Population Genetics of the Nomenspecies *Enterobacter cloacae*

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The genetic heterogeneity of the nomenspecies *Enterobacter cloacae* is well known. *Enterobacter asburiae*, *Enterobacter cancerogenus*, *Enterobacter dissolvens*, *Enterobacter hormaechei*, *Enterobacter kobei*, and *Enterobacter nimipressuralis* are closely related to it and are subsumed in the so-called *E. cloacae* complex. DNA-DNA hybridization studies performed previously identified at least five DNA-relatedness groups of this complex. In order to analyze the genetic structure and the phylogenetic relationships between the clusters of the nomenspecies *E. cloacae*, 206 strains collected from 22 hospitals, a veterinarian, and an agricultural center in 11 countries plus all 13 type strains of the genus and reference strain CDC 1347-71^R were examined with a combination of sequence and PCR-restriction fragment length polymorphism (PCR-RFLP) analyses of the three housekeeping genes *hsp60*, *rpoB*, and *hemB* as well as *ampC*, the gene of a class C β-lactamase. Based on the neighbor-joining tree of the *hsp60* sequences, 12 genetic clusters (I to XII) and an unstable sequence crowd (xiii) were identified. The robustness of the genetic clusters was confirmed by analyses of *rpoB* and *hemB* sequences and *ampC* PCR-RFLPs. Sequence crowd xiii split into two groups after *rpoB* analysis. Only three strains formed a cluster with the type strain of *E. cloacae*, indicating that the minority of isolates identified as *E. cloacae* truly belong to the species; 13% of strains grouped with other type strains of the genus, suggesting that the phenotypes of these species seem to be more heterogeneous than so far believed. Three clusters represented 70% of strains, but none of them included a type or reference strain. The genetic clustering presented in this study might serve as a framework for future studies dealing with taxonomic, evolutionary, epidemiological, or pathogenetic characteristics of bacteria belonging to the *E. cloacae* complex.

Enterobacter cloacae has become increasingly important as a nosocomial pathogen, accounting for up to 5% of hospital-acquired septicemias, 5% of nosocomial pneumonias, 4% of nosocomial urinary tract infections, and 10% of postsurgical peritonitis cases (18, 35, 39). Besides its clinical significance, *E. cloacae* plays an important role as a pathogen in plants (2, 34) and insects (15) and is ubiquitous in the terrestrial and aquatic environments (22). This diversity of habitats is mirrored by the genetic variety of the nomenspecies *E. cloacae*, which has been demonstrated in several studies by biotyping and serotyping (16, 47), ribotyping and phage typing (46), pulsed-field gel electrophoresis and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) (18), and internal transcribed spacer-PCR and random amplification of polymorphic DNA (RAPD)-PCR (6). The 16S ribosomal DNA (rDNA) sequences of *E. cloacae* did not form a coherent cluster but built a patchy tree, in which the clusters of *E. cloacae* strains interfused with those of *Enterobacter aerogenes*, *Escherichia coli*, *Citrobacter* species, and *Leclercia* species. This reflected the genetic heterogeneity of the nomenspecies but did not allow its systematic classification (44).

Over the last few years, five new species (*Enterobacter asburiae*, *Enterobacter cancerogenus*, *Enterobacter dissolvens*, *Enterobacter kobei*, and *Enterobacter nimipressuralis*) have been reassigned to or split out of the nomenspecies *E. cloacae* (4, 31). *E. dissolvens* is the closest relative to *E. cloacae*, since the DNA relatedness of the two species was up to 82% in DNA-DNA hybridization experiments and they were phenotypically not differentiable (4, 25). *E. nimipressuralis* had a DNA relatedness of up to 67% to *E. cloacae* and differed phenotypically from it by being sucrose negative. *E. asburiae* had a DNA relatedness of 63% to *E. cloacae* and differed from it by being Voges-Proskauer, malonate, melibiose, and L-rhamnose negative (4). *E. hormaechei* also had a DNA relatedness of 63% to *E. cloacae* and differed from it by being D-sorbitol, melibiose, and esculin negative (31). *E. cancerogenus* is considered purely phytopathogenic and was transferred from the genus *Erwinia* to the genus *Enterobacter* as a senior synonym of *Enterobacter taylorae* (13). It had a DNA relatedness of 61% to *E. cloacae* and differed from it by being, inter alia, ornithine decarboxylase negative and D-arabinose positive. *E. kobei* had a DNA relatedness of 28% to *E. cloacae* and of 72% to CDC enteric Group 69. It differed from *E. cloacae* only by being Voges-Proskauer negative (24). Most of these species have a DNA relatedness of over 60% to *E. cloacae* and form the so-called *E. cloacae* complex.

