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Evolution and Spread of a Multidrug-Resistant *Proteus mirabilis* Clone with Chromosomal AmpC-Type Cephalosporinases in Europe▽

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Proteus mirabilis **isolates obtained in 1999 to 2008 from three European countries were analyzed; all carried chromosomal AmpC-type cephalosporinase bla_{CMY} genes from a *Citrobacter freundii* origin (bla_{CMY-2}-like genes). Isolates from Poland harbored several bla_{CMY} genes (bla_{CMY-4}, bla_{CMY-12}, bla_{CMY-14}, bla_{CMY-15}, and bla_{CMY-38} and the new gene bla_{CMY-45}), while isolates from Italy and Greece harbored bla_{CMY-16} only. Earlier isolates with bla_{CMY-4} or bla_{CMY-12}, recovered in France from Greek and Algerian patients, were also studied. All isolates showed striking similarities. Their bla_{CMY} genes resided within IS*Ecp1* transposition modules, named Tn*6093*, characterized by a 110-bp distance between IS*Ecp1* and bla_{CMY}, and identical fragments of both *C. freundii* DNA and a ColE1-type plasmid backbone. Moreover, these modules were inserted into the same chromosomal site, within the *pepQ* gene. Since ColE1 plasmids carrying IS*Ecp1* with similar *C. freundii* DNA fragments (Tn*6114*) had been identified earlier, it is likely that a similar molecule had mediated at some stage this DNA transfer between *C. freundii* and *P. mirabilis*. In addition, isolates with bla_{CMY-12}, bla_{CMY-15}, and bla_{CMY-38} genes harbored a second bla_{CMY} copy within a shorter IS*Ecp1* module (Tn*6113*), always inserted downstream of the *ppiD* gene. Sequence analysis of all mobile bla_{CMY-2}-like genes indicated that those integrated in the *P. mirabilis* chromosome form a distinct cluster that may have evolved by the stepwise accumulation of mutations. All of these observations, coupled to strain typing data, suggest that the bla_{CMY} genes studied here may have originated from a single IS*Ecp1*-mediated mobilization-transfer-integration process, followed by the spread and evolution of a *P. mirabilis* clone over time and a large geographic area.**

Acquired cephalosporinases of the AmpC type constitute a significant source of resistance to most of the newer-generation β-lactams in *Enterobacteriaceae* (3, 18, 29). Similar to their natural precursors in several Gram-negative species, e.g., *Aeromonas* spp., *Enterobacter* spp., *Morganella morganii*, or *Citrobacter freundii*, these enzymes hydrolyze penicillins, most cephalosporins, and aztreonam and are poorly inhibited by β-lactam inhibitors (5, 18). Usually, acquired AmpCs are expressed constitutively, conferring resistance to all of their substrates and inhibitor combinations, although resistance levels depend on the amounts of enzymes produced, as well as the presence of other resistance mechanisms (18, 29). The acquired *ampC* genes have escaped from the chromosome of some species to plasmids, following mobilizations mediated by such elements as IS*26*, IS*Ecp1*, or IS*CR1* (14, 21, 24, 27, 34). The identity of these elements, their location with respect to the *ampC* gene, and the size of the DNA fragment mobilized are diagnostic of specific escape events. Mobilizations of the *C. freundii ampC* have given rise to bla_{CMY} genes observed in many species worldwide (1, 18, 29). With more than 40 genes identified thus far in this family, in which bla_{CMY-2} is the most common, this is the largest group of mobile *ampC*s (18, 29). bla_{CMY-2} may also be the precursor of other bla_{CMY} genes through the accumulation of neutral mutations (1).

IS*Ecp1* has played an important role in mobilizing bla_{CMY-2}-like genes, since it is often found at their 5′ flanks (14, 21, 23, 26, 32). It may transpose together with adjacent DNA fragments on the 3′ side, producing transposition modules of various sizes. This process utilizes sequences similar to the IS*Ecp1* inverted right repeat (IRR), and such alternative IRRs mark precisely the modules' 3′ ends (20). IS*Ecp1* has been identified 116 bp upstream from bla_{CMY-2}, bla_{CMY-4}, bla_{CMY-7}, bla_{CMY-21}, and bla_{CMY-23} genes in plasmids of the IncA/C and IncI1 groups disseminated worldwide among *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*, and *Proteus mirabilis* (14–16, 26, 32, 36, 37). It has also been found 110 bp upstream from bla_{CMY-5}, bla_{CMY-31}, and bla_{CMY-36} genes in highly similar ColE1-type plasmids in *Klebsiella oxytoca* (pTKH11), *S. enterica* serovar Newport (pA172), and *K. pneumoniae* (pH 205) from Sweden, the United States, and Greece, respectively (38, 39).

Chromosomal bla_{CMY} genes (bla_{CMY-3}, bla_{CMY-4}, and bla_{CMY-12}) in *P. mirabilis* were first observed sporadically in France, in patients with Greek and Algerian origins (4, 11). Later, *P. mirabilis* isolates with IS*Ecp1* close to bla_{CMY-2}-like genes in their chromosome were also found in Poland (bla_{CMY-4}, bla_{CMY-12}, bla_{CMY-14}, bla_{CMY-15}, and bla_{CMY-38}) (13, 21), Italy (bla_{CMY-16}) (9, 23), and Greece (25). The present international molecular epidemiology study aimed to compare all of the above *P. mirabilis* isolates, in order to assess the clonality of bla_{CMY-2}-like gene sequences, their transposition

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TABLE 1. *P. mirabilis* isolates: basic information from previous studies and the present study

