Four Variants of the *Citrobacter freundii* AmpC-Type Cephalosporinases, Including Novel Enzymes CMY-14 and CMY-15, in a *Proteus mirabilis* Clone Widespread in Poland

Elżbieta Literacka, Joanna Empel, Anna Baraniak, Ewa Sadowy, Waleria Hryniewicz, and Marek Gniadkowski*

National Institute of Public Health, Warsaw, Poland

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Twenty-nine *Proteus mirabilis* isolates from 17 Polish hospitals were analyzed. The isolates were resistant to a variety of antimicrobials, and their patterns of resistance to β -lactams resembled those of the constitutive class C cephalosporinase (AmpC) producers. Indeed, β -lactamases with a pI of ~ 9.0 were found in all of the isolates, and they were subsequently identified as four AmpC-type cephalosporinases, CMY-4, -12, -14, and -15, of which the two last ones were novel enzyme variants. The enzymes were of *Citrobacter freundii* origin and were closely related to each other, with CMY-4 likely being the evolutionary precursor of the remaining ones. The *bla*_{CMY} genes were located exclusively in chromosomal DNA, within EcoRI restriction fragments of the same size of ~ 10 kb. In the CMY-12- and -15-producing isolates, an additional fragment of ~ 4.5 kb hybridized with the *bla*_{CMY} probe as well, which could have arisen from a duplication event during the evolution of the genes. In all of the isolates, the *ISEcp1* mobile element, which most probably is involved in mobilization of the *C. freundii* *ampC* gene, was placed at the same distance from the 5' ends of the *bla*_{CMY} genes, and sequences located between them were identical in isolates carrying each of the four genes. These data suggested that a single chromosome-to-chromosome transfer of the *ampC* gene from *C. freundii* to *P. mirabilis* could have initiated the spread and evolution of the AmpC-producing *P. mirabilis* in Poland. The hypothesis seems to be confirmed by pulsed-field gel electrophoresis typing, which revealed several cases of close relatedness between the *P. mirabilis* isolates from distant centers and showed an overall similarity between the majority of the multiresistant isolates.

Although not as prevalent as extended-spectrum β -lactamases (ESBLs), acquired class C cephalosporinase (AmpC)-type cephalosporinases over time have become an important source of enterobacterial resistance to newer-generation β -lactams (31, 37). These are derivatives of enzymes specific for such organisms as *Enterobacter cloacae*, *Citrobacter freundii*, *Morganella morganii*, *Hafnia alvei*, and *Acinetobacter baumannii* (30, 37). They usually confer resistance to penicillins, cephalosporins, and monobactams and to inhibitor, mainly clavulanic acid, combinations (28, 31, 37). Because of the lack of simple and specific detection tests, there is little information on their prevalence in nosocomial *Enterobacteriaceae* populations. Their remarkable representation was already observed in U.S. hospitals in the first half of the 1990s (1), and this has been confirmed by more recent studies (20, 32). In one of these, the AmpC producers accounted for 2.6% of *Klebsiella pneumoniae* blood isolates in 30 centers in 23 states (20). A comparable frequency of ESBLs and acquired AmpCs (1.6%) was observed in *Escherichia coli* in a hospital in Taiwan (46).

Genes coding for the acquired cephalosporinases are usually located in transferable plasmids, and several lines of evidence indicate that mobilization of the species-specific chromosomal *ampC* genes is mediated by transposable elements (37). Among these are transposons containing the *ISEcp1* insertion sequence, found recently in the vicinity of 5' ends of various

β -lactamase genes, including the *ampC*-type *bla*_{CMY-4} gene (10, 39; P. D. Stapleton, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1457, p. 132, 1999). Of the more than 20 acquired AmpC-type enzymes identified so far (30, 37), the group related to the *C. freundii* cephalosporinase is the biggest, with the CMY-2-BIL-1-LAT-2 variant being likely the common ancestor of the remaining family members (7, 9).

For a relatively long time, *Proteus mirabilis* was one of the most β -lactam-susceptible organisms of the clinically important enterobacteria (28). However, in recent years, a wide variety of β -lactamases have been identified in *P. mirabilis* isolates worldwide, including ESBLs and AmpC-type cephalosporinases (11, 18, 37). Whereas data from several countries have already demonstrated a high prevalence of ESBLs in *P. mirabilis* (18, 34, 35, 36, 38, 45), isolates with acquired AmpC-type enzymes seem to be rare. Interestingly, relatively frequently they had the *ampC*-type genes located in chromosomal DNA (14, 22). In this work we have analyzed 29 *P. mirabilis* isolates from 17 hospitals, all of which were found to express chromosomally encoded cephalosporinases of *C. freundii* origin and which were the first enterobacterial isolates with acquired AmpC β -lactamases to be studied in Poland.

MATERIALS AND METHODS

Clinical isolates. Twenty-nine multiresistant *P. mirabilis* clinical isolates were analyzed in this study (Table 1). They were identified between 1998 and 2001 in 17 medical centers of 16 different cities located all over Poland. Eight of the isolates, recovered in a hospital in Grajewo (in the northeastern part of the country), were selected from a set of 34 *P. mirabilis* isolates from this center that had been sent to the National Institute of Public Health (NIPH) in Warsaw for

* Corresponding author. Mailing address: National Institute of Public Health, ul. Chełmska 30/34, 00-725 Warsaw, Poland. Phone: 48 22-851 43 88. Fax: (48) 22-841 29 49. E-mail: gniadkow@cls.edu.pl.

