of Standard Biological Parts (47), which additionally carries the red marker mRFP1 in the cloning site to facilitate screening for plasmids with the desired insert and a $Cam^r$ marker. Briefly, the insert (*E. coli* MG1655 *cysB* gene with the native promoter and terminator) was PCR amplified with primers containing XbaI and PstI restriction enzyme cleavage sites, respectively. The insert and the plasmid were first cleaved with Fast Digest XbaI and PstI according to the manual provided by Thermo Fisher and then ligated with Ready-To-Go T4 DNA ligase (GE Health Care Life Sciences). The resulting plasmids were transformed into MG1655 by electroporation and then spread on Cam-supplemented MH agar plates for selection of plasmid uptake. PCR was used to confirm the introduction of *cysB* into the plasmid in strain DA37697.

For introduction of the plasmid into the clinical strains, it was prepared from DA37697 and then transformed into strains DA14710, DA14713, DA14718, DA14719, and DA24686 and the *cysB* knockout strain DA28439 as described above, resulting in strains DA37700 to DA37713. The Amd MICs for these clinical strains containing the *cysB* plasmid were then determined as described above.

**Fitness measurements.** Overnight cultures in MH medium were diluted to ~$1 \times 10^6$ to $3 \times 10^6$ CFU/ml in the same medium, and then 300 μl was added in quadruplicate to a 100-well honeycomb plate. Medium blanks and the reference strain *E. coli* MG1655 were added (also in quadruplicate) to each plate to enable calculation of the relative growth rate and subtraction of the absorbance value of the medium. Growth of the samples at 37°C with shaking for 16 h was monitored with a Bioscreen C Analyzer (Oy Growth Curves Ab. Ltd.). Measurements of optical density at 600 nm ($OD_{600}$) were taken every 4 min. Calculations are based on $OD_{600}$ values between 0.02 and 0.1 wherever growth was observed to be exponential. All of the experiments were run three separate times. The R statistical software (48) was used to calculate relative growth rates by dividing the generation time of the parental strain by the generation time of the mutants from the same experiment. A *t* test was performed to detect any significant difference in fitness between the *E. coli* laboratory-isolated mutants and the clinical isolates. The same Bioscreen protocol was used for measurements of fitness in urine.

Fitness in different concentrations of Amd was also examined the same way for a subset of *E. coli* reconstructed mutants, clinical isolates, and strain MG1655 (DA5438, DA14719, DA24678, DA24686, DA28432, DA28434, DA28436, DA28438, DA28439, DA29705, and DA29712), except that the samples were in triplicate. The Amd concentrations were in a dilution series ranging from 10 to 300 mg/liter for the $Amd^r$ strains and from 0.032 to 2 mg/liter for *E. coli* MG1655.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the sequences of the *alaS, argS, aroK, aspS, crp, cyaA, cysB, cysE, ftsA, ftsQ, ftsZ, galE, gltX, ispA, lon, mrdA, mrdB, mreB, mreC, mreD, ppa, rcsB, rcsC, rcsD, rcsF, rplL, rpoB, slt, spoT, thrS, ubiE,* and *ubiX* genes from the clinical $Amd^r$ strains are KP670466 to KP670849 (see Table S3 in the supplemental material).

## RESULTS

***In vitro* isolation of $Amd^r$ mutants and MIC determinations.** Selection of independent mutants was carried out on MH agar plates supplemented with Amd at 4, 8, 16, or 32 mg/liter. Colonies differing in size and morphology (e.g., mucoidy) and from different selective concentrations were picked to ensure that a wide range of $Amd^r$ mutants were isolated. The Amd MICs for isolated *E. coli* mutants ranged between 4 and 96 mg/liter, compared to the MIC of 0.125 mg/liter for *E. coli* MG1655, the EUCAST epidemiological cutoff of ≤1 mg/liter, and the EUCAST clinical breakpoints of ≤8 mg/liter for sensitivity and >8 mg/liter for resistance. The mutation frequencies were $8 \times 10^{-8}$ to $2 \times 10^{-5}$, depending on the concentration of Amd used in the selection plate (the lowest frequency was at the highest concentration, and the highest frequency was at the lowest concentration). The Amd MICs for the 12 clinical Swedish isolates whose whole genomes have been sequenced, as well as the additional five clinical isolates from other European countries used in this study, were in the same range as those for the laboratory-selected mutants (4 to 64 mg/liter) (see Table 4).

**Whole-genome sequencing and genetic reconstruction of laboratory-isolated mutants.** The whole genomes of 19 indepen-

**TABLE 2** Characteristics of Amd$^r$ laboratory-selected *E. coli* mutants

| Gene | No. of mutants | No. of different mutations[a] | MIC(s) |
|---|---|---|---|
| *thrS* | 4 | 4 | 16–48 |
| *aspS* | 7 | 6 (3 in promoter) | 12–16 |
| *gltX* | 2 | 2 | 16–32 |
| *ppa* | 3 | 2 | 96 |
| *mrdA* | 2 | 1 | 12–64 |
| *ubiE* | 1 | | 12 |
| *ubiX* | 1 | | 32 |
| *ispA* | 1 | | 12 |
| *rplL* | 1 | | 8 |
| *rpoB* | 1 | | 32 |
| *spoT* | 1 | | 24 |
| *cysB* | 1 | | 24 |

[a] For the specific mutations in these genes, see Table S1 in the supplemental material.

