

Mechanisms of Decreased Susceptibility to Cefpodoxime in *Escherichia coli*

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Cefpodoxime is one of five antimicrobial agents recommended by the National Committee for Clinical Laboratory Standards for screening isolates of *Klebsiella* spp. and *Escherichia coli* for extended-spectrum β -lactamase (ESBL) production. In a prior study, we noted that among 131 *E. coli* isolates for which the MIC of at least one extended-spectrum cephalosporin (ESC) or aztreonam was ≥ 2 $\mu\text{g/ml}$ (suggesting the presence of ESBL production), there were 59 isolates (45.0%) for which the MIC of cefpodoxime was 2 to 4 $\mu\text{g/ml}$ (i.e., a positive ESBL screening test), but the MICs of ceftazidime, cefotaxime, and ceftriaxone were ≤ 1 $\mu\text{g/ml}$ (below the ESBL screening breakpoint). Thus, the results appeared to be false-positive ESBL screening tests. These 59 isolates were divided into five phenotypic groups based on the susceptibility patterns of the organisms to a variety of β -lactam agents and further characterized. The first group (32 isolates) all produced a TEM-1 β -lactamase, and changes in the major outer membrane proteins were detected in representative strains. The second group (18 isolates) lacked *bla*_{TEM} but showed a number of porin changes; some also showed a modest elevation in production of the AmpC chromosomal β -lactamase. In the third phenotypic group (seven isolates) all expressed an OXA-30 β -lactamase. Some also harbored altered porins. The two remaining phenotypes each had a distinct pattern of porin changes with or without β -lactamase production. These data indicate that several factors are associated with decreased susceptibility to cefpodoxime in *E. coli*, but none of the mechanisms are related to ESBL production. Current screening methods produced false-positive ESBL results for these isolates. Such isolates should not be classified as containing ESBLs, nor should interpretations of ESCs or aztreonam susceptibility be changed to resistant on test reports for these isolates.

Resistance to β -lactam agents in *Escherichia coli* is often mediated by the acquisition of plasmid-encoded β -lactamases (3, 14). These may be either broad-spectrum β -lactamases, such as TEM-1 or SHV-1, that confer resistance only to penicillins and narrow-spectrum cephalosporins, or extended-spectrum β -lactamases (ESBLs), including those derived from TEM-1, SHV-1, OXA-10, or CTX-M, that also confer resistance to extended-spectrum cephalosporins and aztreonam (ATM) (3, 14). Mutations in the attenuator or the weak promoter of the chromosomal *ampC* β -lactamase gene can lead to enhanced production of AmpC, resulting in resistance to penicillins and extended-spectrum cephalosporins (4, 12). Changes in major outer membrane protein (OMP) profiles, such as decreased production of OmpC or OmpF, may also result in low-level β -lactam resistance (9, 15, 29).

Most studies of β -lactam resistance in *E. coli* focus on strains showing high levels of resistance, particularly to extended-spectrum cephalosporins (4, 22). Few studies have investigated the mechanisms of low-level resistance. However, low-level resistance, while of arguable clinical relevance, does have an impact on the accuracy with which clinical laboratories identify ESBL-producing *E. coli* strains when using the NCCLS screening tests (21). The screening breakpoint for identifying potential ESBL-producing strains is ≥ 2 $\mu\text{g/ml}$ for cefpodoxime

(CPD), ceftazidime (CAZ), ceftriaxone, cefotaxime (CTX), and ATM, significantly lower than the suggested resistance breakpoints of 32 to 64 $\mu\text{g/ml}$ (21). NCCLS guidelines recommend that organisms with a positive screening test for ESBL production (MIC of ≥ 2 $\mu\text{g/ml}$ for any one of the five agents listed above) undergo additional testing using both CTX and CAZ, with and without clavulanic acid (the NCCLS recently changed the CPD screening breakpoint to ≥ 8 $\mu\text{g/ml}$). If a clavulanic acid effect (MIC reduced by ≥ 3 dilutions) is observed, the test interpretations for penicillins and cephalosporins should be changed to resistant. However, if confirmation testing is not performed on strains that have a positive screening test, then strains that do not produce ESBLs may be falsely reported as resistant to penicillins and cephalosporins. A survey of laboratories participating in the CDC Active Bacterial Core Surveillance program indicates that less than 50% of laboratories perform ESBL confirmatory tests, suggesting a potentially significant problem in overreporting of resistance (6).