TABLE 1. *P. mirabilis* isolates

Isolate	Yr of isolation	Center (city)	Specimen	PFGE type	β -Lactamase(s) (pI)	<i>bla</i> _{CMY} PCR ^a	CMY variant	EcoRI genomic DNA band(s) (kb) hybridizing with <i>bla</i> _{CMY}
318	1999	Częstochowa	Wound swab	A12	~9.0, 5.6	+	CMY-15	~10, ~4.5
9487	1999	Częstochowa	Wound swab	A13	~9.0, 5.6	+	ND ^b	ND
777	2000	Gdańsk	Urine	A16	~9.0, 5.6	+	ND	ND
27	1999	Grajewo	Urine	A1	~9.0, 5.6	+	CMY-12	~10, ~4.5
5010	1999	Grajewo	Urine	A7	~9.0, 5.6	+	CMY-12	~10, ~4.5
6184	1999	Grajewo	Urine	A4	~9.0, 5.6	+	ND	ND
6195	1999	Grajewo	Urine	A3	~9.0, 5.6	+	ND	ND
6210	1999	Grajewo	Urine	A6	~9.0, 5.6	+	CMY-12	~10, ~4.5
6226	1999	Grajewo	Intubation tube	A5	~9.0, 5.6	+	ND	ND
6227	1999	Grajewo	Wound swab	A2	~9.0, 5.6	+	ND	ND
6422	1998	Grajewo	Urine	B	~9.0, 5.6	+	CMY-12	~10, ~4.5
8345	1999	Kłobuck	Urine	A22	~9.0, 5.6	+	CMY-12	~10, ~4.5
864	2001	Lublin	Sputum	A11	~9.0, 5.4	+	CMY-4	~10
6103	1999	Maków Maz.	Urine	A17	~9.0, 5.6	+	CMY-15	~10, ~4.5
2135	2000	Olsztyn	Pus	A18	~9.0, 5.4	+	CMY-15	~10, ~4.5
3010	2000	Olsztyn	Urine	A19	~9.0, 5.4	+	ND	ND
6523	1999	Rzeszów	Urine	A24	~9.0, 5.6	+	CMY-15	~10, ~4.5
8709	1999	Sosnowiec	Blood	A21	~9.0, 5.6	+	CMY-12	~10, ~4.5
1671	2000	Suwałki	Urine	A20	~9.0, 5.6	+	CMY-15	~10, ~4.5
6769	1999	Świecie	Urine	A23	~9.0, 5.6	+	CMY-15	~10, ~4.5
1180	2000	Warsaw 1	Urine	A8	~9.0, 5.4	+	ND	ND
1181	2000	Warsaw 1	Urine	A8	~9.0, 5.4	+	ND	ND
1662	2000	Warsaw 1	Pus	A9	~9.0, 5.6	+	CMY-15	~10, ~4.5
6735	1999	Warsaw 2	Urine	C	~9.0, 5.4	+	CMY-14	~10
2384	2001	Wejherowo	Wound swab	A15	~9.0	+	CMY-15	~10, ~4.5
7767	1999	Wolomin	Wound swab	A25	~9.0, 5.4	+	CMY-15	~10, ~4.5
1243	2000	Zawiercie	Pus	A14	~9.0, 5.6	+	CMY-15	~10, ~4.5
2014	2000	Zawiercie	Urine	A13	~9.0, 5.4	+	CMY-15	~10, ~4.5
1608	2000	Zielona Góra	Urine	A10	~9.0, 5.4	+	CMY-15	~10, ~4.5
5653 ^c	1999	Poznań		D		—		
7491 ^c	1999	Ostrów Maz.		E		—		
7882 ^c	1999	Warsaw 3		F		—		
6519 ^c	1999	Rzeszów		G		—		
22317 ^d		Paris				+	CMY-4 ^e	

^a + and —, positive and negative PCR result, respectively.

^b ND, not determined.

^c β -Lactam-susceptible *P. mirabilis* isolate from a Polish hospital, used only as an unrelated control in the PFGE analysis (see Materials and Methods) and as a negative control in the *bla*_{CMY} PCR.

^d CMY-4-producing *P. mirabilis* isolate from Hôpital Tenon, Paris, France (22), used only as a positive control in the *bla*_{CMY} PCR.

