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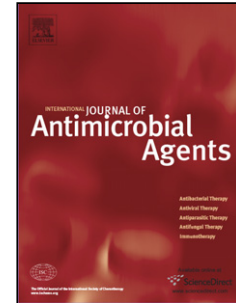
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## High-level ciprofloxacin resistance among hospital-adapted *Enterococcus faecium* (CC17)

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## ABSTRACT

Hospital-adapted *Enterococcus faecium* differ from their colonising variants in humans and animals by additional genomic content. Molecular typing based on multilocus sequence typing (MLST) allows allocation of isolates to specific clonal complexes (CCs), such as CC17 for hospital-adapted strains. Acquired ampicillin resistance is a specific feature of these hospital isolates, especially in Europe. A few recent reports have described acquired high-level ciprofloxacin resistance as a supposed feature of hospital-adapted *E. faecium* strains. In the present retrospective analysis, ciprofloxacin minimum inhibitory concentrations (MICs) of 609 clinical isolates from German hospital patients (1997–2007) were determined and a breakpoint for high-level resistance was deduced (>16 mg/L). Acquired high-level ciprofloxacin resistance was distributed among isolates of 26 different MLST types (all CC17), indicating a wide prevalence of this acquired resistance trait among the hospital-adapted *E. faecium* population. High-level ciprofloxacin resistance was linked to *gyrA* and *parC* mutations in 98 investigated isolates. Eleven different allele types or combinations thereof were identified. Their allocation to specific MLST and pulsed-field gel electrophoresis (PFGE) types revealed differences in the emergence and spread of corresponding mutations and strains.

## 1. Introduction

Isolates of *Enterococcus faecium* are considered facultative pathogens that appear as infectious agents only under specific predispositions and in patients with severe underlying diseases or disorders. Results from recent molecular typing and characterisation experiments, including microarray-based genome comparisons, revealed insights into the population structure within *E. faecium*. Epidemic isolates from hospital patients belong to a specific clonal group [clonal complex (CC) 17]. Among representatives of that group, distinct markers appear to be enriched or exclusively distributed, such as the collagen-adhesin genes *acm* [1–3] and *scm* [4], genes encoding other matrix-binding or pili-associated proteins [4–7], a gene encoding an enterococcal surface protein *esp* [8], a putative hyaluronidase *hyl<sub>Efm</sub>* [9] and genomic islands of unknown functions [10,11]. Ampicillin resistance appears as a resistance marker for hospital *E. faecium*, at least in Europe [12–16], and high-level ciprofloxacin resistance appears to be increasingly distributed among hospital isolates of *E. faecium* and *Enterococcus faecalis* [17–20].

Isolates of the enterococcal species *E. faecalis* and *E. faecium* show generally low susceptibilities to ciprofloxacin and modern fluoroquinolones. The Clinical and Laboratory Standards Institute defines ciprofloxacin resistance in enterococci as a minimum inhibitory concentration (MIC) of  $\geq 4$  mg/L [21]. Other international bodies such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST; [http://www.escmid.org/research\\_projects/eucast/](http://www.escmid.org/research_projects/eucast/)) do not specify a breakpoint for the combination of fluoroquinolones and enterococci. However, certain epidemic strains of *E. faecium* and *E. faecalis* infecting hospital patients may show increased MICs to ciprofloxacin (acquired ‘high-level resistance’) [17,18,22–25]. Colonisation often

precedes subsequent infection by facultative pathogenic bacteria such as multiresistant and vancomycin-resistant isolates of *E. faecium* and *E. faecalis*, and treatment of patients with fluoroquinolones, an antibiotic class increasingly used among hospital patients [26,27], appears as an independent risk factor for acquisition of these strains [28–30]. Increased non-susceptibility to ciprofloxacin and modern fluoroquinolones in hospital-adapted enterococcal strains may actively facilitate colonisation and subsequent infection or may simply reflect the antibiotic pressure exerted among the nosocomial setting in general.

The targets of fluoroquinolones are topoisomerases II and IV, and mutational changes among genes encoding mainly subunits A and to a lesser extent also subunits B are associated with increased MICs to ciprofloxacin and other fluoroquinolones [31,32]. Topoisomerase II (DNA gyrase) appears to be the primary target in Gram-negative bacteria and topoisomerase IV is the primary target in Gram-positive bacteria. Corresponding in vitro selection models were also described for enterococci; however, results are somehow conflicting regarding the primary target in *Enterococcus* spp. and the necessity of specific mutations in one or both A subunit genes to confer what is specified as high-level fluoroquinolone resistance [33–37].

Only a few studies exist where ciprofloxacin resistance mutations in *E. faecium* have been investigated to a greater extent and were linked to specific clonal types and strains [17,19]. In these studies, a breakpoint of high-level ciprofloxacin resistance is suggested of >16 mg/L [19] or ≥64 mg/L (worldwide collection of strains) [17], which was confirmed by molecular analysis of mutations in *gyr* and *par* subunit genes.