Two DNA-DNA hybridization studies have so far partly analyzed the genetic clustering of the *E. cloacae* complex by examining local isolates with *E. cloacae* phenotypes (21, 25), delineating the following genogroups. One group contained both the *E. cloacae* and *E. dissolvens* type strains. A second formed around reference strain CDC 1347 41^R and was not ascribed to a species (42). A third formed around the *E. hormaechei* type strain and contained seven biogroups. A fourth was found around the *E. asburiae* type strain and consisted of three biogroups. Finally, one group in the study of Grimont and Grimont (21) and three in that of Lindh and Ursing (25)

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as well as up to 11 ungroupable strains were not assigned to type or reference strains. However, the hybridization studies of Grimont and Grimont (21) and Lindh and Ursing (25) are difficult to compare. Both authors examined collections of strains recovered at local hospitals in Paris and Copenhagen, respectively. Beside others, this might be the reason for the partly different results of the authors. For example, Grimont and Grimont (21) reported 30% of study strains clustering with the *E. asburiae* type strain, while none of those of Lindh and Ursing (25) clustered with it. Grimont and Grimont (21) found a genetic group of nitrogen-fixing strains (group 5), whereas Lindh and Ursing (25) did not find such a group but reported almost 10% ungroupable strains. An extensive, population genetic-based classification of *E. cloacae* remains elusive.

Population genetic studies have proven to be extremely useful for getting insights into the epidemiology and evolution of the pathogenicity of bacterial species, as exemplified in previous studies on the species (3) and on the genus level (14). The objectives of this study were to describe the genetic structure of the nomenspecies *E. cloacae* and to explore the phylogenetic relationships between its genetic groups and related species. For this purpose, we analyzed 225 strains with *E. cloacae* phenotypes from 22 European regions and all type strains of the genus *Enterobacter* with a combination of partial sequencing of three housekeeping genes and PCR-restriction fragment length polymorphism (PCR-RFLP) of a further protein-coding gene.

MATERIALS AND METHODS

Bacterial test collection. The 241 isolates were sent to us from the United States and from 22 microbiology centers located in 10 European countries from Norway to Mallorca, Spain (north-south axis), and Poland to France (west-east axis). They were recovered from clinical specimen and collected in Austria (Innsbruck 10 strains), Belgium (Brussels 10 strains), France (Marseille 9 strains), Germany (Aachen 12 strains, Berlin 12 strains, Frankfurt 14 strains, Freiburg 9 strains, Gelsenkirchen 11 strains, Hannover 10 strains, Heidelberg 10 strains, Jena 10 strains, Kiel 11 strains, Munich 28 strains, Regensburg 9 strains, and Tübingen 12 strains), Norway (Bergen 4 strains), Poland (Cracow 10 strains), Scotland, United Kingdom (Lanarkshire 6 strains), Spain (Mallorca 6 strains), Switzerland (Lausanne 9 strains), and Sweden (Stockholm 10 strains, Växjö 6 strains). Two isolates were recovered from cucumbers in the United States (34). Eleven isolates from animals were kindly provided by a veterinarian microbiologist of Bavaria, Germany, and were isolated from a monkey, two dogs, a cat, two rabbits, a guinea pig, a wild mouse, a bat, a duck, and a parrot. All isolates were identified as *E. cloacae* by phenotypic methods with API20E (BioMérieux, Marcy-l'Etoile, France) or Crystals (BBL) plus the typical antibiotic susceptibility pattern of *Enterobacter* species or by automated systems like Vitek I, Vitek II, or Micronaut. At our laboratory, the API20E code and an extended antibiotic susceptibility pattern was reevaluated for all isolates. Only one isolate per patient, plant and animal was included in the study.

All 13 type strains of the genus *Enterobacter* were used as points of reference. They are listed in Table 1. Reference strain CDC 1347-71[R] (42) was included as a reference for DNA relatedness groups 2 of Lindh and Ursing (25) and Grimont and Grimont (21). Type and reference strains were obtained from the American Type Culture Collection and the Collection de l'Institut Pasteur (CIP). In addition, the published *hsp60* sequence of *Enterobacter aerogenes* (accession number AB008141) and the *hemB* sequence of *E. coli* (accession number L44595) were downloaded from GenBank for direct comparison.

An ERIC-PCR was performed for all isolates (see below). In cases of identical ERIC patterns for two or more isolates, all but one of them was excluded. The remaining study strains are listed in Table 1. Bacteria were routinely grown at 36°C overnight in Luria-Bertani (LB) broth with slow shaking (100 rpm) or on LB-agar plates. For antibiotic susceptibility testing, bacteria were plated onto Mueller Hinton agar. Susceptibility was tested with agar diffusion tests following published guidelines (30).

Biochemical and susceptibility testing. API20E (BioMérieux, Marcy l'Etoile, France) testing was performed following the manufacturer's instructions. The results were interpreted with the Analytical Profile Index (API) database of the ApiLab Plus software (version 3.3.3; BioMérieux, Marcy l'Etoile, France). Antibiotic susceptibilities to ampicillin, ampicillin plus clavulanic acid, cefoxitin, ceftazidime, and cefotaxime were tested. The expression of the chromosomal AmpC cephalosporinase was interpreted as follows: no expression if the strain was susceptible to all antibiotics tested; basal expression if the strain was resistant to ampicillin, ampicillin-clavulanic acid, and/or cefoxitin, hyperexpression if the strain was resistant to ampicillin, ampicillin-clavulanic acid, cefoxitin, ceftazidime, and cefotaxime.