In a previous study (F. C. Tenover, P. M. Raney, P. P. Williams, K. L. Brittain, C. D. Steward, S. K. Fridkin, R. P. Gaynes, and J. E. McGowan, Jr., Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. D1606, 2000) we noted that among 131 *E. coli* isolates for which the MIC of at least one extended-spectrum cephalosporin was ≥ 2 $\mu\text{g/ml}$, 59 (45%) showed reduced susceptibility to CPD (MICs of 2 to 4 $\mu\text{g/ml}$) but remained below the ESBL screening breakpoint for CAZ, CTX, and ceftriaxone (MICs ≤ 1 $\mu\text{g/ml}$). Because of the low

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TABLE 1. Primers used for PCR amplification of β -lactamase genes

Gene	Primer	Sequence (5'→3')	PCR product size (pb)
<i>E. coli ampC</i> promoter	AB1	GATCGTTCTGCCGCTGTG	271
	AmpC2	GGGCAGCAAATGTGGAGCAA	
TEM	Forward	ATGAGTATTCAACATTTCCG	867
	Reverse	CTGACAGTTACCAATGCTCTC	
SHV	Forward	GGTTATGCGTTATATTCGCC	867
	Reverse	TTAGCGTTGCCAGTGCTC	
OXA-1	OXA-1F	ACACAATACATATCAACTTCGC	814
OXA-2	OXA-1R	AGTGTGTGTTTAGAATGGTGATC	704
	OXA-2F	TTCAAGCCAAAGGCACGATAG	
	OXA-2R	TCCGAGTTGACTGCCGGGTTG	
OXA-10	OXA-10F	CGTGCTTTGTAAAAGTAGCAG	651
	OXA-10R	CATGATTTTGGT GGGAATGG	

MICs of CAZ and CTX, no clavulanic effect could be observed. The objective of this study was to characterize the mechanisms of β -lactam resistance in these 59 strains and to determine whether the results of the ESBL screening test with these organisms should be considered false positives.

MATERIALS AND METHODS

Bacterial isolates. Fifty-nine *E. coli* isolates for which the CPD MICs were 2 to 4 μ g/ml but the CAZ, CTX, and ceftriaxone MICs were ≤ 1 μ g/ml were selected from strains submitted from 26 Project ICARE (Intensive Care Antimicrobial Resistance Epidemiology) laboratories during a 4-year period (1995 to 1999). Microorganisms were identified using Vitek GNI+ cards (bioMérieux, Hazelwood, Mo.) or standard biochemical reactions (19). Three *E. coli* isolates from the Centers for Disease Control (CDC) culture collection, two whose MICs of only CPD (CDC10 and CDC110) were increased and one whose MICs to all extended-spectrum cephalosporins (CDC3100) were increased, due to enhanced chromosomal AmpC production, were also included. Control strains for OMPs included *E. coli* MH225 [MC4100 ϕ (*ompC lacZ*⁺)] (8) and *E. coli* C600, a well-characterized laboratory strain.

Susceptibility testing. The MICs of amoxicillin (AMX) (Sigma), ticarcillin (TIC) (GlaxoSmithKline, Collegeville, Pa.), piperacillin (PIP) (Sigma), cefazolin (CFZ) (Eli Lilly and Company, Indianapolis, Ind.), cefuroxime (CXM) (Eli Lilly and Co.), CPD (Pharmacia Upjohn, Peapack, N.J.), cefoxitin (FOX) (Sigma), CTX (Sigma), CAZ (Eli Lilly and Co.), cefepime (FEP) (Bristol-Myers Squibb, Wallingford, Conn.), and ATM (Bristol-Myers Squibb) were determined by broth microdilution according to NCCLS guidelines (20, 21) using cation-adjusted Mueller-Hinton broth (BD Biosciences, Sparks, Md.). Broth microdilution MICs also were determined for several β -lactam- β -lactamase inhibitor combinations, including AMX and clavulanate (CLAV) (GlaxoSmithKline, Philadelphia, Pa.) in a 2:1 ratio; CFZ, CXM, CPD, CTX, CAZ, and FEP each with 4- μ g/ml CLAV (fixed concentration); and PIP with a fixed 4- μ g/ml concentration of tazobactam (TZ) (Wyeth-Ayerst Pharmaceuticals, Pearl River, N.Y.). *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC 700603 were used as control strains.

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis was performed as previously described using the enzyme *BlnI* (35). Fragment patterns were interpreted as previously described by Tenover et al. (36).

IEF and specific β -lactamase activity determination. Isoelectric focusing (IEF) was performed using crude cell extracts as previously described (16). Hydrolysis rates (in micromoles of substrate hydrolyzed per minute and per microliter of crude cell extract) were determined using the SoftmaxPRO software for enzyme kinetics (Molecular Devices Corporation, Sunnyvale, Calif.) and a SpectraMAX PLUS spectrophotometer (Molecular Devices Corp.) equipped with a microplate reader. Activity was measured as a decrease in absorbance at 240 nm for a 100 μ M solution of cephaloridine. The experiments were performed in triplicate at 25°C, and the mean value was recorded. Specific cephalosporinase activity (in micromoles of substrate hydrolyzed per minute and per microgram of protein) was determined by normalizing hydrolysis rates against the concentration of total proteins in the crude cell extract. Total protein concentration was determined with the bicinchoninic acid method (BCA protein assay kit; Pierce, Rockford, Ill.) (32). Specific β -lactamase activity in isolates

harboring TEM β -lactamases was performed by measuring the hydrolysis of nitrocephin (observed as a decrease in absorbance at 482 nm) instead of cephaloridine.