^e From reference 22.

molecular typing. Their selection was based on the pulsed-field gel electrophoresis (PFGE) typing results, and each of the isolates included in the study represented a distinct PFGE type or subtype. The other isolates, identified in the remaining institutions (one to three isolates per center), were sent to the NIPH by clinical microbiology laboratories for susceptibility retesting. All of the isolates were recovered from laboratory patients. The majority of the isolates ($n = 18$) were cultured from urine samples; the other clinical specimens were wound swabs ($n = 5$), pus ($n = 3$), blood ($n = 1$), intubation tube swab ($n = 1$), and sputum ($n = 1$). The isolates were reidentified in the NIPH with the ATB ID32E tests (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing. The MICs of various antimicrobials were evaluated by the agar dilution method according to the National Committee for Clinical Laboratory Standards guidelines (33). The following compounds were used: amikacin, aztreonam, and cefepime (Bristol-Myers Squibb, New Brunswick, N. J.); ampicillin, cefotaxime, and gentamicin (Polfa Tarchomin, Warsaw, Poland); cefoxitin, chloramphenicol, and tetracycline (Sigma Chemical Company, St. Louis, Mo.); ceftazidime (Glaxo SmithKline, Stevenage, United Kingdom); ciprofloxacin (Bayer, Wuppertal, Germany); lithium clavulanate (Glaxo SmithKline, Betchworth, United Kingdom); imipenem (Merck Sharp & Dohme, Rahway, N. J.); piperacillin and tazobactam (Wyeth, Pearl River, N.Y.); trimethoprim-sulfamethoxazole (Roche, Basel, Switzerland); and tobramycin (Eli Lilly, Indianapolis, Ind.). In all β -lactam inhibitor combinations the constant concentrations of clavulanate and tazobactam were 2 and 4 μ g/ml, respectively, except for one of the amoxicillin-clavulanate combinations, in which a constant ratio of 2:1 between the two compounds was kept. *E. coli* ATCC 25922 and *P. mirabilis* ATCC 7002 were used as reference strains. The possible production of

ESBLs was checked with the double-disk synergy test (27) with disks containing amoxicillin with clavulanate (20 and 10 μ g, respectively), cefotaxime (30 μ g), and ceftazidime (30 μ g) (Oxoid, Basingstoke, United Kingdom) on Mueller-Hinton agar (Oxoid) plates that were unsupplemented and supplemented with 250 μ g of cloxacillin (Polfa Tarchomin) per ml.

Mating. The cefotaxime resistance transfer experiment was performed as described previously (26), with rifampin-resistant *E. coli* A15 as a recipient strain.

PFGE typing. For the PFGE analysis, total DNAs of the isolates were purified as described by Struelens et al. (41) and digested sequentially with NotI and SfiI restriction enzymes (New England BioLabs, Beverly, Mass.). The resulting DNA fragments were electrophoresed in a DR III contour-clamped homogeneous electric field apparatus (Bio-Rad, Hercules, Calif.). Four *P. mirabilis* clinical isolates that are susceptible to all antienterobacterial β -lactam antibiotics were used as epidemiologically unrelated control strains in the analysis. These isolates were identified in the first half of 1999 in four different hospitals in Poland, including one center from which one multiresistant isolate studied was collected (a hospital in Rzeszów). The PFGE results were interpreted according to the criteria described by Tenover et al. (43).

IEF of β -lactamases and detection of their oxyimino- β -lactam-hydrolyzing activities. Isoelectric focusing (IEF) of β -lactamases was carried out with sonicates of the isolates as described by Bauernfeind et al. (8), using a model 111 Mini IEF Cell (Bio-Rad). β -Lactamases were visualized with 1 mM nitrocefin (Oxoid) and with 0.5 mM nitrocefin with 0.3 mM cloxacillin (Polfa Tarchomin). Following IEF, protein extracts were subjected to the bioassay for cefotaxime- and ceftazidime-hydrolyzing activities as described by Bauernfeind et al. (8). The concentration of the two cephalosporins was 2 μ g/ml.

TABLE 2. DNA primers used in this study

Designation	Target gene	Sequence (5'→3')	Annealing positions ^a	Purpose	Expected amplicon size ^b (bp)	Reference
CF-A	<i>bla</i> _{CMY} ^c	ATCCGGGTATGGCCGT	133–149	PCR, sequencing	836 with CF-B	14
CF-B	<i>bla</i> _{CMY} ^c	GGGTTTACCTCAACGGC	968–952	PCR, sequencing		14
CF-1	<i>bla</i> _{CMY} ^c	ATGATGAAAAAATCGATATG	1–20	PCR, sequencing	1,146 with CF-2	This study
CF-2	<i>bla</i> _{CMY} ^c	TTATTGCAGTTTTCAAGAATG	1146–1125	PCR, sequencing		This study
CF-3	<i>bla</i> _{CMY} ^c	CCTGCTCCTGCATCAG	130–115	PCR, sequencing		This study
CF-4	<i>bla</i> _{CMY} ^c	GCAGATCCCCGATGACG	417–433	Sequencing		This study
CF-5	<i>bla</i> _{CMY} ^c	CAGCGTTTGCTGCGTG	237–222	Sequencing		This study
ALA-4	<i>ISEcp1 tnpA</i>	CTATCCGACAAGGGAG	1224–1240	PCR, sequencing	486 with CF-3 ^d	5
ALA-4-HindIII	<i>ISEcp1 tnpA</i>	CCCAAGCTTCTATCCGACAAGGGAG	1224–1240	Cloning	1,520 with CF-2-EcoRI ^d	This study
CF-2-EcoRI	<i>bla</i> _{CMY} ^c	CCGGAATTCTTATTCAGTTTTCAAG	1146–1129	Cloning		This study
TEM-A	<i>bla</i> _{TEM}	ATAAAATTCTTGAAGAC	–6–11	PCR, sequencing	1,075 with TEM-B	29
TEM-B	<i>bla</i> _{TEM}	TTACCAATGCCTTAATCA	1069–1053	PCR, sequencing		29
TEM-C	<i>bla</i> _{TEM}	CCCCGAAGAACGTTTTTC	385–401	Sequencing		29
TEM-D	<i>bla</i> _{TEM}	CTGCAGCAATGGCAACA	750–766	Sequencing		29
TEM-E	<i>bla</i> _{TEM}	TCGTCGTTTGGTATGGC	732–716	Sequencing		29

^a Position numbering is with respect to the first nucleotides of the coding regions of the *bla*_{CMY} and *ISEcp1 tnpA* genes, or as described by Sutcliffe (42) in the case of *bla*_{TEM} genes. Annealing positions of primers are directed from the 5' end to the 3' end of each primer.