**TABLE 3** Characteristics of reconstructed Amd$^r$ mutants of *E. coli* MG1655 strain DA5438 compared to corresponding laboratory-selected mutants

| Strain | Amd resistance-encoding mutation(s) | MIC (mg/liter) | Relative growth rate |
|---|---|---|---|
| DA5438 | None | 0.125 | 1 |
| DA18488 | *thrS* (E519G) | 48 | 0.31 |
| DA29703 | *thrS* (E519G)[a] | 32 | 0.76 |
| DA18480 | *aspS* (P191S) | 16 | 0.54 |
| DA28434 | *aspS* (P191S)[a] | 24 | 0.61 |
| DA18497 | *gltX* (D180G) | 32 | 0.33 |
| DA30120 | *gltX* (D180G)[a] | 24 | 0.36 |
| DA18467 | *ubiE* (del[b] bp 346–349 → FS[c]) | 12 | 0.46 |
| DA28436 | *ubiE* (del bp 346–349 → FS)[a] | 12 | 0.54 |
| DA18483 | *ubiX* (del bp 246 → FS) | 32 | 0.37 |
| DA29707 | *ubiX* (del bp 246 → FS)[a] | 32 | 0.40 |
| DA18491 | *ispA* (IS*1* insertion) | 12 | 0.35 |
| DA30122 | *ispA* (IS*1* insertion)[a] | 16 | 0.33 |
| DA18477 | *rplL* (promoter −10 and duplication) | 8 | 0.62 |
| DA29710 | *rplL* (promoter −10)[a] | 48 | 0.34 |
| DA18481 | *ppa* (D11A) | 96 | 0.48 |
| DA28432 | *ppa* (D11A)[a] | 96 | 0.43 |
| DA18482 | *rpoB* (H447L), *araB* (I450L) | 32 | 0.56 |
| DA29712 | *rpoB* (H447L)[a] | 48 | 0.61 |
| DA18493 | *spoT* (S305P) | 24 | 0.75 |
| DA28438 | *spoT* (S305P)[a] | 32 | 0.76 |
| DA18489 | *mrdA* (D389G), *trxB* (G37D) | 64 | 0.30 |
| DA18490 | *mrdA* (D389G) | 16 | 0.41 |
| DA29705 | *mrdA* (D389G)[a] | 16 | 0.51 |
| DA29715 | *trxB* (G37D)[a] | 0.064 | 0.92 |
| DA29717 | *mrdA* (D389G),[a] *trxB* (G37D)[a] | 64 | 0.29 |

[a] Reconstructed mutant.
[b] del, deletion.
[c] FS, frame shift.

dently selected Amd$^r$ *E. coli* mutants were sequenced. To identify the Amd resistance-encoding mutations, genome assembly was done and the results were compared to the *E. coli* MG1655 genome. Of the 19 mutants, 15 had only one mutation and the other 4 had two mutations. Most of the mutants had mutations in genes that have previously been found to confer Amd resistance, such as *mrdA* (the gene encoding PBP2), *spoT*, *rpoB*, and tRNA synthetase-encoding genes (Table 2 shows the mutated genes and the number of strains with the specific mutations; see Table S1 in the supplemental material). Although some other tRNA synthetases have been connected to Amd resistance before, these specific ones (*thrS*, *aspS*, and *gltX*) were novel. Two strains had the same mutation in *mrdA* but different MICs (12 and 64 mg/liter, respectively). The strain with the higher MIC had an additional mutation in *trxB*, which encodes thioredoxin reductase.

Some previously unidentified Amd resistance-encoding genes were also found, i.e., the *ubiE*, *ubiX*, and *ispA* genes, whose products are part of the respiratory chain; *ppa*, the gene encoding inorganic pyrophosphatase; and the *rplL* gene, which encodes ribosomal protein L7/L12. The *rplL* mutation was also associated with a large duplication of 38 kbp that included the *rplL* gene. To confirm that these genes confer Amd resistance, genetic reconstructions were made to introduce each specific mutation singly into the *E. coli* MG1655 background by means of linear transformation by λ Red recombineering. The MICs for the reconstructed strains and the original mutants and their growth rates are compared in Table 3 and discussed below.

**Whole-genome sequencing of clinical isolates.** To identify potential Amd resistance-encoding mutations in clinical isolates, the whole genomes of 12 Amd$^r$ *E. coli* UTI isolates from a collection of UTI isolates obtained in Sweden from 2005 to 2007 were sequenced and assembled to the *E. coli* MG1655 genome. Potential Amd resistance-encoding genes in these clinical strains, including those previously discovered, as well as the new ones identified here, were compared to those in 17 *E. coli* reference genomes. Nonsynonymous point mutations in known Amd resistance-encoding genes that were found in the UTI isolates but not in the reference strains were considered potential Amd resistance-conferring mutations.