Conjugation, cloning, and DNA sequence analysis of β -lactamase genes. Conjugation experiments were performed using a rifampin- and streptomycin-resistant variant of *E. coli* strain HB101 as the recipient. Transconjugants were selected on Mueller-Hinton agar (Difco) containing 100- μ g/ml ampicillin and a 100- μ g/ml concentration of either rifampin or streptomycin. β -Lactamase genes were cloned using plasmid pBGS18⁻, which contains a kanamycin resistance marker (33). Plasmid DNA, including pBGS18⁻ DNA, was purified from *E. coli* hosts using the QIAfilter plasmid midi kit (QIAGEN, Chatsworth, Calif.). The DNA was digested with *EcoRI* or *BamHI* as described below, ligated overnight with T4 DNA ligase, and transformed into CaCl₂-treated *E. coli* DH5 α competent cells. DH5 α transformants harboring the pBGS18⁻ plasmid with the inserted β -lactamase gene were selected on Mueller-Hinton agar containing 100- μ g/ml ampicillin and 50- μ g/ml kanamycin. β -Lactamase genes were subcloned by digesting transformant plasmids with *EcoRI*, *BamHI*, or *HindIII*, reducing the size of the insert containing the β -lactamase gene to 2 to 4 kb. The resulting DNA inserts from the subclones were sequenced in both directions using universal primers, and with primers specific for the coding region. DNA sequencing reactions were performed using ABI Prism dRhodamine terminator cycle sequencing (PE Applied Biosystems, Foster City, Calif.). Products from sequencing reactions were purified on Centri-Sep spin columns (Princeton Separations, Adelphia, N.J.) before analysis on an ABI Prism 377 DNA sequencer (PE Applied Biosystems).

PCR amplification of *ampC* promoter and attenuator regions. Primers AB1 and ampC2 (Table 1) were used to amplify the *E. coli ampC* promoter and attenuator regions as previously described (5). The forward and reverse sequences were determined using products from two independent PCRs as templates.

PCR amplification of β -lactamases. PCR amplification of *bla*_{TEM} and *bla*_{SHV} β -lactamase genes was performed as previously described (28). Analysis of the DNA sequences of oxacillin-hydrolyzing β -lactamases (OXAs) in GenBank were used to design specific primers for amplification of the most common subgroups in the OXA family of β -lactamases (Table 1). Primers OXA-1F and OXA-1R were designed to amplify an 814-bp fragment of the gene encoding the OXA-1 β -lactamase and the related OXA-4 and OXA-30 genes. Primers OXA-2F and OXA-2R amplify a 704-bp fragment of the OXA-2 gene and the closely related genes OXA-3, OXA-15, and OXA-21. Primers OXA-10F and OXA-10R amplify a 651-bp fragment of the OXA-10 gene and the related genes OXA-7, OXA-11, OXA-13, OXA-14, OXA-16, OXA-17, OXA-19, and OXA-28. Each PCR mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, a 0.1 μ M concentration of each primer, a 200 μ M concentration of each deoxynucleoside triphosphate, 2.5 U of *Taq* polymerase, and 1 μ l of crude cell lysate in a final volume of 100 μ l. The PCR conditions were 96°C for 5 min, 35 cycles of 96°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by a final extension cycle of 72°C for 10 min. The specificity of each set of primers was confirmed using DNA templates from a laboratory collection of *E. coli* strains harboring OXA-1, OXA-2, OXA-3, OXA-4, or OXA-7 β -lactamases.

OMP profile determination. OMPs were isolated from selected strains as previously described (9, 28). Briefly, cells were grown to mid-log phase in Luria-Bertani broth or nutrient broth and harvested by centrifugation. Cell pellets were washed and resuspended in sodium phosphate buffer, pH 7.0, and the cell walls

TABLE 2. β -Lactam resistance phenotypes for 59 *E. coli* isolates for which the MIC of cefpodoxime is ≥ 2 and that of ceftazidime is ≤ 1 $\mu\text{g/ml}^a$