DNA preparation. Bacterial DNA was prepared for PCR by quick-heat lyses. Bacteria were grown in LB to an optical density at 600 nm of 1.0. Then 100 μl of the culture was centrifuged (2 min at 10,000 rpm). The pellet was diluted in 1 ml of distilled water and boiled for 10 min.

ERIC-PCR analysis. ERIC primers annealing to enterobacterial repetitive intergenic consensus regions were used as previously described (18). Amplification reactions were carried out in an Applied Biosystems GeneAmp 2700 PCR thermocycler. Thin-walled PCR tubes were purchased from Applied Biosystems (Branchburg, N.J.). Primers ERIC 1and ERIC 2 (Table 2) were used and synthesized by Metabion, Munich, Germany. Reactions were performed in a total volume of 30 μl under standard conditions (500 pmol of each primer, 200 μM nucleotide mix, 10 mM Tris-Cl [pH 8.3], 5 mM KCl, 1.5 mM MgCl$_2$, and 2.5 U of standard *Taq* polymerase). After a 10-min heating period at 95°C, 35 amplification cycles were run with denaturation at 95°C for 1 min, annealing at 45°C for 1 min, and extension at 72° for 1 min followed by a final extension time of 15 min at 72°C. ERIC-PCR products were visualized after electrophoresis in 3% agarose gels in 1× TAE buffer, staining with ethidium bromide, and exposure to UV light. In each run, ERIC-PCR was repeated for strain EN114 in order to control the standardization of the conditions. The ERIC patterns of EN114 were identical for all three PCR runs performed. Patterns of different strains were compared by visual inspection. The patterns were interpreted as identical if an identical number of bands of identical sizes was found.

PCR assays. The following PCR assays were used: Hsp60, amplifying the *groEL* homologue coding for the 60-kDa heat shock protein; RpoB, amplifying the RNA polymerase beta subunit (*rpoB*) gene; HemB, amplifying the porphobilinogen synthase gene (*hemB*), which codes for a single-pathway enzyme in the early heme biosynthesis cascade; and AmpC, amplifying the chromosomal gene of the Bush type 1 cephalosporinase of *E. cloacae*, which has recently been crystallized for *E. cloacae* strain 99 (26). The PCR primers listed in Table 2 were designed based on conserved regions of the alignment of gene homologues of different *Enterobacteriaceae*. Since only very few sequence data are available for *Enterobacter* species, the sequences used belonged to species of other genera, too. The sequences of the following GenBank accession numbers were used for the design of the PCR primers: for Hsp60, *Escherichia coli* M11294, *E. amnigenus* AB008140, *E. asburiae* AB008137, *E. gergoviae* AB008139, and *Yersinia enterocolitica* X82212; for HemB, *Escherichia coli* L44595, *Salmonella enterica* serovar Typhimurium AE008712, and *Shigella flexneri* AE016978; and for AmpC, *E. asburiae* AJ311172, *E. cloacae* AB016611, *E. cloacae* AF411144, *E. cloacae* D44479, and *E. cloacae* X08081. The RpoB primers were taken over as previously published (29).

Single sets of primers could be used for Hsp60 and RpoB PCRs. Different sets of primers were needed for HemB PCR (sets HemB, HemBu, and HemB_all) and AmpC PCR (sets AmpC and AmpCu) because conserved regions were not identical in all sequences and for some clusters PCR products could not be achieved with one of the primer sets alone. PCR products of HemB, HemBu, AmpC, and AmpCu were sequenced unidirectionally with the forward primers for representatives of each strain cluster in order to verify that the correct target gene had been amplified. The *hemB* sequences obtained in this first step were aligned. Universal HemB_all primers were designed corresponding to their most homologous regions. PCR products were purified with the Nucleospin PCR cleaning kit (Macherey and Nagel, Dueren, Germany) before further processing.