All of the Amd resistance-encoding genes are listed in Fig. 1, and all of the mutations found in those genes are listed in Table 4. The occurrence of the known Amd resistance-encoding genes (i.e., those identified in laboratory selections) in the resistant clinical isolates were distributed as follows. (i) Mutations in the *lon*, *mrdA*, *mrdB*, *ppa*, *ubiX*, *ispA*, *rplL*, *ftsA*, *mreB*, *mreD*, and *aroK* genes were absent from all of the clinical isolates. (ii) Mutations in the *rcsB*, *rcsC*, *rcsD*, *rcsF*, *yrfF*, *thrS*, *gltX*, *aspS*, *argS*, *alaS*, *ubiE*, *spoT*, *cysE*, *ftsQ*, *ftsZ*, *mreC*, *cyaA*, *crp*, *galE*, *rpoB*, and *slt* genes were present in a few strains (one to five). (iii) Mutations in *cysB* were found in all of the clinical isolates (Table 4). In summary, genes involved in cell division and elongation (*mrdAB*, *ftsQAZ*, and *mreBCD*) or cell respiration (*ubiEX*, *ispA*), the Rcs genes (*rcsBCDF* and *yrfF*), and those encoding the tRNA synthetases were mutated in a few clinical isolates, whereas the *cysB* gene was mutated in all of them, suggesting that *cysB* is of particular importance in generating Amd resistance in clinical *E. coli* isolates. Furthermore, five additional Amd$^r$ clinical isolates obtained from several European countries had a mutated *cysB* gene (as shown by localized sequencing of the *cysB* genes); thus, all 17 of the clinical Amd$^r$ isolates had *cysB* mutations. The *cysB* mutations were of different types; many were point mutations leading to either an amino acid change or a stop codon, several were frame shifts, and there was also one deletion and one IS*1* insertion (Table 4).

**Amd resistance is caused by *cysB* mutations that prevent production of cysteine.** To confirm that the *cysB* mutations identified confer Amd resistance, several tests were performed. First, a *cysB*

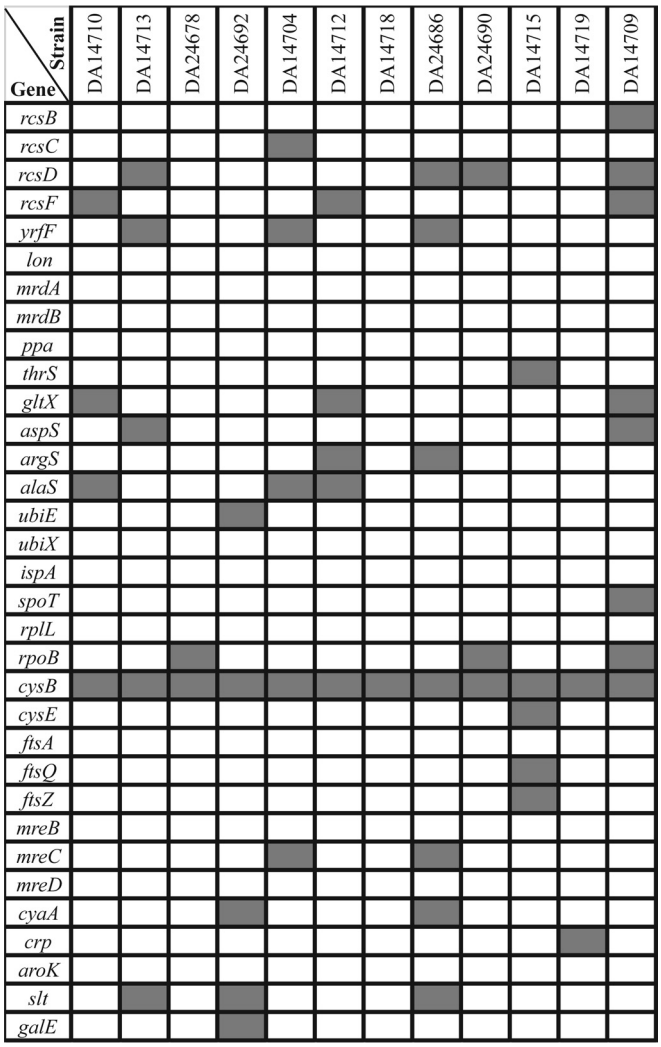


**FIG 1** Putative Amd resistance-encoding genes in clinical strains. Genes marked by gray shading have a mutation that is not present in any of the reference genomes. Specific mutations are listed in Table 4.

knockout mutation was constructed and introduced into laboratory strain MG1655. Inactivation of *cysB* conferred a Amd$^r$ phenotype where the *cysB* knockout mutant for which the MIC was 24 mg/liter (compared to 0.125 for the *cysB*$^+$ strain), showing that inactivation of the CysB protein causes resistance. This had previously been shown in *S. enterica* serovar Typhimurium (31). Second, cysteine auxotrophy screening of some 40 Amd$^r$ *E. coli* strains originating from MG1655 in the initial selection of Amd$^r$ mutants was done with M9 glucose and M9 glucose-cysteine plates. One strain, DA24053, was a cysteine auxotroph. The *cysB* gene was PCR amplified and sequenced, showing that the strain had a deletion of bp 148 in *cysB*. The Amd MIC for this strain was 24 mg/liter, which is identical to that for the *cysB* knockout strain.