Strain	Yr of isolation[a]	Country	City	Specimen	CMY variant	Other β-lactamase(s)	ISEcp1 modules	Ribotype	Source or reference
PL 6735/99	1999	Poland	Warsaw	Urine	CMY-14	TEM-1	Tn*6093*	R5	21
PL 1662/00	2000	Poland	Warsaw	Pus	CMY-15	TEM-2	Tn*6093*, Tn*6113*	R5	21
PL 27/00	1999	Poland	Grajewo	Urine	CMY-12	TEM-2	Tn*6093*, Tn*6113*	R5	21
PL 1376/00	2000	Poland	Zielona Góra	Urine	CMY-45	TEM-1	Tn*6093*	R6	This study
PL 864/01	2001	Poland	Lublin	Sputum	CMY-4	TEM-1	Tn*6093*	R5	21
PL 1455/04	2004	Poland	Łódź	Urine	CMY-38	TEM-2	Tn*6093*, Tn*6113*	R5	13
IT NO-051/03	2003	Italy	Novara	Cutaneous ulcer	CMY-16	TEM-1	Tn*6093*	R5	9
IT VA-1017/03	2003	Italy	Varese	Urine	CMY-16	TEM-1	Tn*6093*	R5	9
IT VA-832/05	2005	Italy	Varese	Urine	CMY-16	TEM-1	Tn*6093*	R5	23
IT VA-070/06	2006	Italy	Varese	Urine	CMY-16	TEM-1	Tn*6093*	R5	23
IT VA-414/06	2006	Italy	Varese	Urine	CMY-16	TEM-1	Tn*6093*	R5	23
IT LC-10/08	2008	Italy	Lecco	Blood	CMY-16	TEM-1	Tn*6093*	R5	This study
GR 17/04	2004	Greece	Thessaloniki	Urine	CMY-16	VIM-1, TEM-1	Tn*6093*	R5	35
GR 15184/05	2005	Greece	Thessaloniki	Urine	CMY-16	VIM-1, TEM-1	Tn*6093*	R5	35
GR 15315/05	2005	Greece	Thessaloniki	Urine	CMY-16	VIM-1, TEM-1	Tn*6093*	R5	35
GR 2506/07	2007	Greece	Athens	Sputum	CMY-16	VIM-1, TEM-1	Tn*6093*	R5	This study
GR 2530/07	2007	Greece	Athens	Urine	CMY-16	VIM-1, TEM-1	Tn*6093*	R5	This study
GR 2720/08	2008	Greece	Athens	Sputum	CMY-16	VIM-1, TEM-1	Tn*6093*	R5	25
22317	–	France/Greece	Paris	Bile	CMY-4	TEM-1	Tn*6093*	R7	11
PLAR	–	France/Greece	Paris	Feces	CMY-4	TEM-1	Tn*6093*	R7	11
34955	–	France/Algeria	Paris	Urinary catheter	CMY-12	TEM-2	Tn*6093*, Tn*6113*	R5	11
GR-485-S	2007	Greece	Athens	Urine	No	None	No	R4	This study
GR-20A-S	2007	Greece	Athens	Urine	No	None	No	ND[b]	This study
GR-99-S	2007	Greece	Athens	Pus	No	None	No	R1	This study
GR-106-S	2007	Greece	Athens	Pus	No	None	No	R1	This study
GR-20-S	2008	Greece	Athens	Pus	No	None	No	R2	This study
GR-28-S	2008	Greece	Athens	Pus	No	None	No	R2	This study
GR-59-S	2008	Greece	Athens	Sputum	No	None	No	R3	This study

[a] –, The date of isolation was not provided in the reference paper (11).
[b] ND, not determined.

modules, and insertion regions, as well as of the bacterial chromosomes themselves.

MATERIALS AND METHODS

Bacterial strains. Twenty-one CMY-producing *P. mirabilis* isolates were studied (Table 1). Eighteen had been recovered between 1999 and 2008 in hospitals of different cities of Poland, Greece, and Italy and were selected (six isolates per country) from larger groups of isolates partially described previously (9, 13, 21, 23, 25, 35). The Polish isolates belonged to various pulsed-field gel electrophoresis (PFGE) patterns and harbored all of the bla_{CMY} genes identified in *P. mirabilis* in that country thus far; the Italian and Greek isolates represented different PFGE patterns and/or clinical centers where these organisms have been studied to date. The three remaining *P. mirabilis* strains with chromosomal bla_{CMY}s, kindly provided by G. Arlet, were isolated in hospitals in Paris from patients originating either from either Greece or Algeria. Strains 22317 and PLAR (Greece) were among the earliest CMY-4 producers reported, while strain 34955 (Algeria) was the first CMY-12 producer ever identified (11). Seven AmpC-negative, epidemiologically unrelated *P. mirabilis* isolates were included for comparative typing; these had been collected from different Greek hospitals from 2006 to 2008.

Detection and sequencing of bla_{CMY} genes. The entire coding regions of bla_{CMY} genes were amplified with the primers ampC1 and ampC2 (11) and directly sequenced by using several bla_{CMY}-specific primers (Table 2). bla_{CMY} sequences were compared for evolutionary relationships by using the MEGA v3.1 software (19). Subsequently, the sequences, prealigned in Mauve 2.3.0 (10), were analyzed by using CloneFrame 1.1 software (12), and the results were visualized by using SplitsTree 4.10 (17) as a consensus network. DNA sequencing in all experiments of the present study was performed either in-house, using the Applied Biosystems technology and equipment (Foster City, CA), or by an external facility (Macrogen, Inc., Seoul, Korea).

Detection of the IS*Ecp1* element. The IS*Ecp1* element was detected upstream of bla_{CMY} genes by PCR with the primers ecpF2 and ampC5 (Table 2), which were also used for sequencing the spacer region between IS*Ecp1* and the bla_{CMY} genes.

Assays of bla_{CMY} locus number and localization. The number of loci containing bla_{CMY} genes was assessed by hybridization of bla_{CMY} and IS*Ecp1* probes with chromosomal DNA digested with EcoRI and HindIII restriction enzymes (MBI Fermentas, Vilnius, Lithuania), as described previously (21). The bla_{CMY} (1,143-bp) and IS*Ecp1* (181-bp) probes were obtained by amplifying DNA from isolate PL 1662/00 with the primer pairs ampC1/ampC2 and ecpF2/ecpR1 (Table 2), respectively. Chromosomal versus plasmidic localization of bla_{CMY} genes was assessed by I-CeuI analysis (22), as described previously (9).