^b PCR product sizes are shown only at the forward primer in each of the primer pairs used.

^c *bla*_{CMY} gene of *C. freundii* origin.

^d The size of the product is specific for the isolates in this study.

PCR identification and sequencing of *bla*_{CMY} genes. Total DNA preparations of the isolates were obtained with the genomic DNA Prep Plus kit (A&A Biotechnology, Gdynia, Poland) and used for PCR amplification of *bla*_{CMY} genes. Initially, the reactions were run with primers CF-A and CF-B, which anneal inside *C. freundii*-type AmpC cephalosporinase genes (Table 2). The entire *bla*_{CMY} gene coding regions were amplified with primers CF-1 and CF-2, which anneal to the very 5' and 3' ends of the *C. freundii ampC* gene coding region, respectively (Table 2). The PCR conditions were as those described previously (14). The amplicons containing the complete *bla*_{CMY} coding regions were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and directly sequenced on both strands by using primers CF-A, CF-B, CF-1, CF-2, CF-3, CF-4, and CF-5 (Table 2). Sequencing was performed with the use of an ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster City, Calif.).

PCR and sequencing of *bla*_{TEM} genes. The *bla*_{TEM} genes were amplified with primers TEM-A and TEM-B and sequenced with primers TEM-A, TEM-B, TEM-C, TEM-D, and TEM-E (Table 2) as described previously (26).

Analysis of the locations of *bla*_{CMY} genes. The locations of *bla*_{CMY} genes were studied by two approaches. In the first of these, undigested total DNAs of the isolates were electrophoresed by PFGE, blotted onto a Hybond N+ membrane (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom), and hybridized with a probe specific for *bla*_{CMY} genes. The probe was the ~1.1-kb PCR product obtained with primers CF-1 and CF-2 and total DNA of isolate 1243 (*bla*_{CMY-15}) as a template. Probe labeling, hybridization, and signal detection were performed with the ECL random-prime labeling and detection system (Amersham Pharmacia Biotech). In the second approach, total DNAs of the isolates, cut with the EcoRI restriction enzyme (MBI Fermentas, Vilnius, Lithuania), were resolved by conventional electrophoresis, blotted onto Hybond N+ membranes, and hybridized with the *bla*_{CMY} probe as described above.

Detection of the *ISEcp1* insertion sequence in the vicinity of *bla*_{CMY} genes. The *ISEcp1* element was detected in the 5'-adjacent regions of *bla*_{CMY} genes by PCR. Primers ALA-4 and CF-3, which anneal to the 3' end of the *ISEcp1*-specific *tnpA* gene and to the proximal fragment of the *bla*_{CMY} gene coding region, respectively, were used (Table 2). Sequencing of the resulting amplicons was performed with the same primers.

Cloning of the *bla*_{CMY} genes. The *bla*_{CMY} genes were amplified with primers ALA-4-HindIII and CF-2-EcoRI (Table 2). The ALA-4-HindIII primer anneals to the same fragment of the *ISEcp1* element as primer ALA-4 but contains the HindIII restriction site at its 5' end. Similarly to CF-2, the CF-2-EcoRI primer corresponds to the 3' end of the *bla*_{CMY} gene coding region and is provided with the EcoRI site at its 5' end. The amplicons were cut with HindIII and EcoRI and cloned into the pGB2 plasmid (19). *E. coli* DH5α transformants were obtained by electroporation in a Gene Pulser II apparatus (Bio-Rad) and selected on Luria-Bertani plates (Sigma Chemical Company) supplemented with 2 μg of ceftazidime and 20 μg of streptomycin (Polfa Tarchomin) per ml. The resulting

constructs were sequenced with primers ALA-4-HindIII, CF-A, CF-4, CF-2-EcoRI, CF-B, and CF-5 and confirmed to have the proper sequences.

Nucleotide sequence accession numbers. Sequences of the coding regions will appear in the GenBank database under accession number AJ555823 for *bla*_{CMY-15} and accession number AJ555825 for *bla*_{CMY-14}.