PCR assays were performed with a Perkin-Elmer Gene Amp PCR System 2400 or an Applied Biosystems GeneAmp PCR System 2700 and *Taq* Gold DNA polymerase (Perkin-Elmer, Branchburg, N.J.) under standard conditions (see ERIC-PCR). Reactions were carried out in a total volume of 50 μl and started after a preheating time of 10 min at 95°C. Samples were subjected to 30 cycles of 30 s at 95°C, 30 s at annealing temperature, and 30 min of extension at 72°C. Cycles were followed by a final elongation step of 15 min at 72°C. Annealing temperatures were optimized by increasing the annealing temperature stepwise by 2°C, starting at 6°C below the lowest T_m of the primer sets used, until specific, single-banded PCR products were obtained. Due to the relatively high variability of the genes, PCRs of the *ampC* and *hemB* genes were only applicable at

TABLE 1. Strains analyzed and their characteristics

Cluster	Strains[a]	No. of strains analyzed			Amino acid alterations[b]			*ampC* PCR-RFLP
		hsp60	*rpoB*	*hemB*	Hsp60 (91 aa)	RpoB (336 aa)	HemB (72 aa)	
I	*E. asburiae* ATCC 35953$_c^{T+\$}$, 501R3$_h^{*+\$}$, E6$_h^{*+\$}$, EN299$_c^+$, EN373$_a^+$, EN485$_c^+$, EN497$_b$, EN539$_b^+$, EN640$_k^\$$	9	7	4	*E. asburiae* ATCC 35953^T: A466→T	501R3: D42→Y	None	g
						EN373: V28→G EN347: S298→A		
II	*E. kobei* ATCC BAA-260$_a^{T+\$}$, CDC 1347 71$_k^{R+\$}$, EN24$_d^{+\$}$, EN326$_a^+$, EN330$_b$, EN355$_b^+$, EN478$_a$, EN488$_b$, EN499$_c^+$, EN529$_d$, EN536$_d^{+\$}$, EN549$_d$, EN632$_b^{\nwarrow}$, EN634$_a$	14	7	4	None		None	a
III	EN114$_b^{+\$}$, EN125$_c$, EN213$_b$, EN261$_b$, EN286$_b$, EN304$_c^+$, EN310$_k$, EN313$_k^+$, EN317$_k$, EN318$_k$, EN322$_g^+$, EN329$_a^+$, EN333$_c$, EN336$_c$, EN341$_b$, EN343$_g^+$, EN344$_b$, EN346$_b$, EN353$_g$, EN356$_g$, EN362$_b$, EN372$_a$, EN374$_a$, EN376$_k^+$, EN378$_k$, EN387$_b^\$$, EN389$_g$, EN396$_g$, EN397$_g$, EN398$_g$, EN399$_b$, EN401$_b$, EN402$_b$, EN404$_c$, EN467$_g$, EN472$_g$, EN476$_g$, EN482$_a$, EN483$_g$, EN491$_k$, EN492$_k^+$, EN494$_b$, EN498$_b$, EN500$_c$, EN521$_b$, EN523$_a$, EN525$_a^\$$, EN527$_c$, EN528$_g$, EN531$_c$, EN532$_g$, EN535$_g$, EN548$_b$, EN560$_c$, EN563$_g$, EN565$_e$, EN608$_b^{\nwarrow}$, EN611$_b^{\nwarrow}$	58	9	3	EN492: G439→C	EN521: L193→M	None	b
					EN343: M447→V EN348: V499→A	EN492: E137→K; V141→L EN521: L193→M		
IV	EN19$_c^+$, EN117$_f^{+\$}$, EN316$_k^{+\$}$, EN345$_c^+$, EN347$_b^+$, EN496$_b^+$, EN519$_g^+$, EN533$_d^{+\$}$, EN538$_f^+$	9	9	3	All: N468→K	EN19: K2→1; T315→P	None	g
						EN538: V1→A		
V	EN36$_a^+$, EN119$_c^{+\$}$, EN124$_c^+$, EN128$_f$, EN303$_c^+$, EN319$_g^+$, EN338$_c^+$, EN340$_b^{+\$}$, EN342$_g^+$, EN479$_b$, EN493$_k$, EN495$_b$, EN517$_g$, EN561$_g^\$$	14	8	3	All: N465→M	EN124: K2→1	None	h
VI	EN5$_b$, EN18$_f^{+\$}$, EN306$_c$, EN312$_k$, EN314$_k^+$, EN332$_b$, EN334$_c$, EN351$_b$, EN360$_g$, EN366$_b^\$$, EN368$_b$, EN375$_k$, EN386$_d$, EN409$_g$, EN489$_b$, EN508$_c^{+\$}$, EN510$_a$, EN514$_c$, EN516$_g$, EN518$_g$, EN526$_b$, EN547$_c$, EN552$_b$, EN556$_c$, EN558$_b$, EN609$_f^{\nwarrow}$, EN631$_g^{\nwarrow}$, EN637$_k$	28	3	3	All: N468→K	None	None	d
VII	*E. hormaechei* ATCC 49162$_b^{T+\$}$, EN280$_b^+$, EN291$_b^{+\$}$	3	3	2	None	None	None	b
VIII	EN30$_b^{+\$}$, EN285$_g$, EN288$_g^+$, EN290$_a$, EN292$_g$, EN293$_c$, EN305$_d^+$, EN311$_k$, EN315$_k$, EN320$_g$, EN323$_g$, EN325$_c$, EN327$_a$, EN331$_b^{+\$}$, EN349$_c$, EN352$_b^+$, EN359$_c$, EN365$_a^+$, EN369$_a^\$$, EN370$_a$, EN371$_a$, EN380$_k$, EN384$_c$, EN390$_a$, EN391$_b^+$, EN395$_d^+$, EN400$_b$, EN403$_g$, EN405$_b$, EN407$_g^{+\$}$, EN410$_b$, EN466$_b$, EN468$_c$, EN469$_c$, EN474$_c$, EN477$_c^+$, EN480$_g$, EN481$_b$, EN484$_g$, EN486$_a$, EN487$_c$, EN490$_b$, EN513$_c$, EN515$_g$, EN530$_g$, EN545$_c$, EN550$_b$, EN551$_c$, EN553$_c$, EN554$_a$, EN559$_b$, EN562$_g$, EN610$_c^{\nwarrow}$, EN613$_b^{\nwarrow\$}$, EN628$_f^{\nwarrow}$, EN630$_g^{\nwarrow\$}$, EN635$_k$, EN636$_k$, EN638$_k$	59	12	6	None	EN288: L5→M	None	d
						EN391: V1→A; V84→L		
IX	EN25$_a^{+\$}$, EN363$_g^{+\$}$, EN364$_g^+$, EN520$_b^{+\$}$, EN524$_a^+$	5	5	3	None	None	None	c
X	*E. nimipressuralis* ATCC 9912$_h^{T+\$}$, EN633$_g^{\nwarrow+\$}$	2	2	2	None	None	None	k
XI	*E. cloacae* ATCC 13047$_a^{T+\$}$, EN90$_b^{+\$}$, EN287$_c^{+\$}$, EN475$_g^{+\$}$	4	4	4	All: T430→S	None	None	No product
XII	*E. dissolvens* ATCC 23373$_h^{T*+\$}$, EN361$_g^{+\$}$, EN408$_d^{+\$}$	3	3	3	All: A468→T	EN408: S168→F	None	e