Partial cloning and sequencing of IS*Ecp1*-bla_{CMY} modules. DNA from all Polish and French isolates was digested with EcoRI and HindIII, and ligated into plasmid pHSG398 (33). *E. coli* DH5α electroporants were selected with 2 μg of cefotaxime/ml and 25 μg of chloramphenicol/ml (Sigma-Aldrich, St. Louis, MO). Recombinant plasmids were checked for inserts of ~4.2 kb ("main bla_{CMY} locus") and ~3.1 kb ("additional bla_{CMY} locus"). Sequencing of the inserts was performed using primers specific for insert-flanking vector sequences, IS*Ecp1* and bla_{CMY}s (Table 2), and other primers designed according to the accumulating sequence. Sequences were analyzed with the DNASTAR Lasergene software (Madison, WI).

Characterization of entire IS*Ecp1*-bla_{CMY} modules and their chromosomal insertion site: "main locus." The structure and site of integration of IS*Ecp1*-bla_{CMY} transposition modules within the *P. mirabilis* chromosome at the "main locus" was initially investigated by inverse PCR. Total DNA (1 μg) of isolate IT NO-051/03 was digested by AgeI (New England Biolabs, Inc., Beverly, MA). The digestion mixture, purified with the Wizard SV Gel and PCR Cleanup system (Promega, Madison, WI), was diluted 1:10 and then self-ligated by using T4 DNA ligase (Promega). The ligation mixture (2 μl) was then used in a PCR with the primers blc/F and C12-tnpA/r (7) (Table 2) to amplify the adjacent regions of the IS*Ecp1*-bla_{CMY} module. Sequences flanking the module were compared to the genomic sequence of *P. mirabilis* strain HI4320 (GenBank accession no. AM942759) (28), using the microbial BLAST tool (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). For the remaining isolates, the module structure and the integration site were analyzed by PCR mapping using primers targeting various regions (Table 2 and Fig. 1), designed based on sequences of the inverse PCR product and the *P. mirabilis* HI4320 locus *pepQ* (positions 3872453 to 3873787) (28).

TABLE 2. Oligonucleotides used in this study

Primer	Gene	Oligonucleotide sequence (5′–3′)	Position[a]	Purpose	Source or reference
ampC1	bla_{CMY}	ATG ATG AAA AAA TCG TTA TGC	1–21*	PCR, sequencing	11
ampC2	bla_{CMY}	TTG CAG CTT TTC AAG AAT GCG C	1122–1143*	PCR, sequencing	11
ampC5	bla_{CMY}	CAG CGT TTG CTG CGT G	222–237*	PCR, sequencing	This study
E4/F	bla_{CMY}	TGG GTT CAG GCC AAC ATG GAT GC	757–779*	PCR mapping, sequencing	This study
E7/R	bla_{CMY}	TGC CAG CAT CAC GAT GCC AAG G	1059–1080*	PCR mapping, sequencing	This study
ecpF2	3′ end of IS*Ecp1*	GTT GCT CTG TGG ATA ACT TG	2084–2102†	PCR, sequencing	39
ecpR1	IS*Ecp1*	CCT AAA TTC CAC GTG TGT	2247–2264†	PCR	This study
ISEcp1/F	*tnpA* of IS*Ecp1*	CAT GCT CTG CGG TCA CTT C	959–977†	PCR mapping	This study
E8/F	Downstream of bla_{CMY}	CCA GGA TAT TGG GCC TC	3534–3550†	PCR mapping	This study
blc/R	*blc*	GAC AAC CAG GAA TGC AGC	3647–3664†	PCR mapping	This study
sugE/R	*sugE*	GCC TGA TAT GTC CTG GAT CGT	4448–4468†	PCR mapping	This study
ORF6/R	*orf6*	CTT CAT CCC TAT CAT CGC CA	5634–5653†	PCR mapping	This study
ORF6e/R	*orf6*	AAT CAG CAA TAA CAT CAC CAT G	5770–5791†	PCR mapping	This study
mobB/R	*mobB*	TAG AGC AGC AGA AGC CAG CT	6205–6224†	PCR mapping	This study
RNAIIp/R	RNAII	TCA TTC CAC GCC TGA CAC TC	6741–6760†	PCR mapping	This study
pepQ/F	*pepQ*	CAC CTG TTG ATT ATT GGC AT	3872704–3872723‡	PCR mapping, sequencing	This study
pepQ/R	*pepQ*	TCA GGC TAA ATG TAA GTC TC	3873769–3873787‡	PCR mapping, sequencing	This study
blc/F	*blc*	CTG TGG ATC CTT TCA CGG A	4025–4043†	Inverse PCR	This study
C12-tnpA/r	*tnpA* of IS*Ecp1*	TAT TCT GAA GAG TCC AAG GAA	1921–1901†	Inverse PCR	6
ppiD/F	Intergenic region PMI0120/*ppiD*	GCG ATT ACT GAA TGC CAT C	150710–150692‡	PCR mapping, sequencing	This study
ppiD/R	*ppiD*	CAA CGG CAG AAC AGC TTG	150366–150383‡	PCR mapping, sequencing	This study

[a] *, Position numbering is according to the first nucleotide of the coding sequence of the bla_{CMY} gene; †, position numbering is according to the sequence of plasmid pH 205 (GenBank acc. no. EU331426) (39); ‡, position numbering is according to the sequence of the *P. mirabilis* HI4320 genome (GenBank accession no. AM942759) (28).

Analysis of the insertion site for IS*Ecp1*-bla_{CMY} modules: "additional locus." Sequences 3′ to the IS*Ecp1*-bla_{CMY} modules of the "additional locus," determined by sequencing of cloned fragments, were compared to the genomic sequence of *P. mirabilis* HI4320 (28). The matching sequence of the *ppiD* region was then used to design primers for mapping the modules in the spacer between the *ppiD* gene (positions 148619 to 150496) and the PMI0120 open reading frame (ORF; 150751 to 151125) (Table 2 and Fig. 1) and for sequencing the 5′ junction.