Third, to further demonstrate the role of the *cysB* mutations in resistance in the clinical isolates, the mutated *cysB* genes from a subset of the clinical Amd$^r$ strains (DA14710, DA14713, DA14718, and DA24686) were genetically transferred into the *E. coli* MG1655 background by recombineering (generating strains DA34895, DA34896, DA34896, and DA34897, respectively). The effects of these mutations on the MIC and fitness were measured by Etest and growth rate determination. The MICs conferred by the different mutated *cysB* genes were 12 mg/liter for DA34897, 24 mg/liter for DA34896 and DA34898, and 32 mg/liter for DA34895, demonstrating that these *cysB* mutations are sufficient to confer high-level Amd resistance. The *cysB* mutations in strains DA34895 and DA34898 imposed severe fitness costs, with relative growth rates of 0.58 and 0.65, respectively. The fitness costs of the *cysB* mutations in strains DA34896 and DA34897 were lower, with both strains having a relative growth rate of about 0.9. Finally, to determine if the *cysB* mutant phenotype is recessive or dominant, we complemented a subset of the clinical *cysB* mutants (strains DA14710, DA14713, DA14718, DA14719, and DA24686) with a plasmid carrying the functional *cysB*$^+$ gene from MG1655. Etests of the resulting strains (DA37700 to DA37713) showed that the Amd MIC for all of them was reduced to the MIC for MG1655 (0.125 mg/liter), compared to the between 16 and 64 mg/liter determined for these strains before plasmid introduction. When *cysB* knockout strain DA28439 was also transformed with the

**TABLE 4** Putative Amd resistance-encoding mutations in the clinical isolates used in this study

| Strain | MIC (mg/liter) | Mutation(s) in known Amd resistance-encoding gene(s)[a] | *cysB* mutation |
|---|---|---|---|
| DA14704 | 32 | *rcsC* (T714N), *yrfF* (R50H), *alaS* (A515T), *mreC* (Q324P, del[b] nt[c] 975–1035) | V12G |
| DA14709 | 4 | *rcsB* (K192T), *rcsD* (A254T), *rcsF* (P26S), *gltX* (D301N), *aspS* (K347N), *spoT* (T350I), *rpoB* (G544S) | IS*1* insertion |
| DA14710 | 64 | *rcsF* (P48S), *gltX* (S65N, T287A), *alaS* (D712E) | G241E |
| DA14712 | 32 | *rcsF* (P48S), *gltX* (S65N, T287A), *argS* (Y200C), *alaS* (D712E) | FS[d] after aa[e] 160 |
| DA14713 | 64 | *rcsD* (P252Q), *yrfF* (D307N), *aspS* (S580G), *slt* (S2266T, P544L) | Q103stop |
| DA14715 | 16 | *thrS* (P7H), *ftsQ* (E262G), *ftsZ* (V331A) | del nt 278–309 |
| DA14718 | 32 | | FS after aa 161 |
| DA14719 | 16 | *crp* (T8A, S17F) | FS after aa 87 |
| DA24678 | 48 | *rcsD* (P490L), *rpoB* (E625A) | FS after aa 161 |
| DA24686 | 32 | *rcsD* (P252Q), *yrfF* (D307N), *argS* (E326K), *mreC* (S69L, A327T, P352S), *cyaA* (Q655L, L693P), *slt* (T142I) | K76stop |
| DA24690 | 32 | *rcsD* (P490L), *rpoB* (E625A) | Q4stop |
| DA24692 | 48 | *ubiE* (S5L), *cyaA* (del nt 1428–1434), *slt* (G58R, G540S), *galE* (E67K) | FS after aa 242 |

[a] Not found in reference genomes.
[b] del, deletion.
[c] nt, nucleotide.
[d] FS, frame shift.
[e] aa, amino acid.

**TABLE 5** Effects of cysteine addition on Amd resistance of clinical isolates[a]

| Strain | Relevant phenotype | Growth on MM | | Amd MIC (mg/liter) | | |
|---|---|---|---|---|---|---|
| | | M9 | M9 + Cys | No Cys | 0.3 mM Cys | 0.75 mM Cys |
| DA5438 | $Cys^+$ | Yes | Yes | 0.125 | 0.125 | 0.125 |
| DA28439 | $Cys^{-b}$ | No | Yes | 24 | 2 | 0.25 |
| DA14704 | $Cys^-$ | No | Yes | 32 | 0.125 | 0.125 |
| DA14709 | $Cys^-$ | No | Yes | 4 | 2 | 0.38 |
| DA14710 | $Cys^-$ | No | Yes | 64 | 8 | 0.125 |
| DA14712 | $Cys^-$ | No | Yes | 32 | 16 | 0.5 |
| DA14713 | $Cys^-$ | No | No | 64 | 4 | 0.125 |
| DA14715 | $Cys^-$ | No | Yes | 16 | 2 | 0.19 |
| DA14718 | $Cys^-$ | No | Yes | 32 | 8 | 0.38 |
| DA14719 | $Cys^-$ | No | Yes | 16 | 0.5 | 0.094 |
| DA24678 | $Cys^-$ | No | Yes | 48 | 16 | 0.38 |
| DA24686 | $Cys^-$ | No | Yes | 32 | 16 | 0.19 |
| DA24690 | $Cys^-$ | No | Yes | 32 | 24 | 0.125 |
| DA24692 | $Cys^-$ | No | Yes | 48 | 6 | 0.19 |

[a] Shown are growth on M9 glucose minimal medium (MM) with or without cysteine added and Amd Etest MICs on MH plates with different concentrations of cysteine. DA5438 is *E. coli* MG1655, DA28439 is a constructed *cysB* knockout strain, and all of the other strains are $Amd^r$ clinical *E. coli* isolates with *cysB* mutations.
[b] *cysB* knockout.

plasmid, a similar result was obtained; the MIC was reduced from 24 to 0.125 mg/liter.