In the present study, we investigated a representative collection of 609 clinical strains of *E. faecium* from German hospital patients representing individual infections or outbreaks collected between 1997 and 2007. The breakpoint for high-level ciprofloxacin resistance was specified and the annual changes in the MIC<sub>50</sub> and MIC<sub>90</sub> values (MICs at which 50% and 90% of all tested isolates were inhibited, respectively) within the test period were determined. In addition, the corresponding resistance phenotype was linked to mutations in *gyrA* and *parC* genes in a subset of 98 invasive isolates. The clonal background of the considered isolates was resolved by multilocus sequence typing (MLST) and partly by pulsed-field gel electrophoresis (PFGE), suggesting different routes of emergence and spread of high-level ciprofloxacin resistance among these hospital-adapted *E. faecium* isolates.

## 2. Materials and methods

### 2.1. Bacterial strains

Our 'Nosocomial Infections' unit (Robert Koch Institute, Germany) hosts the German National Reference Centre for Staphylococci and serves in addition as a Focal Laboratory for Enterococci. In general, enterococcal isolates sent to us are pre-selected for being vancomycin-resistant and multiresistant or with the suspicion of clonal spread (outbreak). Our strain collection includes more than 8000 enterococcal isolates from the early 1990s until now, the majority of which are clinical strains. The subset of strains investigated here includes clinical *E. faecium* isolated from 1997 to 2007. They were chosen as being representative by originating from different clinical laboratories or university medical centres from all over Germany. Only one isolate per hospital at a time was allowed (excluding copy isolates). Based on these criteria, approximately 55 *E. faecium* isolates per year were included summing to a total of

609 isolates. A subset of 156 isolates was MLST typed, originating exclusively from invasive infections/blood cultures. Mutations in the genes *gyrA* and *parC* were resolved for 98 of the 156 MLST typed isolates. For the purpose of comparison, altogether 74 commensal *E. faecium* isolates originating from two independent prevalence studies among healthy persons from the community in two German federal states in 2002 and 2004 were included [38,39].

### 2.2. Antibiotic susceptibility testing

Susceptibility to 19 antibiotics was routinely determined by microbroth dilution using IsoSensitest (until 2006) or Mueller–Hinton broth (from 2007 onwards, according to the German standard DIN58940; data not presented). MICs for ciprofloxacin were determined in a separate dilution (1–1024 mg/L) using Mueller–Hinton broth to determine high-level resistance. MIC<sub>50</sub> and MIC<sub>90</sub> values were defined as the MICs at which 50 and 90% of the investigated isolates were inhibited, respectively.

### 2.3. DNA isolation

Genomic DNA was prepared using a DNA extraction kit (DNeasy Tissue Kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. An initial cell wall lysis step was added dissolving the cell pellet in TES buffer [10 mM Tris, 0.5 mM ethylene diamine tetra-acetic acid (EDTA), 10% sucrose (pH 8.0)] plus 10 mg/mL lysozyme (Roche Applied Science, Mannheim, Germany) followed by incubation at 37 °C for 30 min.



#### 2.4. Polymerase chain reaction (PCR)

PCR was performed with Ready-To-Go™ PCR Beads (GE Healthcare, Freiburg, Germany) according to the manufacturer's instructions. Approximately 0.5 µL of isolated genomic DNA (ca. 10 ng) and primers (200 nM each) were added. The presence of the resistance genes *vanA* and *vanB* was tested in a multiplex PCR [40]. Primers and PCR conditions to amplify fragments of the corresponding subunits A (*gyrA*, *parC*) and B (*gyrB*, *parE*) were chosen as described recently [17] and were as follows: *gyrA*-F, 5'-CGGGATGAACGAATTGGGTGTGA; *gyrA*-R, 5'-AATTTTACTCATACGTGCTTCGG; *gyrB*-F, 5'-TGAAATTCTTGCTGGAAAAC; *gyrB*-R: 5'-CAACAATAGGACGCATGTAAC; *parC*-F, 5'-TTCCCGTGCATTTTCGATCAGTACTTC; *parC*-R, 5'-CGTATGACAAAGGATTCSGTAAATC; *parE*-F, 5'-GTCCGTAAAGCAATCAAAG; and *parE*-R, 5'-CTTTATATAAAGGCGGTAACG. These primers cover the so-called 'quinolone resistance-determining region' (QRDR).

#### 2.5. Pulsed-field gel electrophoresis typing

Genomic DNA for PFGE analysis was isolated, digested with restriction endonuclease *Sma*I and treated as described recently [40]. A 1% agarose gel concentration and a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA) were used for PFGE. The ramped pulsed times were as follows: 1–11 s for 15 h and 11–30 s for 14 h at 14 °C. *Sma*I-digested *Staphylococcus aureus* NCTC 8325 was used as a size marker on all PFGE gels. Composite trees were generated using a band-based similarity coefficient (Dice) and unweighted pair group method with arithmetic mean (UPGMA) clustering (BioNumerics v. 5.1; Applied Maths, Sint-

Martens-Latem, Belgium). Definition of clusters and subgroups was according to Morrison et al. [41], based on an 82% similarity of pattern.