Molecular typing. Ribotyping was performed after HincII (New England Biolabs) digestion of genomic DNA, as described by Pignato et al. (30). For PFGE, total *P. mirabilis* DNA in agarose plugs was prepared as described previously (8), digested with NotI and SfiI (New England Biolabs), and electrophoresed using a CHEF III Bio-Rad apparatus (Hercules, CA). All electrophoretic patterns were analyzed using GelCompar v4.1 (Applied Maths NV, Sint-Martens-Latem, Belgium), using the Dice coefficient and clustering by UPGMA (unweighted pair-group method with arithmetic averages), with 1% tolerance in band position differences. For the NotI+SfiI PFGE patterns, a similarity cutoff of 80% was applied for discerning clusters of related isolates (31).

Antimicrobial susceptibility testing. The MICs of β-lactams were evaluated by the agar dilution method according to Clinical and Laboratory Standards Institute criteria (6). For the isolates tested earlier, the methodology was described in previous reports (9, 11, 21, 23, 25, 35).

Nucleotide sequence accession numbers. Sequences of the bla_{CMY-45} gene coding region and of the Tn*6113* (bla_{CMY-12}) and Tn*6093* (bla_{CMY-16}) modules will appear in the EMBL database under accession numbers FN546177, FR716828, and FM995219, respectively.

RESULTS

Sequence and location of the bla_{CMY} genes in *P. mirabilis* isolates. A total of 21 *P. mirabilis* isolates producing CMY-2-like β-lactamases from different countries and periods were investigated (Table 1). The sequences of the bla_{CMY} genes were already known in five isolates from Poland (bla_{CMY-4}, bla_{CMY-12}, bla_{CMY-14}, bla_{CMY-15}, and bla_{CMY-38}), five isolates from Italy (bla_{CMY-16}), and three isolates from France (although from patients of Greek and Algerian origins [bla_{CMY-4} and bla_{CMY-12}, respectively]) (9, 11, 13, 21, 23). PCR and sequencing showed that the remaining eight isolates carried either bla_{CMY-16} (the recent isolates from Greece and the IT LC-10/08 isolate from Italy) or a new allele (the PL 1376/00 isolate from Poland), named bla_{CMY-45}, which differs from bla_{CMY-2} (2) by four point mutations: $C_{418}A$ (Gln140Lys), $G_{511}T$ (Ala171Ser), $T_{661}C$ (Trp221Arg), and $G_{1019}T$ (Gly340Val).

Comparison to other known bla_{CMY-2}-like alleles indicated that those carried by our *P. mirabilis* isolates form a distinct cluster of more closely related sequences (Fig. 2). Sequence comparisons also suggested that the present study's alleles may have evolved from bla_{CMY-4} by stepwise mutations; bla_{CMY-12} was notable in that it shared, on the one hand, the $G_{511}T$ (Ala171Ser) mutation with bla_{CMY-16} and bla_{CMY-45}, and, on the other, the $G_{1088}A$ (Ser363Asn) mutation with bla_{CMY-15} and bla_{CMY-38} (Fig. 2B).

I-CeuI analysis proved (or confirmed) that the bla_{CMY} genes were chromosome-borne in all studied isolates (data not shown). PCR and sequencing assays revealed (or confirmed) the presence of an IS*Ecp1* element inserted 110bp upstream of the bla_{CMY} genes in all isolates (thus correcting the value of 106 bp previously published for bla_{CMY-4}, bla_{CMY-12}, bla_{CMY-14}, and bla_{CMY-15} [21]). Southern blot experiments carried out with genomic DNAs digested with EcoRI (which cuts inside IS*Ecp1*) and HindIII (which does not cut inside either IS*Ecp1* or any of the bla_{CMY}s) and using either a bla_{CMY} or a IS*Ecp1* probe yielded a hybridizing band of ~4.2 kb with all isolates ("main bla_{CMY} locus") and another band of ~3.1 kb with the isolates carrying bla_{CMY-12}, bla_{CMY-15}, and bla_{CMY-38} ("additional bla_{CMY} locus"). These results revealed (or confirmed) that, while in most isolates the bla_{CMY} gene was present in a single copy, two copies were present in isolates with bla_{CMY-12},