To determine if it was the absence of cysteine or the CysB protein itself that conferred the resistance phenotype, we examined how addition of cysteine influenced the Amd resistance of the clinical isolates and the *cysB* knockout mutant. The $Mec^r$ clinical isolates and the *cysB* knockout strain (DA28439) were grown on M9 plates with or without 0.3 mM cysteine to screen for cysteine auxotrophy. The *cysB* knockout and the clinical strains could grow on minimal plates only when they were supplemented with cysteine (Table 5). One clinical strain grew on minimal medium neither without nor with cysteine and was assumed to have another, unknown, auxotrophy. The Amd MICs on MH medium supplemented with two different concentrations of cysteine (0.3 and 0.75 mM) were also measured for the clinical isolates and the *cysB* knockout. When supplemented with cysteine, these strains lost most of their resistance to Amd (Table 5); a similar result has previously been obtained with *S. enterica* serovar Typhimurium (31). In fact, at the higher concentration, the MICs for all of the clinical isolates were in the range of the *E. coli* MG1655 MIC and well below the clinical breakpoint for Amd (8 mg/liter). This result demonstrates that it is the lack of cysteine, and not the absence of the CysB protein, that confers Amd resistance.

**Fitness of $Amd^r$ strains.** Fitness was measured as the growth rate relative to that of *E. coli* MG1655 by determining $OD_{600}$ over time in the Bioscreen analyzer. The change in OD at exponential growth was recalculated to obtain the growth rate. In MH medium without Amd, all of the resistant mutants isolated in the laboratory were less fit than MG1655; the relative growth rates ranged from 0.25 to 0.7 (Fig. 2). The $Amd^r$ *E. coli* UTI isolates also had lower fitness in MH medium than MG1655 did, with relative growth rates ranging from 0.52 to 0.83 (Fig. 3). The clinical strains had significantly higher growth rates in MH medium than the *E. coli* laboratory isolates (*t* test, $P = 6 \times 10^{-6}$). In urine, however, all of the laboratory-isolated $Amd^r$ mutants had lower fitness than MG1655 (relative growth rates between 0.26 and 0.81), but all of the clinical isolates had significantly higher fitness than MG1655 (relative growth rates between 1.22 and 1.7) (Fig. 3). The clinical strains also had significantly higher growth rates in urine than the *E. coli* laboratory isolates (*t* test, $P < 2 \times 10^{-16}$).

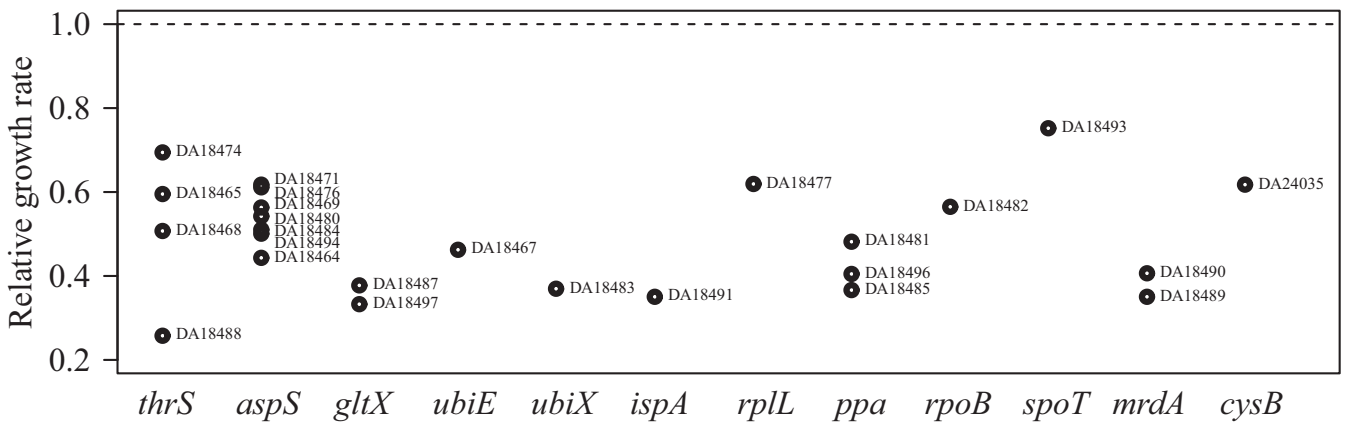


**FIG 2** Fitness levels (relative growth rates) of laboratory-selected *E. coli* mutants, where *E. coli* MG1655 is set to 1.0. Strain names and mutated genes are shown.

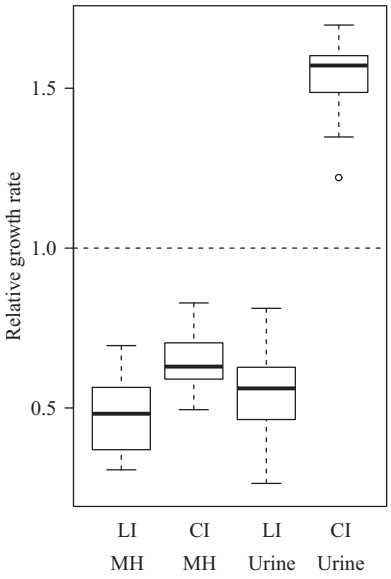


**FIG 3** Box plot of fitness of laboratory-selected *E. coli* isolates (LI) and clinical isolates (CI) in MH medium and urine, showing (from top to bottom) the maximum fitness value, the upper quartile, the median, the lower quartile, and the minimum fitness value. A single outlier is shown as a circle. Fitness was measured as the relative growth rate, where MG1655 strain DA5438 was set to 1.0. *t* tests showed significant differences in fitness between the *E. coli* laboratory-isolated mutants and the clinical isolates in both MH medium ($P = 6 \times 10^{-6}$) and urine ($P < 10^{-16}$).