### *2.6. DNA sequencing*

Sequencing reactions were performed according to the manufacturer's recommendations for cycle sequencing of PCR products (Applied Biosystems, Darmstadt, Germany). Sequence files were read, evaluated, aligned and compared with the reference set of alleles using DNASTAR's Lasergene 8.0 software (SeqMan 8.0; MegAlign 8.0, EditSeq 8.0).

### *2.7. Multilocus sequence typing*

PCRs amplifying the seven loci used for MLST were done according to the reference MLST database (<http://efaecium.mlst.net/>). Sequencing reactions were performed according to the manufacturer's recommendations for cycle sequencing of PCR products (Applied Biosystems). Sequence files were read, evaluated, aligned and compared with the reference set of alleles using sequencing software Lasergene 8.0 from DNASTAR (SeqMan 8.0; EditSeq 8.0), TraceEditPro v. 1.1.1 from Ridom (<http://www.ridom.de>) and via the official MLST webpage (<http://efaecium.mlst.net/>).

### *2.8. Multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA)*

VNTRs were amplified according to Top et al. [42] using modifications given at the corresponding website of the University of Utrecht, The Netherlands (<http://www.mlva.umcutrecht.nl>). MLVA profiles were assigned using the corresponding tool at the webpage (see above).

## 2.9. Statistics

Mathematical calculations and basic statistical analyses were performed with software package EpiCompare 1.0 (Ridom).

## 3. Results

### 3.1. Distribution of ciprofloxacin minimum inhibitory concentrations

The strain collection of *E. faecium* sampled between 1997 and 2007 consisted of 3303 mostly hospital isolates from 179 clinical or university microbiological laboratories from all over Germany. A representative subset of 609 *E. faecium* isolates from this collection (median 53/year, excluding copy isolates) was chosen for further analysis to determine MICs for ciprofloxacin. After plotting MIC values against the number of tested strains at that MIC, a bimodal distribution was observed, suggesting a breakpoint of >16 mg/L for defining high-level ciprofloxacin resistance (Fig. 1). When MIC<sub>50</sub> and MIC<sub>90</sub> data were displayed, an almost constant annual increase was observed (Table 1), with the number of isolates belonging to the group of supposed high-level ciprofloxacin-resistant isolates also increasing annually (Table 1). For the collection of isolates from 2003 onwards, the MIC<sub>50</sub> and MIC<sub>90</sub> values were constantly above the suggested breakpoint for high-level ciprofloxacin resistance, illustrating the high prevalence of this feature among clinical isolates of *E. faecium* from this time onwards. A subset of 156 blood culture isolates was typed using MLST. The data showed the distribution of high-level ciprofloxacin resistance (MIC > 16 mg/L) among 26 MLST types (Table 2), all of which were allocated to CC17 using eBURST (not shown). This finding indicates that high-level ciprofloxacin

resistance did not emerge and disseminate among a limited number of a few MLST types but is rather distributed among the general hospital-adapted *E. faecium* population (CC17). In particular, early representatives (isolated before 2002) of major MLST types such as sequence type ST17 were not high-level ciprofloxacin resistant, whereas later isolates expressed higher MICs (Fig. 2a). In line with that, isolates of ST192 that were highly prevalent among German hospitals only during recent years were all high-level ciprofloxacin-resistant ( $n = 27$ , from 10 different diagnostic laboratories, isolated 2004–2007; see below). For the purpose of comparison, altogether 74 *E. faecium* isolates from two prevalence studies among non-hospitalised persons were investigated. All commensal isolates displayed MICs below the suggested breakpoint for acquired high-level ciprofloxacin resistance (MICs  $\leq 8$  mg/L; see also Table 1).

### 3.2. Screening for *gyrA/B* and *parC/E* mutations

A subset of 98 MLST-typed invasive *E. faecium* isolates was selected for molecular analysis of *gyrA/B* and *parC/E* mutations. Tested isolates spanned a range of ciprofloxacin MICs from  $\leq 1$  mg/L to 512 mg/L, with the vast majority of isolates considered as high-level ciprofloxacin-resistant ( $n = 77$  with MICs of  $>16$  mg/L). Supposed mutations in the QRDR of *gyrA* and *parC* alleles were determined (Table 3). Isolates possessing MICs of  $<16$  mg/L did not possess any mutational changes in *gyrA* or *parC*. The most frequent mutations in isolates with ciprofloxacin MICs of  $\geq 16$  mg/L appeared at a position in *gyrA* encoding for Ser-83 and a position in *parC* encoding for Ser-80 (*E. coli* numbering) (Table 3). Occasionally, a position in *gyrA* encoding for Glu-87 was modified (4 of 98 isolates). Mutations at a position in *parC* encoding Glu-84 or any of the other described modifications [17,19,20,33] were not

identified in the present set of strains. If mutations appeared, both targets *gyrA* and *parC* were always affected; no clinical isolate possessed single mutations in either *gyrA* or *parC*. Corresponding fragments of subunits B (*parE/gyrB*) were investigated from a subset of 33 isolates, including 26 isolates with ciprofloxacin MICs of  $\geq 16$  mg/L. It was known from previous studies in enterococci that both targets were not affected; we also did not identify any relevant mutation in *gyrB* or *parE* genes.