TABLE 1—*Continued*

Cluster	Strains[a]	No. of strains analyzed			Amino acid alterations[b]			*ampC* PCR-RFLP
		hsp60	*rpoB*	*hemB*	Hsp60 (91 aa)	RpoB (336 aa)	HemB (72 aa)	
XII	*E. dissolvens* ATCC 23373$_h$[T☆+$], EN361$_g$[+$], EN408$_d$[+$]	3	3	3	All: A468→T	EN408: S168→F	None	e
Xiii	EN28$_a$[+], EN385$_b$[+], EN509$_a$[+], EN512$_a$[+], EN612$_f$[↗]	5	4	0	All: A466→T	EN28: V1→E EN509: E167→Q; T218→S; L219→V		f + i
Type strains	*E. amnigenus* ATCC 3072$_k$[T], *E. cancerogenus* ATCC 33241$_h$[T☆+], *E. cowanii* CIP 107300$_a$[T], *E. gergoviae* ATCC 33028$_c$[T+], *E. intermedius* ATCC 33110$_h$[T], *E. pyrinus* ATCC 49851$_h$[T☆], *E. sakazakii* ATCC 29544$_b$[T]	7	2	0				
Total		220	78	40				

[a] All strains for which *hsp60* was analyzed. T, type strain. R, reference strain of genomic group 2 (21, 25); + strains for which *rpoB* and *ampC* were analyzed in addition to *hsp60*; $, strains included in the *hemB* tree of Fig. 3; ☆, environmental strains isolated from plants; ↗, strains isolated from animals. Footers indicate materials from which strains were isolated: a, blood cultures and primarily sterile fluids like cerebrospinal fluid; b, respiratory tract; c, urogenital tract; d, peritoneal swabs, ascites, gall bladder; e, invasive medical devices; f, stool; g, skin swabs, wounds, abscesses; h, isolates from plants or legumes; k, not specified.

[b] With reference to the majority of strains.

annealing temperatures of 50°C. Otherwise, the following annealing temperatures were chosen: 57.5°C for *hsp60* and 59°C for *rpoB*. PCR products were visualized in 2.0% agarose gels after electrophoresis and ethidium bromide staining.

DNA sequencing. PCR-amplified DNA was sequenced with the dye terminator method in both directions. To prepare cycle sequencing reactions, 0.2 μg of purified DNA was added to a 20-μl reaction solution containing 4 μl of BigDye dye terminator sequencing mix and 0.7 μl of a 5-pmol primer solution. Cycle sequencing was performed in the Applied Biosystems GeneAmp PCR System 2700. Cycle parameters were 25 cycles with an initial 96°C denaturation step of 10 s, followed by an annealing step at 57°C of 10 s and an extension step of 4 min at 60°C. Sequences were determined by electrophoresis with the ABI Prism 377 DNA sequencer. About 1% of base pairs sequenced resulted in a nontypeable character. They have been corrected, based on the reverse sequences, with the SeqMan (DNASTAR) program.