	MIC ($\mu\text{g/ml}$)				
	Major phenotypes			Minor phenotypes	
	A	B	C	D	E
n strains (%)	32 (54.2%)	18 (30.5%)	7 (11.9%)	1 (1.7%)	1 (1.7%)
β -Lactamase pI	5.4	≥ 8.5	7.2		5.4, 5.7, 6.8
AMX	>64	32->64	>64	4	>64
AMX-CLAV	16-32	16-32	32->64	4	32
TIC	>128	8-32	>128	8	>128
PIP	>128	<4-8	>128	16	>128
PIP-TZ	8->128	<4-8	≥ 128	16	16
CFZ	16->256	4-64	16-32	2	16
CFZ-CLAV	2-4	2-16	4-8	1	2
CXM	16-32	8-32	32-128	16	128
CPD	2-4	2-4	4-8	4	2
FOX	16->32	8-32	8-32	16	16
CAZ	0.5-1	0.5-1	0.25-1	0.5	1
CAZ-CLAV	0.5	0.5-1	0.12-0.5	0.5	0.5
CTX	0.5	0.25-1	1	1	0.5
CTX-CLAV	0.25-0.5	0.12-0.5	0.25	0.5	0.5
FEP	0.12-0.5	0.06-0.25	2-4	0.25	0.5
FEP-CLAV	0.12	0.06-0.12	0.12-0.5	0.12	0.12

^a NCCLS breakpoints (S/I/R): AMX, CAZ, CXM, CFZ, FEP, FOX, $\leq 8/16/\geq 32$; CPD, $\leq 2/4/\geq 8$; CTX, $\leq 8/16-32/\geq 64$; PIP, TIC, $\leq 16/32-64/\geq 128$

were disrupted by treatment with lysozyme and sonication. Total membrane proteins were separated from cell debris by differential centrifugation. Sarkosyl-insoluble OMPs were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through 10% polyacrylamide-8 M urea gels. *E. coli* strains C600 and MH225 (OmpC⁻) were used as controls.

RESULTS

Fifty-nine *E. coli* isolates for which the CPD MICs were 2 to 4 $\mu\text{g/ml}$ were divided into five phenotypic groups (groups A to E) based on β -lactam susceptibility profiles, IEF results, and PCR assays for *bla*_{SHV}, *bla*_{TEM}, and *bla*_{OXA}. Characteristics of phenotypic groups A to E are presented in Table 2. Representative isolates from each group were selected for further study.

Characterization of isolates with phenotype A. The 32 isolates in this group were obtained from 11 hospitals with a maximum of eight isolates from a single hospital. No more than two isolates from any hospital had the same pulsed-field gel electrophoresis (PFGE) profile (data not shown). Isolates in phenotype A were identified by the following characteristics: (i) positive for *bla*_{TEM} by PCR and positive for a β -lactamase with pI 5.4, consistent with a TEM-type enzyme; (ii) high MICs for penicillin-type agents (AMX, TIC, and PIP); (iii) high MICs for FOX; (iv) low-level resistance to β -lactam- β -lactamase-inhibitor combinations (AMX-CLAV and PIP-TZ); and (v) low-level resistance to CFZ and CXM. Analysis of OMPs from six strains showed a variety of patterns, including isolates with modifications in both OmpC and OmpF. Two strains, EC410 and EC3437 (from two different hospitals), were investigated further. OmpF was missing in both EC410 (data not shown) and EC3437 (Fig. 1). An additional OMP, larger than OmpC, was produced by EC3437.

Conjugation experiments between EC410 or EC3437 and *E. coli* HB101 revealed a transferable β -lactamase with a pI of 5.4 that was associated with a carriage of a plasmid of approximately 150 kb. The antimicrobial resistance profiles of

transconjugants from both isolates (Table 3) were typical of strains expressing a TEM-1 β -lactamase. The transconjugants were resistant to penicillins, but the MICs of cephalosporins (including those of CFZ, CXM, and FOX) and β -lactam- β -lactamase inhibitor combinations remained low. The MICs of CAZ and CTX remained close to the baseline levels (0.5 and 0.12 $\mu\text{g/ml}$, respectively) for the host strain. Thus, the mechanisms associated with the isolates of phenotype A appear to be production of TEM-1 β -lactamase in conjunction with modification of porins.

Strain EC3006 had an antimicrobial resistance pattern similar to those of the other strains from phenotype A, but with a higher resistance to CFZ. The CFZ MIC was markedly reduced by CLAV (Table 2). PCR was positive for *bla*_{TEM}, and IEF revealed a TEM-type β -lactamase with a pI of 5.4. Penicillin and narrow-spectrum cephalosporin resistance was transferred to *E. coli* HB101 by conjugation; however, the MICs of several β -lactams, including CFZ, CXM, FOX, and CPD (Table 4), were significantly lower than those of the EC3600 parent, suggesting that alteration of the porins in the parent strain contributed to the higher level of resistance. A significant CLAV effect was observed for CFZ. A 25-kb *Bam*HI fragment harboring a β -lactamase gene was cloned from the HB101 transconjugant into pBGS18⁻ plasmid, and an approximately 3-kb *Eco*RI-*Hind*III fragment from this plasmid was isolated and subcloned. DNA sequence analysis revealed an open reading frame with a nucleotide sequence identical to the TEM-1 β -lactamase (data not shown). Further analysis revealed a *bla*_{TEM-1B} promoter (2, 13) which is characterized by a C-to-T nucleotide substitution at position 32 (177 bp upstream of the coding region). This substitution is found in TEM-2 β -lactamase and its derivatives and is known to create two overlapping promoter sequences, resulting in a 10-fold increase in penicillin resistance (7) Assays of specific enzymatic activity