### 3.3. Pulsed-field gel electrophoresis

The most prevalent MLST types in our collection of 98 strains were ST17 ( $n = 28$ ; 29%), ST192 ( $n = 27$ ; 28%) and ST18 ( $n = 20$ ; 20%). A different prevalence of ciprofloxacin MICs and QRDR mutations among isolates of identical MLST types prompted us to type isolates from selected STs with a highly discriminative method such as *Sma*I macrorestriction PFGE. Isolates of ST17 and ST192 were compared. The collection of 28 ST17 isolates consisted of strains isolated between 1999 and 2007 with ciprofloxacin MICs between  $\leq 1$  mg/L and 512 mg/L (Fig. 2a). High-level ciprofloxacin-resistant isolates harboured various mutations at positions encoding Ser-83 in *gyrA* or Ser-80 in *parC* (Fig. 2a; Table 3). Individual mutations as well as combinations of *gyrA* and *parC* allele types were very diverse, suggesting an independent development of high-level ciprofloxacin resistance among various ST17 clones. Isolates of ST192 were only prevalent during recent years. All the 27 high-level ciprofloxacin-resistant ST192 *E. faecium* isolates were epidemiologically unlinked and originated from 10 microbiological laboratories; however, all showed similar characteristics such as a combination of identical *gyrA* (Ser-83 [AGT] to Ile-83 [ATT]) and *parC* (Ser-80 [AGC] to Arg-80 [CGC]) mutations (Fig. 2b). In addition, all ST192 isolates possessed a silent/synonymous mutation in *parC* encoding His-75

(CAT to CAC), which appeared almost exclusively among isolates of that MLST type and was found only once in a non-ST192 isolate in our collection (isolate UW6918, ST17). PFGE typing and subsequent fragment analysis using the criteria of Morrison et al. [41] (82% similarity cut-off) revealed 17 clusters among the 28 ST17 isolates [discriminatory index (DI) 0.952; 95% confidence interval (CI) 0.914–0.99] and 11 clusters among the 27 ST192 isolates (DI 0.815; 95% CI 0.689–0.94).

#### 4. Discussion

High-level ciprofloxacin resistance in isolates of *E. faecium* and/or *E. faecalis* was analysed and described in a few recent papers. However, the respective strain collections were mostly limited or restricted in terms of time, geographical coverage or any homogeneity, thus not allowing any specific epidemiological conclusions [20,33,34]. Here, based on a comprehensive collection of clinical *E. faecium* isolated from German hospital patients between 1997 and 2007, we showed a permanent increase in the MICs for ciprofloxacin (Table 1). Based on a bimodal distribution of ciprofloxacin MICs of the 609 representative isolates from that time span, an MIC of >16 mg/L could be deduced as the microbiological breakpoint (Fig. 1) for defining high-level ciprofloxacin resistance in *E. faecium*. This is in agreement with previous reports suggesting a breakpoint of >16 mg/L [19] or  $\geq 64$  mg/L [17] and is also confirmed here by sequence determination of the QRDR in *gyrA* and *parC*. All ( $n = 77$ ) but three isolates possessing MICs of >16 mg/L harboured modifications within the genes *gyrA* and *parC* that have been shown to be linked with ciprofloxacin resistance. Of the eight isolates having an MIC of 16 mg/L, three showed wild-type alleles and five had acquired different mutations in *gyrA* and *parC*.

In our collection of clinical strains, wild-type alleles of *gyrA/B* and *parC/E* were detectable in isolates of different MLST types and with MICs ranging from  $\leq 1$  mg/L to 16 mg/L and also exceptionally with higher MICs of 32 mg/L or 64 mg/L ( $n = 3$ ; Table 3). Isolates with acquired high-level ciprofloxacin resistance possessed mutations in both *gyrA* and *parC*, but MICs differed by several dilutions in isolates harbouring identical *gyr/par* mutations (Table 3). This indicates alternative resistance mechanisms or upregulation and downregulation of genes other than those investigated here but involved in higher expression of fluoroquinolone non-susceptibility, such as efflux pumps or topoisomerase protection genes [36,43–45]. Several authors have also reported double mutations in *parC* and *gyrA* prevalent among high-level ciprofloxacin-resistant clinical isolates of *E. faecium* [19,20,34], whereas other reports demonstrated single *parC* mutations as sufficient to confer elevated ciprofloxacin MICs in natural isolates [17,33]. In some of the previous studies, sequential mutations were introduced by challenging test strains with growing concentrations of fluoroquinolones in vitro [36,37]. Differences in the in vitro selective potential were discussed as being drug-related (due to different fluoroquinolones used), or species- and even strain-dependent [35–37]. Based on our collection of isolates, we could not confirm special features associated with ciprofloxacin resistance in Gram-positive bacteria in general, such as a more susceptible target in genes encoding topoisomerase IV (*parC/E*), and combined with that a sequential accumulation of specific mutations in different targets increasing step by step the MICs for ciprofloxacin [31,36,37].