RESULTS

Antimicrobial susceptibility testing and mating. The MICs of various antimicrobials were determined for the group of *P. mirabilis* isolates; Table 3 shows ranges of the MICs of β-lactam antibiotics. Of the β-lactams tested, all of the isolates were resistant to ampicillin and, despite some variation in MICs, to piperacillin and ceftazidime. The MICs of cefotaxime and ceftazidime were remarkably high as well, and most of the isolates were clearly resistant to these compounds (25 isolates with cefotaxime MICs of 64 to 128 μg/ml and 26 isolates with ceftazidime MICs of 32 to 128 μg/ml). The isolates were almost uniformly susceptible to cefepime, aztreonam, and carbapenems. Only sporadic isolates could be interpreted as having intermediate or low-level resistance to these drugs (two isolates with aztreonam MICs of 16 μg/ml, seven isolates with cefepime MICs of 16 or 32 μg/ml, and three isolates with imipenem MICs of 8 or 16 μg/ml). There was no significant inhibitory effect of combinations of β-lactams with clavulanate at 2 μg/ml (data not shown), but tazobactam reduced the MICs of piperacillin usually by two or three dilutions and in extreme cases by even six or seven dilutions. This meant that all of the isolates were susceptible to piperacillin with tazobactam. Consistent with the susceptibility data, none of the isolates was found to be a putative ESBL producer in any of the variants of the double-disk synergy test (27).

Despite some phenotypic diversity, the isolates demonstrated wide resistance to aminoglycosides, with only 2 isolates susceptible to the four compounds tested (amikacin, gentamicin, tobramycin, and netilmicin) and 16 isolates resistant to them all. Only four and three isolates were susceptible to ciprofloxacin and trimethoprim-sulfamethoxazole, respectively,

TABLE 3. β -Lactam susceptibilities of the *P. mirabilis* isolates and the *E. coli* DH5 α transformants expressing the CMY-4, -12, -15, and -14 β -lactamases

Isolate	MIC (μ g/ml) ^a										
	AMX	AMC (2:1)	PIP	TZP ^b	CTX	CAZ	FEP	FOX	ATM	IPM	MEM
All	>512	32/16–128/64	128–>512	1–64	32–128	16–128	2–32	32–512	0.5–16	1–16	0.06–4
<i>P. mirabilis</i> 864 (CMY-4)	>512	64/32	128	1	32	16	4	64	0.5	2	0.125
<i>P. mirabilis</i> 27 (CMY-12)	>512	64/32	256	32	64	128	16	256	8	4	0.5
<i>P. mirabilis</i> 1662 (CMY-15)	>512	64/32	256	32	128	128	8	512	8	16	4
<i>P. mirabilis</i> 6735 (CMY-14)	>512	64/32	128	8	64	128	32	128	16	2	0.06
<i>E. coli</i> DH5 α (pGBCMY-4)	512	ND ^c	32	2	32	128	0.5	64	32	0.25	ND
<i>E. coli</i> DH5 α (pGBCMY-12)	512	ND	32	4	16	128	0.25	64	32	0.25	ND
<i>E. coli</i> DH5 α (pGBCMY-15)	>512	ND	32	2	16	128	0.25	64	32	0.25	ND
<i>E. coli</i> DH5 α (pGBCMY-14)	512	ND	32	2	16	128	0.5	64	16	0.25	ND
<i>E. coli</i> DH5 α	2	ND	$\leq$ 0.25	$\leq$ 0.25	$\leq$ 0.015	$\leq$ 0.015	0.015	$\leq$ 0.5	$\leq$ 0.015	0.125	ND
<i>E. coli</i> ATCC 25922	4	4/2	1	0.5	0.03	0.25	0.125	8	0.125	0.06	0.03
<i>P. mirabilis</i> ATCC 7002	1	$\leq$ 1/0.5	$\leq$ 0.25	$\leq$ 0.25	$\leq$ 0.015	0.06	0.125	2	$\leq$ 0.015	0.5	0.015

^a AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; PIP, piperacillin; TZP, piperacillin-tazobactam; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; FOX, cefoxitin; ATM, aztreonam; IPM, imipenem; MEM, meropenem. The MICs of amoxicillin, cefotaxime, and ceftazidime with clavulanate (2 μ g/ml) are not shown since there was no effect of clavulanate on the MICs of these compounds.

^b Tazobactam concentration, 4 μ g/ml.

^c ND, not determined.

and they all were resistant to tetracycline and chloramphenicol (data not shown).

None of the isolates produced transconjugants that would be able to grow on medium containing 2 μ g of cefotaxime per ml and 64 μ g of rifampin per ml.

PFGE typing. The 29 multiresistant *P. mirabilis* isolates were typed by PFGE, along with four β -lactam-susceptible clinical isolates of this species recovered in 1999 in different Polish hospitals and used as control strains in the analysis. The results are shown in Table 1. Despite overall diversity, PFGE patterns of the multiresistant isolates revealed several aspects of similarity, and many of them could be classified into a single PFGE type (43). Usually the patterns of the isolates from the same center were similar to each other, as shown, e.g., by isolates from Warsaw 1 (patterns A8 and A9), Zawiercie (A13 and A14), and Grajewo (A1 to A7), which represented a group of 34 isolates of the same patterns identified in this hospital (data not shown). Either indistinguishable or similar PFGE patterns also characterized *P. mirabilis* isolates identified in centers of different cities. This was, for example, the case for isolates from Częstochowa, Zawiercie, and Lublin (A11 to A14); Maków, Olsztyn, Gdańsk, and Wejherowo (A15 to A18); and Grajewo and Warsaw 1 (A5 and A9). The four β -lactam-susceptible *P. mirabilis* isolates included in the analysis produced PFGE patterns that were substantially different from each other and from any of those of the multiresistant isolates.