PCR-RFLP. In order to determine group-specific restriction patterns of *ampC*, PCR products were subjected to analysis of the PCR-RFLP patterns after restriction with *Hae*III (Invitrogen, Groningen, The Netherlands). This restriction enzyme was chosen after computer analysis of the different *ampC* sequences of representative strains (EN30, EN117, EN124, *E. asburiae* type strain, and *E. dissolvens* type strain) with MapDraw (DNASTAR Inc., 1999). Restriction was

performed with 1 μg of DNA in a total volume of 20 μl over 4 h at 37°C. DNA was electrophoresed in 2% or 3% agarose gels, stained with ethidium bromide, and visualized with UV light.

Data analysis. Sequence data were analyzed with MegAlign (DNASTAR Inc., 1999) and PAUP version 4.0b10 (43) on a Macintosh G4 personal computer. Multiple alignment was performed with Clustal V, included in the MegAlign program. With MegAlign, pairwise percent sequence divergence was calculated as 100 times the sum of the residue distances from one strain to the other along the tree divided by the sum of all branch lengths. With Microsoft Excel, mean percent sequence divergences between clusters of sequences and the distinctness parameters *k* were calculated as previously described (32).

With PAUP 4.0b10, neighbor-joining trees (38) were estimated based on pairwise genetic distances on the basis of all substitutions with the Jukes-Cantor distance parameter. A group of strains was called a genetic cluster if the mean distinctness parameter *k* was above 2.0; otherwise it was called a sequence crowd. The significance of branchings was evaluated by bootstrap analysis of 100 replicates. Only groups with frequencies above 50% were kept. Bootstrap values above 80% were considered significant, and the cluster was designated as being strongly supported.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences reported in this paper are: for *hsp60*, AJ417108 to AJ417143,

TABLE 2. PCR assays and primers used

PCR assay	PCR primer	Primer sequence, 5′→3′	T_m (°C)	PCR product (nt)	Reference
ERIC	ERIC-1	ATG TAA GCT CCT GGG GAT TCA C	60.3	Random	18
	ERIC-2	AAG TAA GTG ACT GGG GTG AGC G	62.1		
RpoB	RpoB-F	AAC CAG TTC CGC GTT GGC CTG G	65.8	1,088	29
	RpoB-R	CCT GAA CAA CAC GCT CGG A	58.8		
Hsp60	Hsp60-F	GGT AGA AGA AGG CGT GGT TGC	61.8	341	
	Hsp60-R	ATG CAT TCG GTG GTG ATC ATC AG	60.6		
HemB	HemB-F	GGC AGA CCA TGA CAG ACT TAA T	64	570	
	HemB-R	ACC TGC AGC AGC TGC AAC CA	60		
HemBu	HemBu-F	ACT TCT CAC GGT CAC TGC GGT	61.8	459	
	HemBu-R	CAT SGC ATA CTC ACC GCT CAC	61.8		
HemB_all	HemB_all-F	CG RCG GTT VAG CGG GTT CAT CTG	66.3	237	
	HemB_all-R	TGA AYC TBG GCA AGC AGG CBG T	63.7		
AmpC	AmpC-F	TCT CTT GCT CTG CTC GCC	61.4	647	
	AmpC-R	ACC GCT TTA CCG TCA CGA TAG	59.8		
AmpCu	AmpCu-F	AAA TCC CTT TGC TGT GCC CTG	59.8	657	
	AmpCu-R	CCA GGC GTA ATG CGC CTC TTC	63.7		

Genetic clusters (I-XII)

Sequence crowd (*xiii*)

—— 0.005 substitutions/site

AJ543761 to AJ543910, and AJ567846 to AJ567902; for *rpoB*, AJ543656 to AJ543728 and AJ566948 to AJ566941; for *hemB*, AJ426490 to AJ426522 and AJ567697 to AJ567724.

RESULTS

Strain collection. In order to establish a representative population genetic study, we aimed to examine strains with great genetic variety. Therefore, we collected 241 isolates with *E. cloacae* phenotypes from 22 European medical and veterinarian centers and an agricultural center in the United States. All isolates were twice identified as *E. cloacae*, at the respective centers and at our laboratory by biochemical and susceptibility analyses. Although the isolates derived from different patients, animals, or plants, there was still a theoretical chance that clonal isolates were recovered in the frame of epidemic outbreaks at the centers. Their inclusion would jeopardize the representativeness of the population genetics because pseudoclusters of clonal strains would form.

To eliminate this risk, ERIC-PCRs were performed for all isolates. The good specificity of ERIC-PCR for the detection of identical isolates has recently been demonstrated (18). Comparing the ERIC patterns obtained by visual inspection, 35 isolates showed patterns which were identical to those of other strains (data not shown). They were excluded. In addition to the remaining 206 study strains, all 13 type strains of the genus *Enterobacter* and reference strain CDC 134771^R, which represents genetic group 2 of Grimont and Grimont (21), were included. All 220 strains and their characteristics are listed in Table 1. They were isolated from blood cultures (26 strains), the respiratory tract (59 strains), urogenital tract (38 strains), abdomen (9 strains), superficial swabs (44 strains), artificial devices (7 strains), stool (7 strains), plants (7 strains), and unknown origins (23 strains).