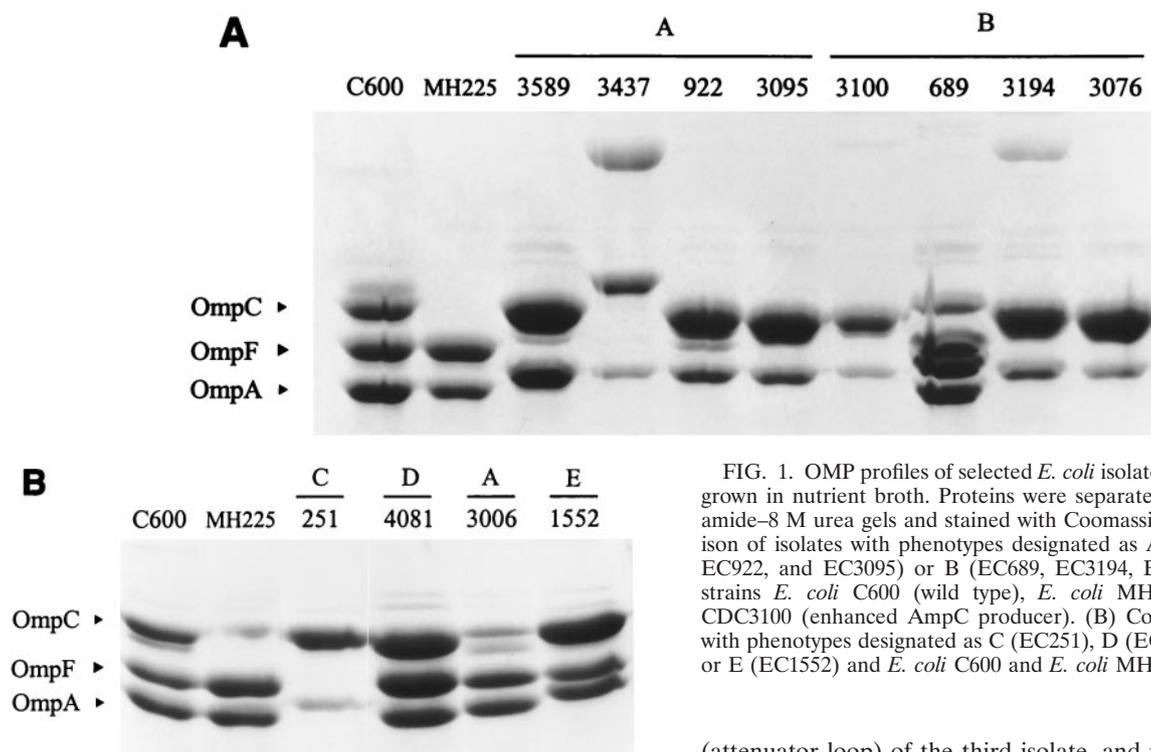


FIG. 1. OMP profiles of selected *E. coli* isolates and control strains grown in nutrient broth. Proteins were separated on 10% polyacrylamide-8 M urea gels and stained with Coomassie blue. (A) Comparison of isolates with phenotypes designated as A (EC3589, EC3437, EC922, and EC3095) or B (EC689, EC3194, EC3076) and control strains *E. coli* C600 (wild type), *E. coli* MH225 (OmpC⁻), and CDC3100 (enhanced AmpC producer). (B) Comparison of isolates with phenotypes designated as C (EC251), D (EC4081), A (EC3006), or E (EC1552) and *E. coli* C600 and *E. coli* MH225 (OmpC⁻).

demonstrated a significant increase in β -lactamase production of EC3006 compared to an isolate producing a classic TEM-1 β -lactamase (350.9 ± 39.5 versus 41.9 ± 4.5 nmol min⁻¹ mg⁻¹, respectively). As previously demonstrated (24, 34, 37), TEM-1 hyperproduction is likely responsible for the higher resistance levels for AMX-CLAV and CFZ found in the EC3006 transconjugant compared with expected MICs of an unmodified TEM-1 β -lactamase. Additionally, the porin profile for EC3006 indicated that the production of OmpC was significantly decreased, and an additional OMP intermediate in size between OmpC and OmpF, was present. The relative quantity of OmpF did not appear to be altered when compared with the OMP profiles of the control strain, *E. coli* C600.