IEF of β -lactamases and detection of their oxyimino- β -lactam-hydrolyzing activities. In the IEF analysis, all of the multiresistant *P. mirabilis* isolates were characterized by a β -lactamase band with a pI of around 9.0, and all but one (isolate 2384) were characterized by another band with a pI of 5.6 (19 isolates) or 5.4 (9 isolates) (Table 1). The pI 9.0 enzymes were not visualized when IEF gels were stained with nitrocefin supplemented with 0.3 mM cloxacillin. Protein extracts of all of the isolates were subjected to the bioassay for cefotaxime-hydrolyzing activity, and the activity was assigned exclusively to the pI 9.0 β -lactamase bands. In 10 selected extracts, the pI 9.0

β -lactamases also demonstrated the ability to hydrolyze ceftazidime under the experimental conditions used.

PCR identification of *bla*_{CMY} genes. The IEF and bioassay results, analyzed in the context of the susceptibility data, indicated that the pI 9.0 β -lactamases could be acquired AmpC-like cephalosporinases, solely responsible for the β -lactam resistance phenotypes of the isolates. In order to check this hypothesis, PCR was performed with total DNAs of 10 selected isolates and primers annealing inside coding regions of *C. freundii*-type *ampC* genes. These initial reactions yielded products of the expected size (Table 2). Subsequently, DNA preparations of all of the isolates were screened with primers CF-1 and CF-2, which correspond to the very ends of the *C. freundii* *ampC* gene coding region. Amplicons of the expected size (Table 2) were obtained for each isolate, which confirmed that they possessed the *ampC*-like genes (*bla*_{CMY} genes) of the *C. freundii* type.

Sequences of *bla*_{CMY} genes. Twenty *bla*_{CMY}-specific PCR products, obtained for the isolates from 16 hospitals in 15 cities, were selected for DNA sequencing. The results are shown in Tables 1 and 4. Four different sequences of the *bla*_{CMY} coding regions were identified. Four isolates from the hospital in Grajewo (all of the isolates from this center selected for sequencing) and two isolates from two other centers (Kłobuck and Sosnowiec) carried the *bla*_{CMY} gene coding sequence, which was identical to that deposited in GenBank under accession number Y16785. The AmpC-type β -lactamase encoded by this sequence has been designated CMY-12 by Decré et al. (22) and CMY-4 by Barlow and Hall (7). The *bla*_{CMY} coding region of a single isolate from a hospital in Lublin was identical to those that appeared in GenBank with the accession numbers Y16782 and Y16783. According to Decré et al. (22), it codes for β -lactamase CMY-4, whereas Barlow and Hall designated this enzyme CMY-3 (7). The remaining sequences corresponded to two novel AmpC-type β -lactamase variants, designated CMY-15 and -14. The majority of differences between them and the *bla*_{CMY-2} gene coding

TABLE 4. Results of sequencing of the *bla*_{CMY} genes

Nucleotide (amino acid) position ^a	Nucleotide (amino acid) ^b					
	<i>bla</i> _{CMY-2} ^c	<i>bla</i> _{CMY-2} ^d	<i>bla</i> _{CMY-4} ^e	<i>bla</i> _{CMY-12} ^f	<i>bla</i> _{CMY-15}	<i>bla</i> _{CMY-14}
511 (171)	G (Ala)			T (Ser)		
661 (221)	T (Trp)		C (Arg)	C (Arg)	C (Arg)	C (Arg)
961 (321)	G (Val)					A (Ile)
1088 (363)	G (Ser)			A (Asn)	A (Asn)	
1140	G	A	A	A	A	A

^a Nucleotide numbering starts with the first nucleotide of the gene coding region.

^b Only the differences in nucleotides and amino acids with respect to *bla*_{CMY-2} (accession number X91840) (8) are shown; amino acids are shown only in the case of substitutions and not for silent mutations.

^c Accession number X91840 (9).

^d Accession number Y16784 (22).

^e Accession numbers Y16782 and Y16783 (22) and isolate 864 (this study).

^f Accession number Y16785 (22) and isolates 27, 5010, 6210, 6422, 8345, and 8709 (this study).

region (X91840) (9) were those present in *bla*_{CMY-4} and *bla*_{CMY-12} as well. The *bla*_{CMY-15} gene, found in 12 isolates from 11 different centers, was the most prevalent and the most widespread of all of the cephalosporinase genes identified in this work. Its coding sequence differed at three nucleotide positions from that of *bla*_{CMY-2}: T661→C, G1088→A, and G1140→A. The first two differences corresponded to amino acid substitutions in the β-lactamase sequence, and these were substitutions Trp to Arg at position 221 (W221R) and Ser to Asn at position 363 (S363N), respectively. The *bla*_{CMY-14} gene was identified in a single isolate from a hospital in Warsaw, and apart from the T661→C (W221R) substitution and the G1140→A silent mutation, it possessed one novel mutation compared with the *bla*_{CMY-2} sequence. This mutation, G961→A, which is responsible for the Val-to-Ile substitution at position 321 (V321I), was observed for the first time in an *ampC*-type gene of *C. freundii ampC* origin.