Molecular systematics analysis. Despite their public health significance, only very few sequence data for housekeeping genes were available for *Enterobacter* species at public databases. Hence, we tried oligonucleotides for the amplification of the gyrase A gene (*gyrA*), gyrase B gene (*gyrB*), or subunit C of topoisomerase IV (*parC*) published for other species (5, 10), but PCR products were not obtained with any of them for all of the study strains. However, we obtained PCR products for all strains by amplifying *hsp60*, *rpoB*, and *hemB*.

Molecular phylogeny based on *hsp60* sequence analysis. The sequences of a portion of 273 nucleotides of the *hsp60* gene were determined for the study set of 220 strains by bidirectional sequencing. All sequences were pairwise aligned; 91 characters (33%) were variable (defined as different in at least one strain). A neighbor-joining tree was constructed with all 220 study strains (Fig. 1). Since *E. aerogenes* is a homotypic synonym to *Klebsiella mobilis* and belongs to another genus, its *hsp60* sequence was used to root the tree; 13 groups of strains were found.

A group of strains was considered a genetic cluster when its mean distinctness parameter *k*, which represents a quantitative measure of distinctness (32), was above 2.0 (Table 3). A *k* parameter above 2.0 indicates that sequence similarity groups can be considered distinct genetic clusters. Otherwise the group was called a sequence crowd. Based on the *k* parameters, 12 clusters (I to XII) and one sequence crowd (xiii) could be distinguished.

Three prominent clusters were found, together representing more than two thirds of the study strains (cluster III, 58 strains; cluster VI, 28 strains; and cluster VIII, 59 strains). None of these clusters included a type or reference strain. Separate clusters formed around the type strains of *E. asburiae*, *E. cloacae*, *E. dissolvens*, *E. hormaechei*, *E. kobei*, and *E. nimipressuralis*. However, these clusters contained only one (0.1%) to 12 (6%) study strains. The results of the bootstrap analysis strongly supported the majority of the clusters with the exceptions of clusters I (63%), containing the *E. asburiae* type strain, VII (76%), VIII (60%) and IX (<50%). Taking clusters VII and VIII together, the resulting metacluster was strongly supported (82%). Sequence crowd xiii had only poor bootstrap support (24%).

In order to get a better understanding of the distances of the clusters, the mean numbers of nucleotide differences along the tree were calculated as parameters of distance (Table 3). Mean percent sequence divergences within the clusters were equal to or below 1.0 (0 to 1.0 ± 0.6), with the exception of cluster I (3.8 ± 2.3), which was due to a relatively high divergence of the *E. asburiae* type strain to the other strains of the cluster. Sequence crowd xiii had a within group divergence of 6.7 ± 1.3. Divergences between the clusters were above 2.5 (2.7 ± 0.5 to 9.1 ±

FIG. 1. Neighbor-joining tree after analyzing 273 nucleotides (91 variable) of the *hsp60* gene of 206 study strains and 14 type and reference strains of the genus *Enterobacter*. The tree was rooted with the *hsp60* sequence of *E. aerogenes* downloaded from GenBank (ENAE, accession number indicated). Type strains are labeled with T, reference strains with R, isolates from plants with a flower symbol, and isolates from animals with a picture of a dog. The animals from which strains were isolated are indicated after strain denominations, as are the abbreviations of the centers (see below) from which the strains originated. If several strains from one center had identical sequences, they were pooled at one spot of the tree, and the number of pooled strains is indicated after the center abbreviation. The scale gives the Jukes-Cantor distance along the branches. Numbers at the nodes of the tree indicate bootstrap values obtained after 100 replicates. A group of strains were considered a genetic cluster (I to XII) if the mean distinctness parameter *k* was above 2.0 (Table 3); otherwise it was called a sequence crowd (xiii). Cluster and sequence crowd denominations are indicated at the right side of the figure. Species abbreviations: ENAM, *E. amnigenus*; ENAS, *E. asburiae*; ENCA, *E. cancerogenus*; ENCL, *E. cloacae*; ENCO, *E. cowanii*; ENDI, *E. dissolvens*; ENGE, *E. gergoviae*; ENHO, *E. hormaechei*; ENIN, *E. intermedius*; ENKO, *E. kobei*; ENNI, *E. nimipressuralis*; ENPY, *E. pyrinus*; ENSA, *E. sakazakii*. Center abbreviations: AUIN, Innsbruck, Austria; BEBR, Brussels, Belgium; FRMA, Marseille, France; GEAA, Aachen, Germany; GEBE, Berlin, Germany; GEFB, Freiburg, Germany; GEFR, Frankfurt, Germany; GEGE, Gelsenkirchen, Germany; GEHA, Hannover, Germany; GEHE, Heidelberg, Germany; GEJE, Jena, Germany; GEKI, Kiel, Germany; GEMU, Munich, Germany; GERE, Regensburg, Germany; GETU, Tuebingen, Germany; NOBE, Bergen, Norway; POKR, Cracow, Poland; SPMA, Mallorca, Spain; SVST, Stockholm, Sweden; SVVA, Vaexjoe, Sweden; SWLA, Lausanne, Switzerland; UKLA, Lanarkshire, Scotland, United Kingdom.