We determined how the growth of subsets of Amd$^r$ clinical isolates and reconstructed mutant strains (DA5438, DA14719, DA24678, DA24686, DA28432, DA28434, DA28436, DA28438, DA28439, DA29705, and DA29712) was affected by increased concentrations of Amd (Fig. 4). All of the resistant mutants had severely reduced growth rates at increased Amd concentrations, and it is notable that none of the resistant isolates could grow at concentrations of >300 mg/liter.

**Cross-resistance in reconstructed *E. coli* mutants.** The Amd$^r$ reconstructed *E. coli* mutants were examined for cross-resistance to other antibiotics by Etest (Table 6). The antibiotics tested were three other β-lactams (meropenem, cefotaxime, and Amp), five antibiotics that are commonly used to treat UTIs (ciprofloxacin, fosfomycin, nitrofurantoin, trimethoprim, and trimethoprim-sulfamethoxazole), and four other antibiotic groups (erythromycin, Cam, Tet, and nalidixic acid). No large differences from *E. coli* MG1655 were seen, except for erythromycin, where several Amd$^r$ mutants showed higher MICs than MG1655 and some few strain-antibiotic combinations showed a lower MIC than MG1655 (e.g., DA28436 and nitrofurantoin, DA28439 and Tet, and DA29712 and fosfomycin).

## DISCUSSION

Amd resistance is rapidly acquired by bacteria in laboratory selections but appears rare in clinical isolates (15, 16, 49). Prior to this work, 30 genes that can confer Amd resistance had been found (Table 1), and here we identified eight additional genes. Thus, the fact that at least 38 genes can cause Amd resistance when mutated (often when inactivated) explains the high frequency of mutation to resistance in laboratory selections and the expectation that resistance would also be common in clinical settings. Here, we compared clinical isolates and laboratory-selected resistant mutants to identify resistance mutations and seek explanations for the low incidence of resistance in clinical isolates.

**New Amd resistance-encoding genes found in laboratory selections.** Many of the mutations that confer Amd resistance cause a ppGpp concentration increase (Table 1) (10, 21–23, 33–37), but the mechanism by which elevated ppGpp levels confer resistance remains unknown. In this study, several new genes with known or potential involvement in ppGpp metabolism were discovered. First, mutations in three genes coding for tRNA synthetases (*thrS*, *aspS*, and *gltX*) conferred resistance. Although these specific tRNA synthetases have not been associated with Amd resistance previously, others have (23), and the resistance caused by these mutations was due to activation of the stringent response and ppGpp synthesis.

Another example of mutants probably owing their resistance to the stringent response is the *ppa* mutants. The product of *ppa* is the enzyme inorganic pyrophosphatase, which catalyzes the cleavage of pyrophosphate ($PP_i$) into two phosphate ions (50, 51). Since $PP_i$ is a product of the stringent response, it is plausible that Amd resistance conferred by *ppa* mutations is connected to the stringent response. In addition, $PP_i$ is also an inhibitor of both cell division and tRNA synthetases, which are also possible routes for Amd resistance (52).

Additional new Amd resistance mutations found were in the ribosomal gene *rplL* and cell respiration genes *ubiE*, *ubiX*, and *ispA*, all of which result in slow growth and might induce the stringent response through the pppGpp synthetase SpoT (38). The *rplL* mutant might also be connected to the stringent response in another, more direct, way. The main pppGpp synthetase RelA binds to the ribosomal protein L11 (38), which interacts with L12 (the product of *rplL*) via L10, and it is conceivable that an L12 mutation could affect RelA function via L10 and L11 (53).

We also found that a mutation in *trxB* could increase the level of resistance of an already resistant *mrdA* mutant, although not conferring any resistance on its own. The gene *trxB* encodes thioredoxin, an enzyme that is involved in the cysteine biosynthesis pathway (54). Inactivation of this pathway has previously, as well as in this study, been shown to confer Amd resistance (31, 32).

**CysB inactivation is the major mechanism of Amd resistance in clinical isolates of *E. coli*.** We observed two important differences between the clinical and laboratory-isolated Amd$^r$ isolates with regard to mutation spectra. First, the spectrum of resistance-causing mutations appeared much narrower in the clinical isolates than in the laboratory-selected isolates, and second, all of the clinical isolates but only one of the laboratory-selected mutants had a mutated *cysB* gene. We sequenced the whole genomes or performed cysteine auxotrophy screening of 62 mutants, and since *cysB* is only 1 of 38 known Amd resistance-encoding genes, many of which confer resistance when having loss-of-function mutations, the observation that 1/62 mutants is defective in *cysB* is close to the expected result.

CysB protein is the major positive regulator of cysteine biosynthesis in *Enterobacteriaceae* (54), and mutations in the cysteine biosynthesis pathway genes *cysB* and *cysE* have previously been shown to confer Amd resistance on laboratory-selected mutants of *S. enterica* serovar Typhimurium (31, 32). Although the mechanism by which *cysB* confers resistance has not been fully elucidated, it is at least partly due to increased intracellular levels of ppGpp (32).