Characterization of isolates with phenotype B. The second group of 18 isolates (phenotype B) also showed multiple PFGE patterns (data not shown). These strains, isolated from nine different hospitals, were characterized by antimicrobial susceptibility profiles that included resistance to AMX but susceptibility or intermediate resistance to TIC and PIP (Table 2). Susceptibility to CFZ, CPD, and FOX varied among the isolates, and no significant CLAV effect was observed. Since a pI band of ≥ 8.5 was observed in the IEF, enhanced AmpC production was initially suspected as a β -lactam resistance mechanism. The cephalosporinase-specific activity of four isolates from four different hospitals showed only a modest 2- to 2.5-fold increase when compared with *E. coli* strain DH5 α (Table 5). DNA sequence analysis of the *ampC* promoter and attenuator regions revealed identical mutations in two isolates at position -76 (upstream from the start codon) and at positions +22, +26, +27, and +32 in the attenuator loop. Nucleotide substitutions were noted at positions -73, -66, -28, and +17

(attenuator loop) of the third isolate, and no mutations were detected in the fourth isolate (Fig. 2).

To investigate whether these nucleotide substitutions contribute to CPD resistance by increasing the production of AmpC, the study isolates were compared with control strains CDC10, CDC110, and CDC3100, which express increased levels of AmpC (Table 5). Increased production of AmpC was associated with MICs of CPD of 8 to 16 μ g/ml (data not shown) and cephalosporinase activity that was increased 14- to

TABLE 3. MICs for EC410 and EC3437 and transconjugants

Antibiotic	MIC (μ g/ml) for:				
	HB101	EC410	HB101/410TC	EC3437	HB101/3437TC
AMX	4	>64	>64	>64	>64
AMX-CLAV	4	16	8	16	4
TIC	≤ 4	>128	>128	>128	>128
PIP	≤ 4	>128	>128	>128	>128
PIP-TZ	≤ 4	≤ 4	≤ 4	32	≤ 4
CFZ	2	16	4	32	4
CFZ-CLAV	1	4	1	4	1
CXM	4	32	4	16	4
CXM-CLAV	2	16	2	16	2
FOX	4	32	4	32	4
CPD	1	2	1	4	1
CPD-CLAV	0.5	2	0.5	2	0.5
CAZ	0.5	0.5	0.5	0.5	0.5
CAZ-CLAV	0.25	0.25	0.25	0.5	0.25
CTX	0.12	0.5	0.12	0.25	0.12
CTX-CLAV	0.12	0.25	0.06	0.25	0.12
ATM	0.25	0.5	0.25	0.5	0.25
ATM-CLAV	0.12	0.5	0.12	0.25	0.25
FEP	0.06	0.12	0.06	0.12	0.12
FEP-CLAV	0.06	0.06	0.06	0.12	0.06

^a Strains EC410, EC3437 and their respective transconjugants harbored a pI 5.4 TEM beta-lactamase (see characterization of phenotype A).

TABLE 4. Resistance profiles of EC251 and EC3006 and their respective transconjugants and clones^a

Antibiotic	MIC ($\mu\text{g/ml}$)							
	HB101	DH5 α	EC251	HB101 251TC	DH5 α (pBG251)	EC3006	HB101 3006TC	DH5 α (pBG3006)
AMX	4	≤ 2	>64	>64	>64	>64	>64	>64
AMX-CLAV	4	≤ 2	32	16	32	32	16	32
TIC	≤ 4	≤ 4	>128	>128	>128	>128	>128	>128
PIP	≤ 4	≤ 4	>128	64	>128	>128	>128	>128
PIP-TZ	≤ 4	≤ 4	128	16	>128	>128	128	>128
CZ	2	1	16	4	32	>256	32	256
CZ-CLAV	1	1	4	1	1	2	1	1
CXM	4	2	32	8	64	32	8	16
CXM-CLAV	2	1	16	4	4	16	4	2
FOX	4	2	32	4	2	16	4	2
CPD	1	0.5	4	1	16	4	1	2
CPD-CLAV	0.5	0.5	2	0.5	0.5	2	0.5	0.25
CAZ	0.5	0.25	0.5	0.25	0.25	1	0.5	0.5
CAZ-CLAV	0.25	0.25	0.25	0.25	0.12	0.5	0.25	0.25
CTX	0.12	0.06	1	0.5	4	0.5	0.12	0.12
CTX-CLAV	0.12	0.06	0.25	0.12	0.12	0.5	0.06	0.06
ATM	0.25	0.06	0.5	0.12	0.5	0.5	0.25	0.12
ATM-CLAV	0.12	0.06	0.5	0.12	0.06	0.25	0.12	0.12
FEP	0.06	0.06	2	1	>32	0.5	0.12	0.5
FEP-CLAV	0.06	0.03	0.12	0.12	0.25	0.12	0.06	0.12

^a EC251 and its corresponding transconjugant (HB101 251TC) and clone [DH5 α (pBG251)] harbored an OXA-30 β -lactamase (see characterization of phenotype C) and EC3006 and its corresponding transconjugant (HB101 3006TC) and clone [DH5 α (pBG3006)] harbored a TEM-1 β -lactamase.