Taking the data already published and that from the present study together, it appears unambiguous that acquired high-level ciprofloxacin resistance is, in addition to acquired ampicillin resistance, increasingly prevalent among hospital strains of *E.*

*faecium*. The absence of acquired high-level ciprofloxacin resistance among commensal *E. faecium* isolates from two outpatient prevalence studies performed during the study period supports this model (Table 1). Screening for high-level ciprofloxacin resistance for all of the enterococcal strains sent to the German Focal Laboratory for Enterococci was introduced in October 2007. From this time until December 2008, in total 376 clinical *E. faecium* isolates were received (from 45 microbiological laboratories representing more than 60 different German hospitals), 364 (97%) of which expressed MICs of >16 mg/L, showing that high-level ciprofloxacin resistance is highly prevalent among clinical *E. faecium* among German hospital patients (>99% were ampicillin-resistant).

The prevalence of QRDR mutations among isolates from various MLST types differed. The supposed clonal divergence based on different QRDR mutations in *gyrA* and *parC*, for instance, among ST17 isolates could be confirmed by PFGE and subsequent cluster analysis (Fig. 2a). In contrast, all ST192 isolates possessed identical *gyrA* and *parC* mutations and results of PFGE analysis revealed a recognisable fragment pattern and a distinct level of clonal relatedness according to the set criteria (Fig. 2b) [41]. Combination of all data for the ST192 isolates suggests a model where high-level ciprofloxacin resistance once emerged among a specific ST192 clone that later on disseminated among several hospitals in different cities and federal states in Germany, before the occasional horizontal acquisition of vancomycin resistance genes (*vanA/B*) (Fig. 2b).

Between 1995 and 1999, a specific *vanA*-type *E. faecium* clone spread country-wide [46,47]. These isolates possessed a specific PFGE and plasmid pattern, were MLST type ST117 and MLVA type MT12, and demonstrated a characteristic marker gene



pattern (*esp*<sup>+</sup>, *hyl*<sub>*Efm*</sub><sup>-</sup>). None of the ST117 isolates investigated (*n* = 5) showed any *gyrA* or *parC* mutation despite reaching MICs of up to 64 mg/L (Table 3). Therefore, if and to what extent acquired high-level resistance to ciprofloxacin indeed supports the successful dissemination of isolates among the nosocomial setting is still debatable and must be resolved in further studies. The rapid spread of this resistance trait within recent years and among various clonal types and only among hospital-adapted isolates (CC17) suggests an obvious benefit for the bacterium in this specific setting. The increased prevalence of high-level ciprofloxacin resistance in clinical *E. faecium* isolates in recent years coincides with an increasing prevalence of vancomycin resistance in *E. faecium* since 2003 in Germany, accumulating in a wider prevalence of a various number of multiresistant and vancomycin-resistant clinical *E. faecium* clones [15,48,49].

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**Competing interests**

None declared.

**Ethical approval**

Not required.

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**Fig. 1.** Bimodal distribution of ciprofloxacin minimum inhibitory concentrations (MICs) for the 609 *Enterococcus faecium* clinical isolates from German hospital patients (1997–2007).

**Fig. 2.** Similarity dendrogram [unweighted pair group method with arithmetic mean (UPGMA)] of *Sma*I macrorestriction analyses resolved by pulsed-field gel electrophoresis (PFGE) of epidemic *Enterococcus faecium* from multilocus sequence type (MLST) (a) ST17 and (b) ST192. An 82% similarity cut-off value has been delineated for reasons of comparison based on the criteria of Morrison et al. [41]. Origin, town from which the isolate originated (please consider that an identical 'town' could represent isolates from different hospitals); year, year of isolation; MIC CIP, minimum inhibitory concentration of ciprofloxacin (mg/L); esp and hyl, results of polymerase chain reaction (PCR) screenings for fragments of the *esp* or *hyl<sub>Efm</sub>* genes; *vanA/B*, presence of *vanA* or *vanB* by PCR. \* Ciprofloxacin MIC of UW6930 (ST192) was 4 mg/L in Mueller–Hinton broth owing to weak growth in Mueller–Hinton in general; the MIC was 32 mg/L in brain–heart infusion broth.

**Table 1**

Annual development of minimum inhibitory concentrations (MICs) for ciprofloxacin among 609 clinical *Enterococcus faecium* isolates from 1997–2007 (I) and 74 commensal *E. faecium* isolates from two outpatient prevalence studies performed in 2002 and 2004 (II)

Collection	Year	No. of isolates	MIC <sub>50</sub> (mg/L)	MIC <sub>90</sub> (mg/L)	% of isolates with MIC ≤ 16 mg/L	% of isolates with MIC > 16 mg/L
I	1997	51	2	4	100	0
	1998	52	2	16	94	6
	1999	61	4	64	85	15
	2000	51	4	256	61	39
	2001	55	32	256	47	53
	2002	35	4	256	54	46
	2003	51	64	256	33	67
	2004	58	128	256	16	84
	2005	53	256	256	4	96
	2006	72	256	256	8	92
	2007	70	128	256	13	87
II	2002	20	1	4	100	0
	2004	54	2	4	100	0

MIC<sub>50/90</sub>, MICs at which 50% and 90% of all tested isolates were inhibited, respectively.