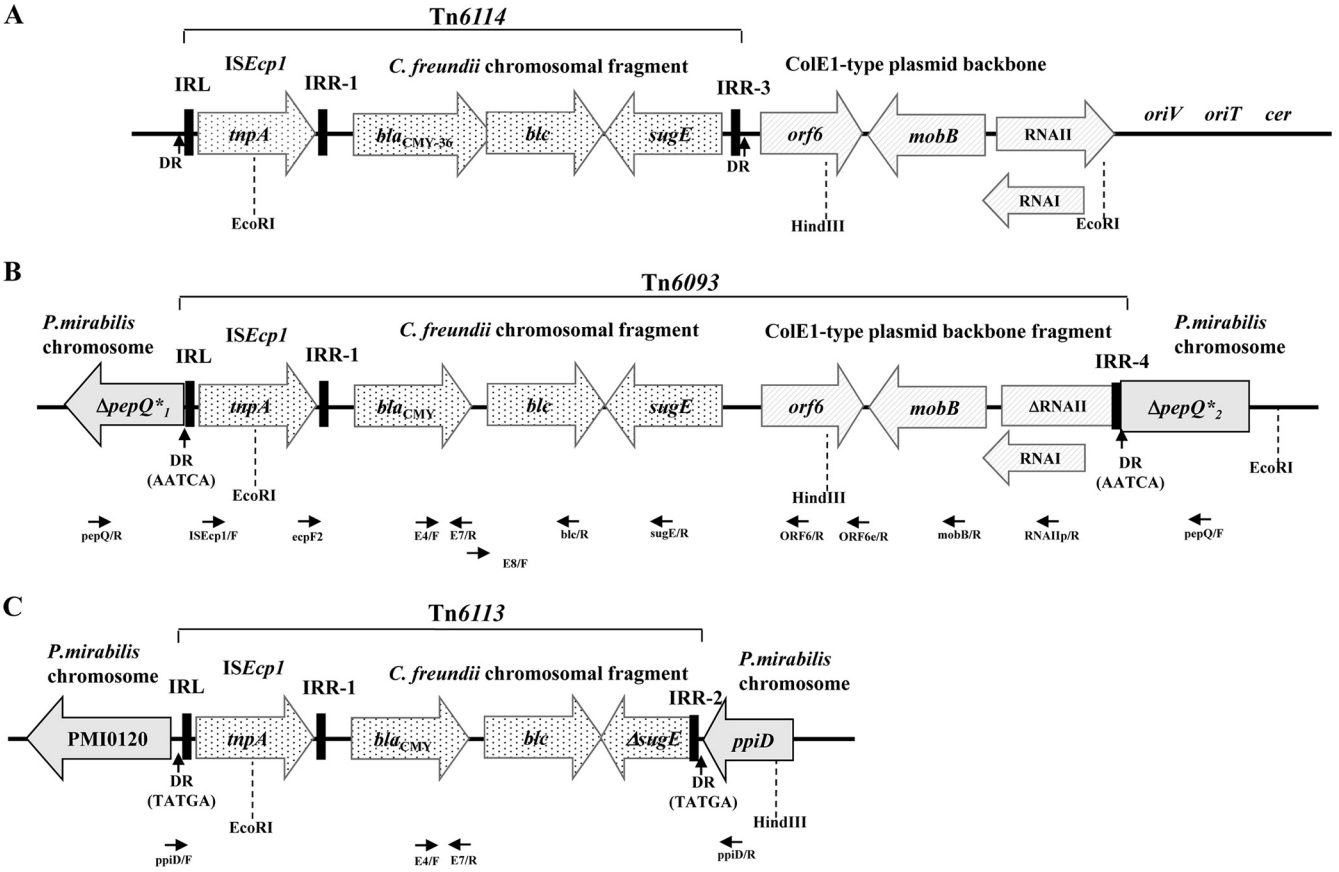

FIG. 1. Schematic representation of IS*Ecp1* transposition modules containing the *C. freundii*-derived bla_{CMY} genes, enzyme restriction sites, and primer hybridization sites used in the analysis of the Tn*6093* and Tn*6113* structures. (A) Tn*6114* module present in the pH 205 plasmid; (B) Tn*6093* module inserted into the *P. mirabilis pepQ* gene ("main locus"); (C) Tn*6113* module inserted between *ppiD* and PMI0120 ORF in the *P. mirabilis* chromosome ("additional locus"). The schemes are aligned according to the IS*Ecp1* position. EcoRI and HindIII sites, used in cloning experiments, are indicated by dashed vertical lines. Black arrows below the Tn*6093* and Tn*6113* schemes represent primers used for PCR mapping and sequencing.

bla_{CMY-15}, and bla_{CMY-38} from Poland and France (Algeria) (21).

Genetic context of the bla_{CMY} genes and their integration sites. The genetic context of the bla_{CMY} genes and their integration sites were investigated by a combination of cloning, inverse PCR, and PCR mapping and sequencing, as detailed in Materials and Methods. The results showed that all of the isolates carried variants of a 6,210-bp IS*Ecp1*-bla_{CMY} module, named Tn*6093*, integrated into the *P. mirabilis pepQ* gene coding for Xaa-Pro dipeptidase (28). In all isolates, Tn*6093* was inserted in the same position within *pepQ* and was flanked by 5-bp direct repeats (AATCA), indicating that the IS*Ecp1*-mobilized module had integrated by transposition into the *P. mirabilis* chromosome. Tn*6093* contained IS*Ecp1*, followed by the *C. freundii*-derived bla_{CMY}, *blc*, and *sugE* genes, a part of the ColE1-type plasmid backbone (regions *orf6*, *mobB*, and ΔRNAII), and terminating with an IS*Ecp1* IRR-like sequence, named IRR-4 (Fig. 1). Tn*6093* matched very well fragments of plasmids pTKH11, pA172, and pH 205 with IS*Ecp1* modules called Tn*6114*, carrying complete bla_{CMY}, *blc*, and *sugE* genes and terminating with the IRR-like motif IRR-3 (Fig. 1) (38, 39). Compared to the homologous part of pH 205 (39), the Tn*6093* variants had, apart from variable mutations within bla_{CMY}s, eight stable single nucleotide polymorphisms: one between IS*Ecp1* and bla_{CMY} and seven downstream of Tn*6114*. The Tn*6093*-containing locus gave rise to the ~4.2-kb band ("main bla_{CMY} locus") recognized in Southern blot experiments with all *P. mirabilis* isolates.

In the isolates with two bla_{CMY} copies, a second IS*Ecp1*-bla_{CMY} module, 3,783 bp long and named Tn*6113*, was inserted into the *P. mirabilis* intergenic spacer between ORF PMI0120 and the *ppiD* gene encoding peptidyl-prolyl *cis-trans* isomerase D (28). In all of these isolates, Tn*6113* was inserted at the same position, 154 bp downstream of *ppiD*, and was flanked by 5-bp direct repeats (TATGA), which indicated that this module also had integrated into the chromosome by transposition. Tn*6113* consisted of IS*Ecp1*, followed by the *C. freundii*-like sequences bla_{CMY}, *blc*, and Δ*sugE*, and terminating with an IRR-like motif inside *sugE*, named IRR-2 (Fig. 1). Therefore, Tn*6113* was shorter by 247 bp than Tn*6114*. The Tn*6113*-containing locus gave rise to the ~3.1-kb band ("additional bla_{CMY} locus") identified in Southern blot experiments with the *P. mirabilis* isolates carrying two bla_{CMY} copies; the same bla_{CMY} allele was always present in both Tn*6093* and Tn*6113* modules.