17-fold when compared with that observed in *E. coli* DH5 α . DNA sequence analysis revealed the following mutations that are known to result in increased transcription of *ampC*: CDC110, a nucleotide substitution at the -32 position; CDC10 and CDC3100; and a 1-bp and a 2-bp insertion between the -35 and the -10 promoter consensus sequences, respectively. Each of the CDC isolates and the study isolates also had several additional nucleotide substitutions when compared with the *E. coli* K-12 sequence (Fig. 2).

The OMP profiles were altered for most of the selected isolates. EC689 showed a decrease in OmpC and an additional OMP between OmpC and OmpF, whereas EC3076 and EC3194 appeared to have lost OmpF (Fig. 1). AmpC-hyperproducing strains from the CDC collection for which the MICs

of only CPD (8 to 16 $\mu\text{g/ml}$) among the extended-spectrum cephalosporins were increased (CDC10 and CDC110) showed no apparent alterations of OMPs. On the other hand, strain CDC3100, for which MICs to all extended-spectrum cephalosporins were increased, appeared to have lost OmpF in addition to the AmpC hyperproduction (Fig. 1). Altogether these results suggest that the β -lactam resistance phenotype in this group of strains is mainly due to the loss of porin expression and not to AmpC hyperproduction.

Characterization of phenotype C. Seven isolates from five hospitals, each with a unique PFGE pattern, were grouped in phenotype C. Each isolate expressed a β -lactamase with a pI of 7.1 to 7.2. PCR assays were positive for *bla*_{OXA} and negative for *bla*_{TEM} and *bla*_{SHV}. Phenotypically, these strains showed high-level resistance to penicillins that was not significantly decreased by the addition of CLAV (Table 2). Resistance to CFZ, CXM, and FOX was variable. CTX MICs of 0.25 to 1 $\mu\text{g/ml}$ were below the resistant breakpoint but were slightly above those for other *E. coli* isolates. A modest CLAV effect was observed with CTX but was more apparent with FEP. Conjugation of *E. coli* strain EC251 with HB101 resulted in the transfer of a β -lactamase gene that was associated with a plasmid of approximately 160 kb. The β -lactamase gene was localized to a 2.3-kb *Hind*III restriction fragment and subcloned into *E. coli* DH5 α (pBG251B3H1). A search of the GenBank database indicated sequence similarity with a fragment of Tn2603 that encodes the OXA-1 β -lactamase (26, 38). PCR amplification with the *bla*_{OXA-1} primers and DNA sequence analysis indicated that each of the isolates in the phenotype C contained the OXA-30 enzyme, which differs from OXA-1 by an Arg131-to-Gly modification (31). In addition to the OXA β -lactamase, analysis of the OMPs from strain EC251 revealed that OmpF was missing (Fig. 1). These data suggest that the

TABLE 5. Mutations in *ampC* promoter and attenuator regions and cephalosporinase specific activity of selected *E. coli* isolates

Strain	Mutations in promoter and attenuator ^a	Cephalosporinase sp act ^b
DH5 α	Same as <i>E. coli</i> K-12 control	1.00
EC689	-73, -66, -28, +17	2.59 \pm 0.26
EC3076	-76, +22, +26, +27, +32	2.04 \pm 0.13
EC3194	-76, +22, +26, +27, +32	2.67 \pm 0.12
EC3358	No mutations detected	2.40 \pm 0.09
Control strains		
CDC10	-73, -28, 1-bp insert between -35 and -10, +17	13.6 \pm 2.2
CDC110	-73, -32, +58	14.6 \pm 1.3
CDC3100	-76, 2-bp insert between -35 and -10, +22, +26, +27, +32	17.2 \pm 3.7

^a Key mutations associated with enhanced AmpC production are indicated by bold type.

^b Specific cephalosporinase activities are relative values based on the activity in the wild type strain DH5 α .

depends on the diffusion rate of the drug across the outer membrane; activity of efflux pumps, such as the AcrAB-TolC system; and the hydrolysis of the drug by the chromosomal AmpC β -lactamase (17, 23). Although present at very low levels, the AmpC β -lactamase plays a key role in the intrinsic resistance of *E. coli* to β -lactam agents (17, 23). Mutations leading to a stronger promoter (12); the acquisition of a stronger promoter by horizontal DNA transfer from other species, such as *Shigella sonnei* (25); or mutations in the transcriptional attenuator (11) of the *E. coli* AmpC chromosomal β -lactamase lead to enhanced production of AmpC which increases the level of resistance to β -lactam agents, including both penicillins and cephalosporins. Acquisition of plasmid-mediated β -lactamases also plays a major role in β -lactam resistance (14). However, porin alterations, as shown in this study, although frequently overlooked as a mechanism of β -lactam resistance, clearly play a key role in β -lactam resistance, especially when present in strains containing β -lactamases (9, 15, 29).