**Table 2**

High-level ciprofloxacin resistance (MIC > 16 mg/L) according to multilocus sequence type (MLST)

MLST	No. of isolates	Town <sup>a</sup>	MIC (mg/L)
16	2	2	32/256
17	28 <sup>b</sup>	16	≤1–512
18	20 <sup>b</sup>	14	≤1–512
65	2	1	128/256
78	11 <sup>b</sup>	11	16–256
80	2	1	32
81	4 <sup>b</sup>	2	≤1–256
117	14 <sup>b</sup>	9	4–256
125	1	1	256
132	1	1	128
192	27 <sup>b</sup>	12	16–512 <sup>c</sup>
202	8 <sup>b</sup>	5	≤1–256
203	5	5	64–128
208	1	1	128
252	2	2	128/256
275	1	1	256
279	1	1	64
282	1	1	128
294	1	1	256
306	1	1	256
371	1	1	128
375	1	1	256
376	1	1	256
377	1	1	256
378	3	2	256
485	1	1	128

MIC, minimum inhibitory concentration.

<sup>a</sup> Town from where isolates originated (hospital laboratory/microbiological laboratory).

<sup>b</sup> Also contains isolates with ciprofloxacin MICs of ≤16 mg/L.

<sup>c</sup> The ciprofloxacin MIC of UW6930 (ST192) was 4 mg/L in Mueller–Hinton broth owing to weak growth in Mueller–Hinton in general; the MIC was 32 mg/L in brain–heart infusion broth.

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**Table 3**

Mutations leading to nucleotide substitutions in the genes *gyrA* and *parC* correlated with their corresponding ciprofloxacin minimum inhibitory concentrations (MICs)

<i>gyrA</i>		<i>parC</i>			Total ( <i>n</i> = 98)	MLST	Ciprofloxacin MIC ( mg/L)									
Ser-83 (AGT)	Glu-87 (GAG)	His-75 (CAT)	Ser-80 (AGC)	Glu-84 (GAA)			≤1	2	4	8	16	32	64	128	256	512
+	+	+	+	+	19	16,17,18,19,81,117,202	6	2	4	1	3	2 <sup>a</sup>	1			
+	Lys (AAG)	+	Ile (ATC)	+	3	16,81,252					2				1	
Arg (AGA)	+	+	Arg (CGC)	+	1	282								1		
Arg (AGG)	+	+	Ile (ATC)	+	2	17									2	
Arg (AGG)	+	+	Arg (AGA)	+	4	17					2				2	
Arg (CGT)	+	+	Ile (ATC)	+	10	17,18,371					1	1	1	3	4	
Ile (ATT)	+		Arg (AGA)	+	1	78									1	

Ile (ATT)	+	+	Arg (CGC)	+	1	17						1					
Ile (ATT)	+		His (CAC) <sup>b</sup>	Arg (CGC)	+	28	17,192 <sup>c</sup>					1	13	8	1	5	
Ile (ATT)	+			Ile (ATC)	+	4	17,202									4	
Tyr (TAT)	+			Ile (ATC)	+	24	17,18,80,117					2				19	3
Tyr (TAT)	Gly (GGG)			Ile (ATC)	+	1	78										1

MLST, multilocus sequence type.

+, wild-type allele.

<sup>a</sup> MIC results were confirmed in repeated experiments.

<sup>b</sup> Synonymous/silent mutation.

<sup>c</sup> Altogether one ST17 and 27 ST192 isolates.

<sup>d</sup> One isolate (UW6930, ST192) grew weakly in general in Mueller–Hinton broth with a ciprofloxacin MIC of 4 mg/L; in brain–heart infusion broth its ciprofloxacin MIC was 32 mg/L.