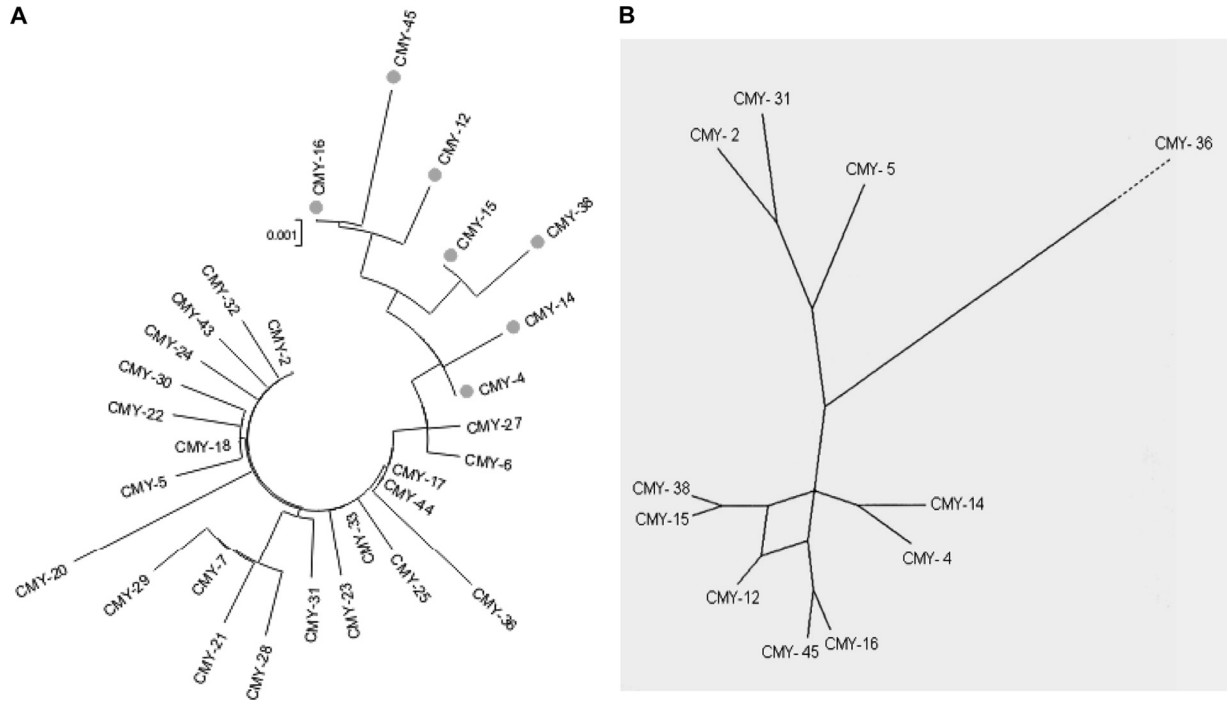

FIG. 2. Sequence comparison of bla_{CMY-2}-like genes. (A) Dendrogram of all mobile bla_{CMY-2}-like gene sequences obtained with the MEGA v3.1 software (19); (B) consensus network of the bla_{CMY-2}-like genes located in IS*Ecp1* modules with a 110-bp distance between IS*Ecp1* and bla_{CMY} (plasmidic Tn*6114*-like, and *P. mirabilis* Tn*6093*-like modules). The consensus network was generated with the SplitsTree 4.10 software (17), after prealignment in Mauve 2.3.0 (10) and analysis by the CloneFrame 1.1 software (12).

Clonality of the bla_{CMY}-carrying *P. mirabilis* isolates. Given the identical structures and insertion sites of bla_{CMY}-carrying elements, we investigated the possible clonality of *P. mirabilis* chromosomes using ribotyping and PFGE of genomic DNA after NotI-SfiI digestion. All bla_{CMY}-carrying isolates belonged to three ribotypes. The main one, R5, grouped 18 isolates, while the remaining three isolates belonged to two other ribotypes (R6, $n = 1$; R7, $n = 2$). In contrast, the six bla_{CMY}-negative control isolates tested, belonged to four distinct ribotypes, R1 to R4 (Fig. 3). As expected, PFGE profiling resulted in a somewhat higher discrimination, nevertheless confirming the considerable relatedness of bla_{CMY}-positive isolates compared to that of bla_{CMY}-negative ones (Fig. 3). Clonal relationships similar to those revealed by ribotyping were also obtained when the I-CeuI typing patterns were compared, as expected, given that this enzyme cuts within *rrn* loci (data not shown).

Antimicrobial susceptibility testing. The β-lactam resistance patterns of the study isolates were typical as for producers of CMY-type cephalosporinases (1, 21, 29), with high-level resistance to penicillins, cefoxitin, cefotaxime, and ceftazidime (Table 3). However, there were significant differences in resistance levels, even among the isolates that produced the same enzyme variant, and this variation concerned both the compounds that are substrates of CMY-like β-lactamases and those that are not (for imipenem, the presence of metallo-β-lactamase VIM-1 in the isolates from Greece was noteworthy). Although high, the MICs for the isolates with double bla_{CMY} gene copies were not consistently higher than those for the isolates with single bla_{CMY} copies.