Locations of the *bla*_{CMY} genes. In order to determine whether the *bla*_{CMY} genes were chromosomal or plasmidic, undigested total DNAs of 12 selected isolates were electrophoresed by PFGE, blotted onto a nylon membrane, and hybridized with the probe corresponding to the *bla*_{CMY-15} coding region. The isolates were derived from 10 hospitals and belonged to different PFGE types or subtypes, and all of the *bla*_{CMY} genes identified were present in this group. For each of the isolates, the probe hybridized exclusively with a single band with the slowest migration, which most probably contained chromosomal DNA (results not shown).

Hybridization of the *bla*_{CMY} probe with total DNA digested with EcoRI was performed for 20 selected isolates that represented almost all of the hospitals, various PFGE types or subtypes, and all of the *bla*_{CMY} genes identified in this work (Table 1). EcoRI has no restriction site within any of the *bla*_{CMY} gene coding regions analyzed in this study. Two hybridizing bands, of ~10 and ~4.5 kb, were obtained in the cases of 18 isolates that contained *bla*_{CMY-12} and *bla*_{CMY-15}. The two single isolates which possessed *bla*_{CMY-4} and *bla*_{CMY-14} genes, respectively, were characterized only by the ~10-kb band. Full correlation of results was observed when total DNAs were digested with the DraI enzyme, which does not cut within the *bla*_{CMY} sequences analyzed here as well (results not shown).

The *ISEcp1* insertion sequence in the vicinity of *bla*_{CMY} genes. In order to check whether the *P. mirabilis bla*_{CMY} genes

were accompanied by the *ISEcp1* elements on their 5' sides, total DNAs of all of the isolates were used in PCR with primers annealing within *ISEcp1* and closer to the 5' end of the *bla*_{CMY} coding regions, respectively. Amplicons of around 500 bp were obtained for all of the isolates, which indicated that *ISEcp1* was indeed present in the vicinity of all of the *bla*_{CMY} genes and that the distance between the element and the beginning of the *bla*_{CMY} coding frames was around 100 bp.

Sequences of five *ISEcp1-bla*_{CMY} amplicons, corresponding to all four different *bla*_{CMY} genes (isolates 27, 864, 1662, and 6735 with *bla*_{CMY-12}, *bla*_{CMY-4}, *bla*_{CMY-15}, and *bla*_{CMY-14}, respectively), were determined and found to be identical to each other. The *ISEcp1* insertion site was located exactly 106 bp upstream from the first nucleotide of the *bla*_{CMY} coding regions. The 106-bp fragment was compared with four sequences that flank *C. freundii ampC* alleles on their 5' sides, deposited in GenBank under accession numbers D13207, D85910, X76636, and X03866. The comparison revealed high homology between the regions analyzed, ranging from 81.1% identity with sequence X76636 to 97.2% (three nucleotide differences) with D13207.

Cloning and expression of the *bla*_{CMY} genes. PCR products encompassing the *bla*_{CMY-4} (from isolate 864), *bla*_{CMY-12} (isolate 27), *bla*_{CMY-15} (isolate 1662), and *bla*_{CMY-14} (isolate 6735) genes together with their 5' adjacent regions were cloned and expressed in the isogenic background of *E. coli* DH5α. Since the 5' regions of the genes were identical, expression levels of the resulting constructs pGBCMY-4, -12, -15, and -14 were expected to be the same, and this made it possible to elucidate the eventual role of the substitutions observed in the enzymes. The *E. coli* transformants were subjected to MIC evaluation with the representative group of β-lactam antibiotics, and their susceptibility patterns were found to follow those of the *P. mirabilis* clinical isolates (Table 3). Although the MICs obtained for the transformants were in general lower than the MICs for the original isolates, some interesting exceptions were observed as well. For example, the MICs of aztreonam were higher for all of the transformants than for the corresponding original isolates, and *E. coli* DH5α(pGBCMY-4) was additionally more resistant to ceftazidime and ceftazidime with clavulanate. What was the most important, however, was that there were no significant differences in MICs between the CMY-4, -12, -15, and -14-producing transformants.

PCR identification of *bla*_{TEM} genes. Amplicons of the expected size (Table 2) were obtained in PCR with primers specific for *bla*_{TEM} genes for all but one isolate. The only negative isolate was 2384, which was the only one that had no pI 5.4 or 5.6 β -lactamase in the IEF analysis. Sequencing was performed for two isolates with the pI 5.4 β -lactamase (isolates 864 and 6735) and two isolates with the pI 5.6 enzyme (isolates 27 and 1662). This revealed that their pI 5.4 and 5.6 β -lactamases were TEM-1 and TEM-2, respectively (www.lahey.org/studies/webt.htm).

DISCUSSION

To our knowledge, fewer than 10 *P. mirabilis* isolates with acquired AmpC-type enzymes have been described until now (14, 21, 22, 25, 44), and, although they were analyzed mainly in France, they had probably emerged in several different countries. Data presented in this work document for the first time a country-wide dissemination of such organisms. Similarly to the majority of the earlier AmpC-producing *P. mirabilis* strains (14, 22, 44), all of the isolates studied here were found to produce AmpC-type enzymes of *C. freundii* origin. These included two known (CMY-4 and -12) and two novel (CMY-15 and (14) variants of that evolutionary lineage. Interestingly, CMY-4 and -12 were originally identified in *P. mirabilis* isolates from Tunisia (44) and France (from an Algerian patient) (22), respectively, and CMY-4 was later found again in two *P. mirabilis* isolates from France (from patients from Greece) (22). With more than 10 variants now, the group of the *C. freundii*-type β -lactamases seems to be the most "successful" among the acquired AmpCs (7, 37).