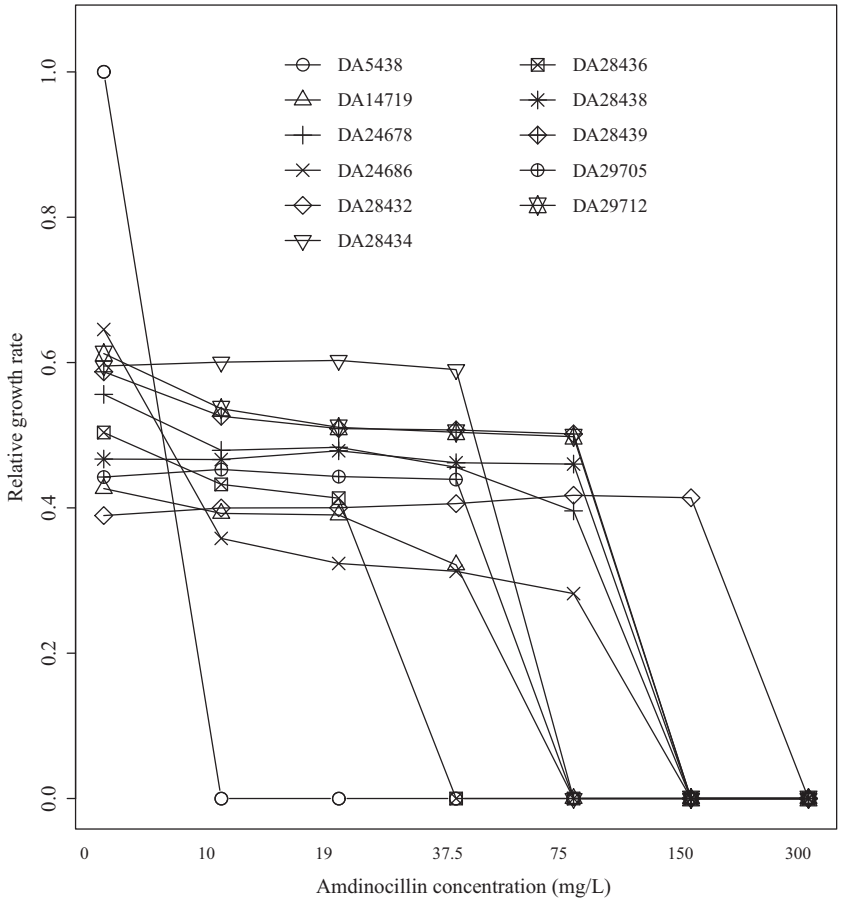


**FIG 4** Relative growth rates of Amd$^r$ reconstructed mutants and clinical isolates as a function of the Amd concentration. DA5438 is *E. coli* MG1655 (with a Amd MIC of 0.125 mg/liter). DA14719 (Amd MIC, 16 mg/liter), DA24678 (Amd MIC, 48 mg/liter), and DA24686 (Amd MIC, 32 mg/liter) are Amd$^r$ *E. coli* clinical isolates. DA28432 (Amd MIC, 96 mg/liter; *ppa* [D11A]), DA28434 (Amd MIC, 24 mg/liter; *aspS* [P191S]), DA28436 (Amd MIC, 12 mg/liter; *ubiE* [deletion of bp 346 to 349 → frame shift]), DA28438 (Amd MIC, 32 mg/liter; *spoT* [S305P]), DA28439 (Amd MIC, 32 mg/liter; *cysB* knockout), DA29705 (Amd MIC, 16 mg/liter; *mrdA* [D389G]), and DA29712 (Amd MIC, 48 mg/liter; *rpoB* [H447L]) are reconstructed *E. coli* Amd$^r$ mutants.

Transfer of different *cysB* mutations (including amino acid substitutions, stop codons, and frame shifts) from clinical isolates into a laboratory strain conferred high-level resistance, demonstrating that Amd resistance in clinical isolates of *E. coli* is conferred by inactivation of the *cysB* gene. Furthermore, introduction of a plasmid containing a functional *cysB*$^+$ gene from strain MG1655 into the clinical strains resulted in loss of Amd resistance, demonstrating that their resistance results from inactivation of the CysB protein and that the resistant mutant phenotype is recessive. When their medium was supplemented with cysteine, the clinical isolates lost their resistance to Amd, indicating that their resistance is due to the absence of cysteine biosynthesis (rather than loss of the CysB activator *per se*). We also found clinical isolates that carried *cysB* mutations with even higher MICs (>256 mg/liter) than that conferred by *cysB* inactivation alone (16 to 32 mg/liter); these mutants are likely to carry additional, unidentified, resistance mutations.

**Why has the frequency of Amd resistance among *E. coli* UTI isolates remained low?** Even though the frequency of mutation to Amd resistance is very high in *in vitro* selections, resistance development appears to be relatively uncommon in clinical settings. We propose that this is due to two factors. First, the majority of the resistant mutants that are found in laboratory selections grow slowly, reducing their ability to become fixed in the bladder. Thus, because of frequent repeated micturition, bacteria residing in urine need to grow above a certain rate to be stably maintained, at least during planktonic growth (55). In other words, among all of the mutant types that can be found *in vitro*, only a small subset (including the *cysB* mutant) are fit enough to be stably maintained in the bladder. In line with this idea, the type of resistance mutation found in clinical isolates, *cysB*, imposes a relatively low fitness cost whereas many of the other, highly resistant mutants found in the laboratory selections (e.g., *mrdA*, *ppa*, *ubiX*, *ispA*, *ubiE*, and some tRNA synthetase mutants) showed severe growth rate reductions. Other factors that reduce the fixation rate of resistant mutants in clinical settings are the facts that all of the Amd$^r$ clinical isolates showed strong growth rate reductions in response to increasing Amd levels and that none of the mutants could grow at a concentration of >300 mg/liter (Fig. 4). Since the concentration of Amd in urine often reaches several hundred mg/liter during treatment (1, 8, 9), this suggests that even highly resistant mutants would have problems remaining in the bladder during treatment since their growth rates are reduced below the threshold level needed for stable maintenance (55).