DISCUSSION

This study follows several reports on the international presence of unusual *P. mirabilis* strains with chromosomal bla_{CMY-2}-like genes. Such isolates were first recovered in the 1990s in France, some of them from Greek and Algerian patients (4, 11); further studies revealed their emergence and spread in Poland (13, 21) and, more recently, in Italy (9, 23) and Greece (25, 35). Comparative analysis of representative isolates from all of the above countries revealed striking similarities among them, regardless of geographic and temporal origins. First, their bla_{CMY} genes resided in transposition modules of the same structure, called Tn*6093*, and composed of IS*Ecp1*, the *C. freundii* chromosomal fragment and a part of the ColE1-type plasmid backbone. Second, Tn*6093* elements in all isolates were integrated into the same site of the *P. mirabilis* chromosomal *pepQ* gene (28). These data are consistent with a single mobilization of these bla_{CMY} genes' ancestor and identified its putative donor to the *P. mirabilis* chromosome. This was most probably a ColE-like plasmid similar to pTKH11, pA172, and pH 205, all of which carry an IS*Ecp1* module with bla_{CMY-2}-like genes, named Tn*6114* (38, 39). Tn*6114*-like elements, extended by plasmid scaffold sequences, formed the Tn*6093* modules. During the putative mobilization-transfer-integration, IS*Ecp1* would have utilized different alternative IRRs while transposing from the *C. freundii* chromosome to the plasmid (IRR-3) and then from the plasmid to the *P. mirabilis* chromosome (IRR-4), thus giving rise to the Tn*6114*- and Tn*6093*-like structures, respectively.

In sequence comparisons, the *P. mirabilis* bla_{CMY} alleles

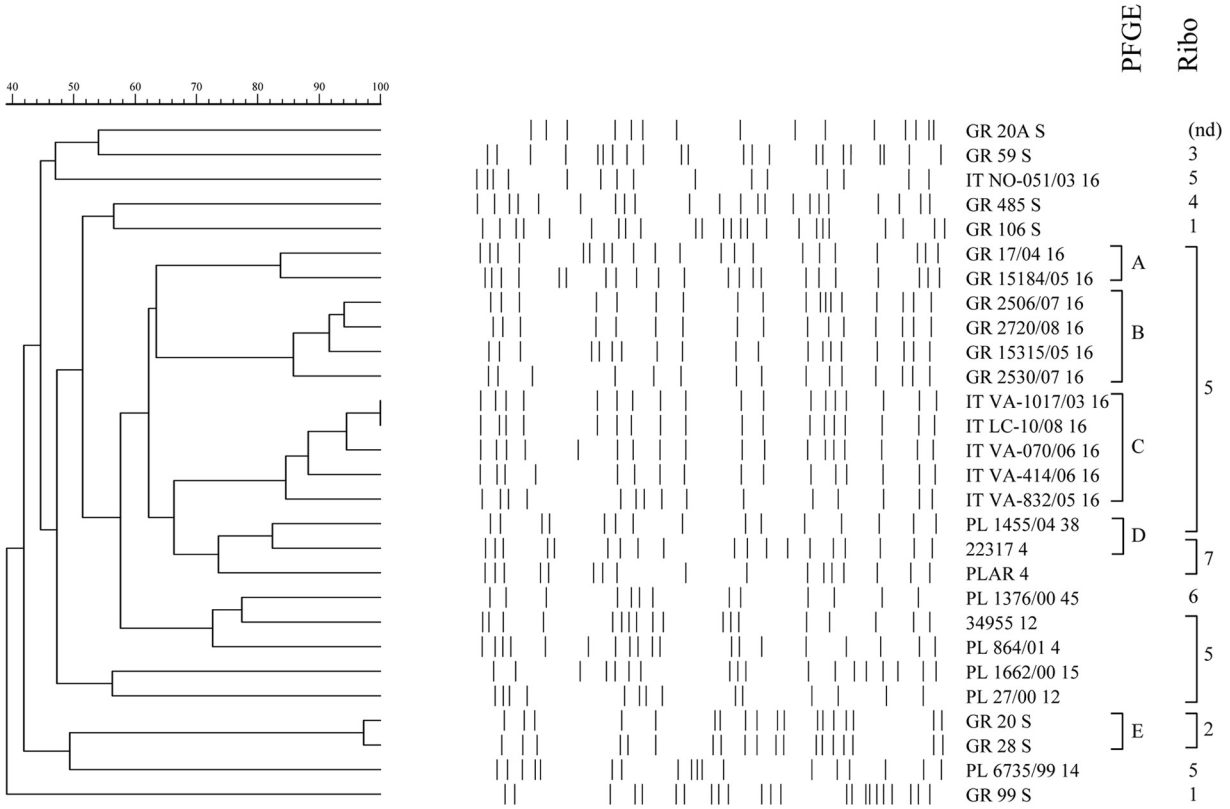

FIG. 3. Dendrogram based on NotI+SfiI PFGE patterns, indicating clusters of the resulting types, as well as ribotypes. The bands arising after NotI+SfiI PFGE for each isolate are shown. The number next to the isolate code denotes the CMY variant. PFGE type clusters (>80% similarity, as per the percent scale at top left) are labeled A to E; ribotypes 1 to 7 are shown to the right of these.

formed a distinct cluster among all bla_{CMY-2}-like genes. This lends further support to the hypothesis of a single mobilization-transfer-integration event, as posited by the data just discussed: according to this, the seven bla_{CMY} alleles studied here will have evolved by the stepwise acquisition of mutations after Tn*6093* chromosomal integration. Allele bla_{CMY-12}, since it shares polymorphisms specific to two separate gene sublineages, may have arisen from recombination between two different alleles. Most of the bla_{CMY} genes identified in the *P. mirabilis* chromosome have only been observed in this genetic environment thus far. Their direct precursor is likely to have been bla_{CMY-4}, identified in early isolates from France/Greece and Poland (although not in a plasmidic Tn*6114*-like module), rather than the bla_{CMY}s (bla_{CMY-5}, bla_{CMY-31}, and bla_{CMY-36}) of the ColE1-like plasmids described to date (38, 39), which all have higher numbers of nucleotide differences. Moreover, several other polymorphisms have been detected between Tn*6093* and the homologous parts of these plasmids. As has been shown in earlier studies on recombinant *E. coli* expressing most of the bla_{CMY} genes studied here, mutations acquired during these genes' evolution were neutral (9, 21); however, the clinical *P. mirabilis* strains themselves varied in resistance levels to β-lactams (9, 21, 23, 25, 35) (Table 3). Such variation could be due to other enzymes, as in the case of the Greek isolates, which produced VIM-1 metallo-β-lactamase (25, 35) (Table 1), or to nonenzymatic mechanisms, e.g., reduction in permeability, as previously proposed (21).