Alteration of the major porin proteins, OmpF and OmpC, may be due to mutations in the regulatory genes, such as *mar* (30) or *ompB* (8), or to alteration of the individual porin genes by insertions, deletions, or point mutations. Interruption of porin genes by insertion sequences is a common cause of porin loss in *Enterobacteriaceae* (10, 27), and the correlation of antimicrobial resistance and porin loss has been demonstrated in several enterobacterial species (1, 10, 18).

The most common mechanism of reduced susceptibility to CPD among the isolates in this study was production of a TEM-1 β -lactamase associated with the loss or alteration of a major porin protein. While the effect on the MICs of CTX and CAZ was minimal, the changes were sufficient to elevate the CPD MICs above the screening breakpoint for ESBLs set by the NCCLS.

The mechanism responsible for the second most common phenotype among the 59 isolates was initially considered to be enhanced production of the chromosomal AmpC β -lactamase. Data supporting this hypothesis included PCR assays that were negative for *bla*_{TEM}, *bla*_{SHV}, and *bla*_{OXA} and detection of a β -lactamase with a pI of 8.3 to 8.5 by IEF. On the other hand, although nucleotide substitutions in the *ampC* promoter or attenuator regions were found, they were not those known to be associated with enhanced AmpC production, and only a modestly enhanced β -lactam hydrolysis (2 to 2.5 times the background levels) was detected. Control strains showing *ampC* activity 14 to 17 times that of wild-type *E. coli* controls did show known mutations in *ampC*, including nucleotide insertions between the -35 and -10 promoter sequences, and a nucleotide substitution at the -32 position (4, 12). In addition, the study strains expressed lower levels of resistance to the extended-spectrum cephalosporins than expected for strains with enhanced AmpC production (14). Thus, alteration of porin proteins, as shown for strains EC689, EC3194, and EC3076, is the resistance mechanism most likely to be responsible for this phenotype. Strains CDC10 and CDC110 with high cephalosporinase activity had higher CPD MICs (8 to 16 μ g/ml) than the study strains but remained susceptible to other extended-spectrum cephalosporins (CAZ \leq 1 μ g/ml), whereas the only strain with high extended-spectrum cephalosporin MICs, CDC3100, had a combination of AmpC hyperproduc-

tion and OmpF loss. These results suggest that many of the previously reported AmpC-producing strains that have higher levels of resistance to extended-spectrum cephalosporins may also have porin alterations. Unfortunately, few investigators determined the OMP profiles of strains that they reported as AmpC-associated resistance (4, 22). Therefore, the significance of this mechanism of resistance in terms of actual challenge for treatment of *E. coli* infections remains unknown.

The third mechanism of reduced susceptibility involved production of an OXA-30 β -lactamase that was also associated with porin changes in some strains. The isolates in this study came from a variety of hospitals in the United States, suggesting that this β -lactamase is widely distributed. The extended-spectrum cephalosporin most affected by OXA-30 is FEP. MICs of FEP were 2 to 4 μ g/ml for isolates in this phenotypic group, in contrast to MICs of 0.06 to 0.12 μ g/ml that are typical for wild-type *E. coli* isolates. In transformants containing the cloned OXA-30 β -lactamase gene, where the enzyme is produced in larger amounts, MICs of FEP and CTX were >32 and 4 μ g/ml, respectively. Thus, inoculum effects may be important.

It is clear that CPD MICs are more heavily influenced by porin changes, enhanced TEM production, and OXA-type β -lactamases than are the MICs of CTX or CAZ. This is likely the reason for the high number of false-positive results obtained when CPD is used for screening *E. coli* isolates for ESBLs. The NCCLS recommends that strains harboring ESBLs be considered resistant to all penicillins and cephalosporins whether the MICs are above the traditionally recommended breakpoints or not (21). We support these recommendations. Recently, the NCCLS changed the CPD screening breakpoint from ≥ 2 to ≥ 8 μ g/ml based on additional data from our laboratory (P. M. Raney, P. P. Williams, J. E. McGowan, Jr., and F. C. Tenover, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. D-182, 2001). In summary, we have characterized a series of *E. coli* isolates that display decreased susceptibility to CPD only, producing what we believe to be false-positive ESBL screening test results. Such isolates should not be classified as ESBLs, nor should they be classified as containing ESBLs, nor should the interpretations of extended-spectrum cephalosporins be changed to "resistant" on susceptibility test reports.

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