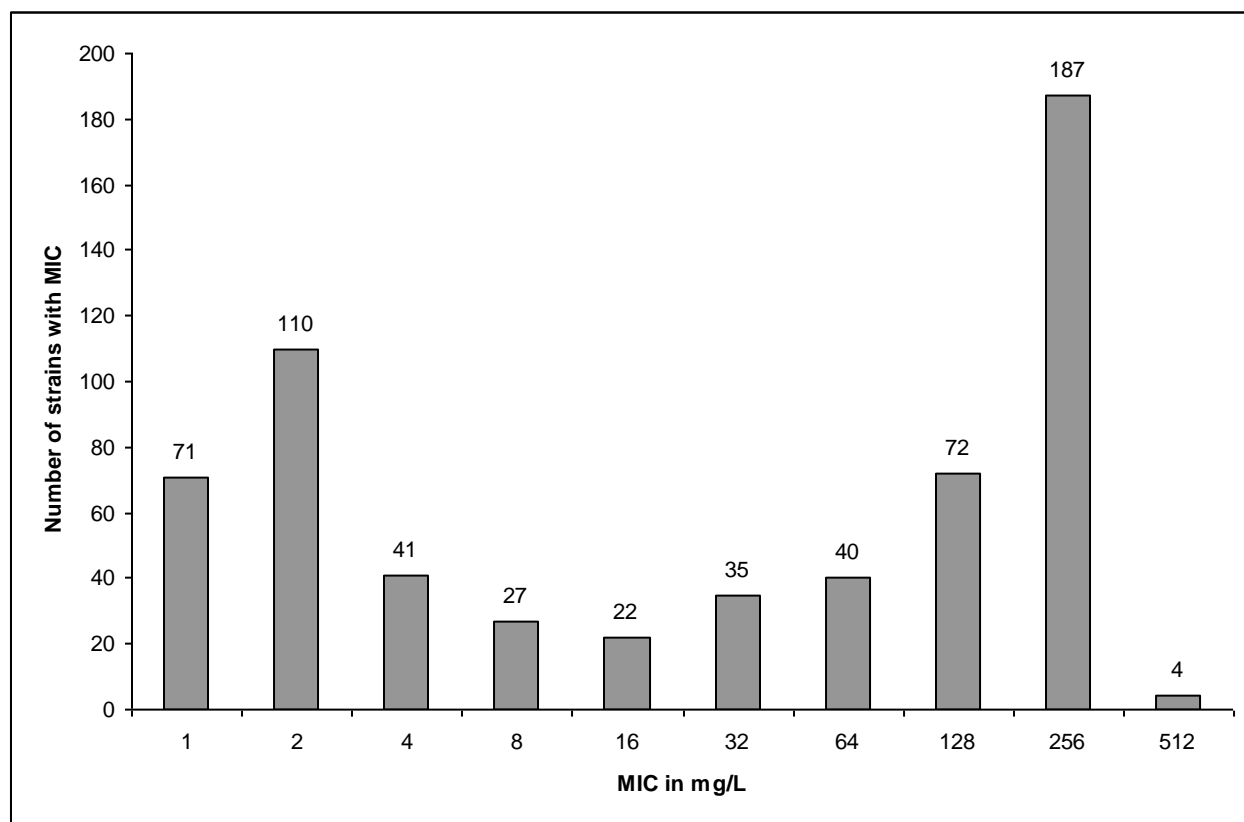


Fig. 1.



Dice (Opt:0.50%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]

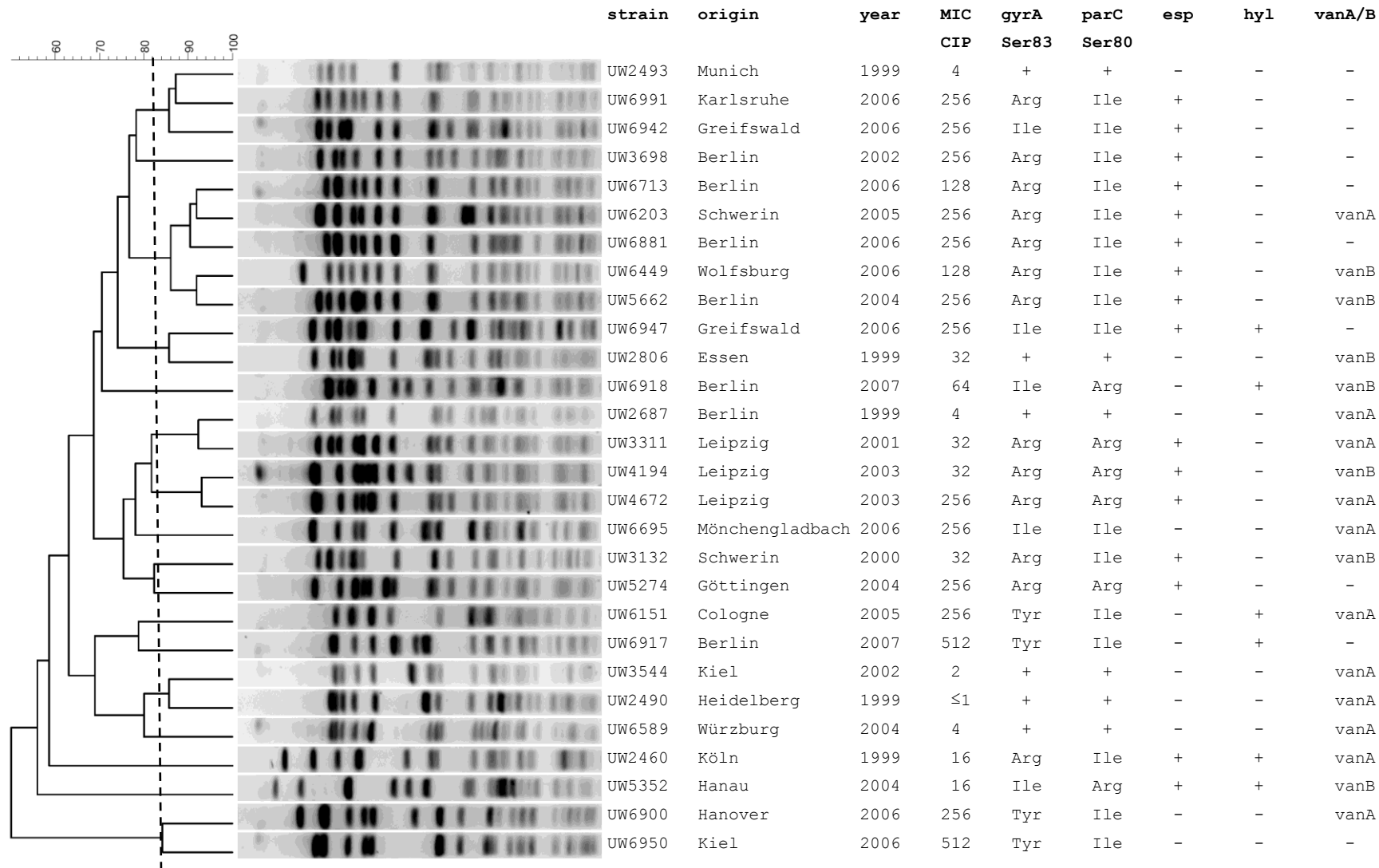
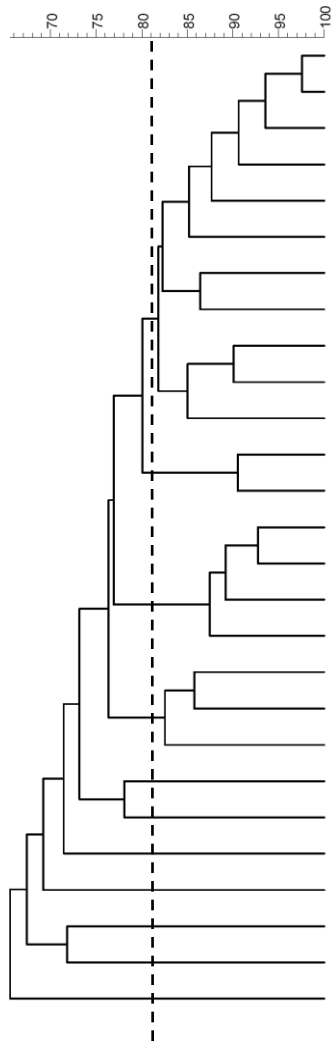