The presence of a second bla_{CMY} module in a subset of our isolates was also intriguing. Previously, Polish isolates had been found to contain two copies of bla_{CMY-12} and bla_{CMY-15} (21). The present study, while confirming these observations, also showed that the French-Algerian isolate with bla_{CMY-12} (11) and the Polish one with bla_{CMY-38} (13) also had two gene copies. These "duplicated" alleles are related: within the bla_{CMY} sequence cluster, bla_{CMY-15} and bla_{CMY-38} form a sublineage, to which bla_{CMY-12} is closely linked (Fig. 2). All "additional bla_{CMY} loci" contained an identical IS*Ecp1* module, Tn*6113*, inserted into the same site, downstream of the *ppiD* gene (28). Most probably, this emerged from a transposition event in which IS*Ecp1* utilized yet another IRR-like motif (IRR-2); however, it is impossible to decide on whether Tn*6113* resulted from a "main bla_{CMY} locus" Tn*6093* or a plasmidic Tn*6114* transposition. At any rate, this integration also probably occurred once, at the origin of the specific sublineage of bla_{CMY}s. Interestingly, the Tn*6093* and Tn*6113* found in the same chromosome always harbored identical bla_{CMY} variants, possibly due to sequence homogenization by a gene-conversion-like mechanism. As reported previously, isolates with double bla_{CMY} copies did not show consistently higher β-lactam resistance levels compared to single-copy isolates (21) (Table 3). This might again have resulted from the already mentioned contributions of β-lactamase- and non-β-lactamase-mediated mechanisms alike on overall resistance.

Addressing the possible clonality of the bla_{CMY}-carrying iso-

TABLE 3. β-Lactam susceptibility of the study *P. mirabilis* isolates

Isolate	Major β-lactamase(s)	MIC (μg/ml)[a]									Source or reference
		AMP/AMX	PIP	TZP	CTX	CAZ	FEP	FOX	ATM	IPM	
Clinical *P. mirabilis* isolates[b]											
PL 6735/99	CMY-14	>512	128	8	64	128	32	128	16	2	21
PL 1662/00	CMY-15	>512	256	32	128	128	8	512	8	16	21
PL 27/00	CMY-12	>512	256	32	64	128	16	256	8	4	21
PL 1376/00	CMY-45	>512	256	16	64	128	4	128	8	2	This study
PL 864/01	CMY-4	>512	128	1	32	16	4	64	0.5	2	21
PL 1455/04	CMY-38	>512	128	32	>128	64	16	128	8	2	This study
IT NO-051/03	CMY-16	>128	64	4	64	32	2	32	1	2	9
IT VA-1017/03	CMY-16	>128	64	4	>128	32	2	32	1	2	9
IT VA-832/05	CMY-16	>128	>128	4	>128	32	2	64	2	4	23
IT VA-070/06	CMY-16	>128	>128	2	128	16	2	32	1	2	23
IT VA-414/06	CMY-16	>128	>128	2	128	16	2	32	1	2	23
IT LC-10/08	CMY-16	>128	>128	4	128	32	4	32	2	2	This study
GR 17/04	CMY-16 + VIM-1	>128	>128	16	>128	32	4	32	2	2	35
GR 15184/05	CMY-16 + VIM-1	>128	>128	16	128	32	8	>128	2	2	35
GR 15315/05	CMY-16 + VIM-1	>128	>128	32	>128	32	16	>128	2	4	35
GR 2506/07	CMY-16 + VIM-1	>128	>128	64	>128	128	16	>128	8	8	This study
GR 2530/07	CMY-16 + VIM-1	>128	>128	64	128	64	4	>128	4	4	This study
GR 2720/08	CMY-16 + VIM-1	>128	>128	16	>128	128	16	>128	4	8	25
22317	CMY-4	>128	64	2	64	32	0.125	32	1	1	11
PLAR	CMY-4	>128	128	4	128	64	0.25	128	1	1	11
34955	CMY-12	>128	32	2	>128	>128	0.25	128	2	0.5	11
ATCC isolates											
E. coli ATCC 25922		4	1	0.5	0.03	0.25	0.125	8	0.125	0.06	21
P. mirabilis ATCC 7002		1	≤0.25	≤0.25	≤0.015	0.06	0.125	2	≤0.015	0.5	21

[a] Abbreviations: AMP, ampicillin; AMX, amoxicillin; ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; IPM, imipenem; PIP, piperacillin; TZP, piperacillin + tazobactam. For AMP/AMX, PIP, and FOX, different highest concentrations of the compounds were used in the MIC determinations in the three laboratories participating in the study (512 or 128 μg/ml). For TZP, the tazobactam concentration was 4 μg/ml.

[b] Most of the isolates were obtained from Poland (PL), Italy (IT), and Greece (GR). Isolates 22317 and PLAR originated also from Greece. Isolate 34955 originated from Algeria.

lates required a method able to assess phylogenetic similarity among organisms collected over a large geographic area and a long period of time. Therefore, in the absence of an MLST scheme for *P. mirabilis*, we used ribotyping. This revealed a significant relatedness among most of our isolates, especially compared to a group of random bla_{CMY}-negative *P. mirabilis* strains. In general, these results were congruent with those obtained with the more discriminatory, PFGE-based methods (using either I-CeuI-digested or NotI-SfiI-digested genomic DNA).

The present study indicates a common origin of *P. mirabilis* strains with acquired and chromosomally inserted bla_{CMY-2}-like genes, spreading in Europe since the 1990s. Their dissemination is probably quite efficient: in Poland, such isolates comprised ~20% of the nosocomial *P. mirabilis* isolates in the mid-2000s (13), while in Italy their progressive spread has also been documented (9, 23). It is therefore likely that they represent a true international clonal group of *P. mirabilis* undergoing continuous diversification over time and place.

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