**TABLE 6** MICs of antibiotics[a] for reconstructed Amd[r] mutants and *E. coli* MG1655

| Strain | Genotype | MIC (mg/liter) of: | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Amd | Mem | Ctx | Amp | Cip | Nit | Fof | Tmp | Sxt | Chl | Nal | Tet | Ery |
| DA5438[b] | Wild type | 0.125 | 0.023 | 0.064 | 2 | 0.016 | 2 | 0.5 | 0.125 | 0.047 | 6 | 3 | 1 | 16 |
| DA28432 | *ppa* (D11A) | 96 | 0.032 | 0.047 | 0.75 | 0.016 | 3 | 0.19 | 0.94 | 0.032 | 4 | 0.5 | 0.75 | 198 |
| DA28434 | *aspS* (P191S) | 24 | 0.047 | 0.047 | 2 | 0.016 | 3 | 0.094 | 0.125 | 0.032 | 6 | 3 | 0.5 | 64 |
| DA28436 | *ubiE* (del[c] bp 346–349 → FS[d]) | 12 | 0.023 | 0.032 | 1 | 0.016 | 2 | 0.094 | 0.75 | 0.064 | 1.5 | 3 | 0.5 | 64 |
| DA28438 | *spoT* (S305P) | 32 | 0.032 | 0.064 | 1.5 | 0.016 | 0.25 | 0.19 | 0.125 | 0.023 | 3 | 2 | 0.094 | 32 |
| DA28439 | *cysB* KO[e] | 32 | 0.064 | 0.047 | 2 | 0.023 | 1 | 0.5 | 0.38 | 0.064 | 4 | 3 | 0.75 | >256 |
| DA29703 | *thrS* (E519G) | 32 | 0.032 | 0.064 | 2 | 0.023 | 4 | 1 | 0.25 | 0.047 | 6 | 3 | 0.75 | 24 |
| DA29705 | *mrdA* (D389G) | 16 | 0.023 | 0.023 | 0.75 | 0.016 | 1.5 | 0.38 | 0.94 | 0.016 | 6 | 3 | 0.5 | 24 |
| DA29707 | *ubiX* (del bp 246 → FS) | 32 | 0.047 | 0.032 | 1 | 0.023 | 1 | 0.094 | 0.25 | 0.047 | 3 | 3 | 1 | 96 |
| DA29710 | *rplL* (promoter −10) | 48 | 0.032 | 0.047 | 0.5 | 0.023 | 2 | 0.125 | 0.125 | 0.032 | 4 | 3 | 0.75 | 24 |
| DA29712 | *rpoB* (H447L) | 48 | 0.047 | 0.064 | 2 | 0.023 | 4 | 0.064 | 0.094 | 0.047 | 4 | 3 | 0.75 | 16 |
| DA29715 | *trxB* (G37D) | 0.064 | 0.023 | 0.047 | 2 | 0.016 | 6 | 1 | 0.19 | 0.047 | 4 | 3 | 1 | 24 |
| DA29717 | *mrdA* (D389G) *trxB* (G37D) | 64 | 0.016 | 0.023 | 0.75 | 0.023 | 2 | 0.19 | 0.38 | 0.032 | 6 | 6 | 0.5 | 48 |
| DA30120 | *gltX* (D180G) | 24 | 0.047 | 0.032 | 1 | 0.016 | 0.75 | 0.25 | 0.094 | 0.032 | 4 | 2 | 0.5 | 96 |
| DA30122 | *ispA* (IS*1* insertion) | 16 | 0.032 | 0.023 | 1 | 0.016 | 2 | 0.094 | 0.125 | 0.032 | 1 | 2 | 0.38 | 96 |

[a] Mem, meropenem; Ctx, cefotaxime; Cip, ciprofloxacin; Nit, nitrofurantoin; Fof, fosfomycin; Tmp, trimethoprim; Sxt, trimethoprim-sulfamethoxazole; Nal, nalidixic acid; Ery, erythromycin.
[b] *E. coli* MG1655.
[c] del, deletion.
[d] FS, frame shift.
[e] KO, knockout.

Finally, an important implication of these findings is that the frequency of mutation to resistance in laboratory selections (as it is often used by the pharmaceutical industry and academic researchers to assess the risk of development of resistance to new antibiotics) might sometimes be a misleading predictor of clinical resistance development. That is, even though the frequency of mutation to Amd resistance in laboratory selections can be very high ($10^{-5}$), resistance development during treatment remains rare in clinical settings. This implies that a high frequency of mutation to resistance in the laboratory need not necessarily prohibit the development of a promising drug candidate. Instead, assessment of mutant fitness in a relevant physiological context might be a more predictive measure of the risk of resistance evolution in real life.

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