The susceptibility patterns of the isolates studied corresponded well to those observed earlier for the CMY-producing *P. mirabilis* isolates (14, 22, 44). Their more specific features were low aztreonam MICs compared to cefotaxime and ceftazidime and a significant effect of tazobactam on piperacillin MICs, which is due to efficient inhibition of CMY enzymes by tazobactam (14, 37). The remarkable diversity in the MICs could result from differences in the genetic backgrounds of the isolates, including their permeability, which, according to earlier observations (2, 4, 13, 17, 40), might have been coresponsible for the resistance to imipenem in sporadic isolates. It is rather unlikely that the quantitative differences in resistance were due to activity of the particular cephalosporinase variants or differences in their expression levels. Genes coding for the four CMYs had identical promoter regions, and upon cloning and expression in the isogenic background, they did not produce any significant differences in β -lactam MICs. These data confirmed the earlier opinion that mutations accumulating in the *C. freundii ampC* alleles and their derivatives are rather neutral (7). The β -lactam susceptibility of the isolates must have been also affected by TEM-type β -lactamases (pI 5.4 or pI 5.6 [TEM-1 or TEM-2, respectively] in representative isolates).

Further analysis of the results gave some insights into the epidemiology of the AmpC-producing *P. mirabilis* in Poland. The central question is whether a *bla*_{CMY} gene of the *C. freundii* origin had once been introduced into a *P. mirabilis* strain which later underwent dissemination and evolution or whether there were multiple transmissions of *bla*_{CMY} genes into the *P.*

mirabilis population or within it. In the PFGE analysis, indistinguishable or closely related AmpC-producing isolates were identified in distant cities, and the majority of them all seemed to form a clonal group. The results of mating and hybridization of the *bla*_{CMY} probe to undigested and PFGE-resolved DNAs of 12 representative isolates indicated that the *bla*_{CMY} genes were located only in their chromosomes. Although already observed in CMY-3-, -4-, and -12-producing *P. mirabilis* (14, 22), the chromosomal location of the acquired *ampC*-like genes is very rare, as it requires several genetic events in order to occur (7, 13, 30, 37). Most probably the mobilization of the *bla*_{CMY} genes from *C. freundii* to *P. mirabilis* was mediated by a transposon formed by the *ISEcp1* element (39). Noteworthy are that in all of the isolates *ISEcp1* was located at the same distance from the *bla*_{CMY} genes and that in four representative isolates the sequences between them were identical. All of these data suggested that there had been a single original source of all of the *bla*_{CMY} genes and that the *P. mirabilis* population studied arose from the single acquisition of a *C. freundii*-type *ampC* gene, its chromosomal insertion, and the clonal spread and evolution of the organism.

Further support of this hypothesis came from the comparison of sequences of the *bla*_{CMY} genes, which could have emerged one from the other by accumulation of point mutations along two separate lineages. The *bla*_{CMY-4} gene might be the default sequence, which by the G961→A (V321I) mutation gave rise to *bla*_{CMY-14} and, alternatively, by the G1088→A (S363N) substitution gave rise to *bla*_{CMY-15} (Table 4). Subsequently, *bla*_{CMY-15} could have evolved directly into *bla*_{CMY-12} by acquisition of the G511→T (A171S) mutation. This view is congruent with the results of hybridization of the *bla*_{CMY} probe to EcoRI-digested genomic DNAs of the isolates. Whereas in the *bla*_{CMY-4}- and *bla*_{CMY-14}-carrying isolates the probe hybridized with a single band of ~10 kb, in all of the isolates with the *bla*_{CMY-15} and *bla*_{CMY-12} genes, an additional band of ~4.5 kb was observed. This indicated that gene duplication could occur in the *bla*_{CMY-4}→*bla*_{CMY-15}→*bla*_{CMY-12} lineage; however, it is not known whether it was *bla*_{CMY-4} or *bla*_{CMY-15} that became duplicated and whether the second gene copy was complete. Overall, the CMY-15- and CMY-12-producing isolates were not more resistant to β -lactams than the CMY-4 and -14 producers, and no heterozygotes were observed during sequencing of the *bla*_{CMY-15} and *bla*_{CMY-12} genes. We might hypothesize that the second gene copy was not complete and was not amplified for sequencing and cloning.

The several arguments discussed above suggested that the population of AmpC-producing *P. mirabilis* in Poland might be a result of the long-lasting spread of a single clone of this organism. The clone has been distributed over the whole country, and this very likely has been due to parallel dissemination of several clone variants, with the CMY-15-producing ones obviously being the most expansive. The large-scale spread of a multiresistant enterobacterial clone has already been reported several times (3, 12, 15, 16, 23), and the transmission of enterobacteria between hospitals in Poland seems to be a common practice (6, 24). The question remains whether the *P. mirabilis* described in this work has been restricted to Poland or has been of wider geographic distribution. The comparison of the Polish isolates with isolates with chromosomal *bla*_{CMY-3},

*bla*_{CMY-4}, and *bla*_{CMY-12} genes from other countries (14, 22) may bring some interesting epidemiological observations.

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