Fig. 2a

Dice (Opt:0.50%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]



Strain	Origin	year	MIC CIP	gyrA Ser83	parC Ser80	parC His75	esp	hyl	vanA/B
UW6951	Kiel	2006	64	Ile	Arg	(CAC)	+	+	-
UW6954	Kiel	2006	64	Ile	Arg	(CAC)	+	+	-
UW6919	Berlin	2007	32	Ile	Arg	(CAC)	+	+	-
UW6929	Ulm	2006	32	Ile	Arg	(CAC)	+	+	-
UW6896	Hanover	2006	32	Ile	Arg	(CAC)	+	+	-
UW6893	Hanover	2006	256	Ile	Arg	(CAC)	+	+	-
UW6933	Ulm	2006	64	Ile	Arg	(CAC)	+	+	-
UW6943	Greifswald	2006	64	Ile	Arg	(CAC)	+	+	-
UW6715	Berlin	2006	32	Ile	Arg	(CAC)	+	+	-
UW6941	Greifswald	2006	64	Ile	Arg	(CAC)	+	+	-
UW5267	Cologne	2004	32	Ile	Arg	(CAC)	+	+	vanB
UW6894	Hanover	2006	32	Ile	Arg	(CAC)	+	+	-
UW6699	Murnau	2006	32	Ile	Arg	(CAC)	+	+	vanB
UW6720	Berlin	2006	512	Ile	Arg	(CAC)	+	+	-
UW6884	Berlin	2006	256	Ile	Arg	(CAC)	+	+	-
UW6711	Berlin	2006	256	Ile	Arg	(CAC)	+	+	-
UW6982	Jena	2006	128	Ile	Arg	(CAC)	+	+	-
UW6718	Berlin	2006	16	Ile	Arg	(CAC)	+	+	-
UW6993	Karlsruhe	2006	32	Ile	Arg	(CAC)	+	+	-
UW6696	Ulm	2006	32	Ile	Arg	(CAC)	+	+	-
UW5909	Freiburg	2004	32	Ile	Arg	(CAC)	+	+	vanA
UW6373	Ulm	2005	32	Ile	Arg	(CAC)	+	+	vanB
UW6986	Jena	2006	32	Ile	Arg	(CAC)	+	+	-
UW6995	Karlsruhe	2006	256	Ile	Arg	(CAC)	+	+	-
UW6915	Berlin	2007	64	Ile	Arg	(CAC)	+	+	-
UW6930	Ulm	2006	32*	Ile	Arg	(CAC)	+	+	-
UW5913	Freiburg	2004	64	Ile	Arg	(CAC)	+	+	vanA

Fig. 2b