TABLE 3. Mean percent sequence divergence within and between the sequence clusters and *k* parameters (given as ratio of between-group divergence to the mean of within-group divergence)[a]

Gene	Cluster	Data for cluster												
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	xiii
hsp60	I	3.8 ± 2.3	2.5	2.9	1.7	3.7	3.4	3.1	3.2	1.9	3.0	2.8	4.3	1.2
	II	4.8 ± 0.5	0 ± 0	31.0	3.4	47.0	>99	>99	18.0	8.6	8.7	17.7	>99	1.8
	III	6.0 ± 0.4	4.6 ± 0.2	0.3 ± 0.2	3.7	25.1	36.2	39.5	13.4	6.8	10	14.7	54.6	1.8
	IV	5.2 ± 1.8	4.5 ± 0.3	5.4 ± 0.3	1.0 ± 0.6	4.1	3.8	4.0	3.4	2.4	6.4	3.5	5.6	1.3
	V	7.5 ± 0.7	7.1 ± 0.2	7.5 ± 0.3	6.1 ± 0.3	0.3 ± 0.2	38.3	38.0	12.7	10.9	11.6	19.2	60.3	2.2
	VI	6.4 ± 0.5	4.9 ± 0.0	5.4 ± 0.2	5.0 ± 0.3	5.8 ± 0.2	0 ± 0	>99	6.4	6.0	15.4	21.7	>99	1.9
	VII	6.0 ± 0.5	5.3 ± 0.0	5.9 ± 0.2	5.2 ± 0.3	5.7 ± 0.2	1.9 ± 0.0	0 ± 0	2.9	6.8	13.1	20.3	>99	1.9
	VIII	7.0 ± 0.5	5.4 ± 0.3	6.0 ± 0.3	5.5 ± 0.4	5.7 ± 0.3	1.9 ± 0.3	0.9 ± 0.3	0.6 ± 0.4	4.3	8.3	10.4	23.6	1.9
	IX	4.6 ± 0.7	3.9 ± 0.6	4.1 ± 0.4	4.3 ± 0.5	6.5 ± 0.6	2.7 ± 0.5	3.1 ± 0.5	3.2 ± 0.5	0.9 ± 0.4	6.3	7.0	15.0	1.4
	X	6.6 ± 0.3	6.1 ± 0.4	5.0 ± 0.2	5.4 ± 0.6	5.8 ± 0.3	5.4 ± 0.4	4.6 ± 0.4	5.4 ± 1	5.0 ± 0.3	0.7 ± 0	12	20.3	1.5
	XI	6.1 ± 1.2	5.3 ± 0.0	6.6 ± 0.2	5.6 ± 0.5	8.6 ± 0.2	6.5 ± 0.0	6.1 ± 0.0	6.3 ± 0.3	5.2 ± 0.6	7.8 ± 0.1	0.6 ± 0.3	16.7	1.9
	XII	8.2 ± 1.7	6.9 ± 0.0	8.2 ± 0.2	7.3 ± 1.0	9.1 ± 0.1	8.5 ± 0.0	7.3 ± 0.0	7.1 ± 0.6	6.7 ± 0.4	7.1 ± 0.2	5.0 ± 0.2	0 ± 0	2.0
	xiii	6.7 ± 1.6	6.0 ± 1.2	6.4 ± 0.9	6.0 ± 2.0	7.7 ± 1.5	6.5 ± 1.6	6.4 ± 1.3	6.9 ± 1.6	5.3 ± 0.9	6.1 ± 1.1	6.9 ± 0.7	6.8 ± 0.7	6.7 ± 1.3
rpoB	I	0.9 ± 0.3	4.0	4.9	2.8	3.7	6.5	5.9	4.6	3.8	8.4	3.8	4.5	1.4
	II	2.4 ± 0.2	0.3 ± 0.2	6.0	5.0	9.1	13.7	11.2	5.1	4.17	14.9	5.1	6.6	1.4
	III	2.7 ± 0.1	1.5 ± 0.1	0.2 ± 0.1	6.7	11.7	9.6	5.6	5.4	2.2	16.5	5.6	8.4	1.8
	IV	2.0 ± 0.2	2.0 ± 0.2	2.4 ± 0.1	0.5 ± 0.2	5.0	11.3	9.3	6.3	4.6	12.2	5.6	6.4	1.5
	V	2.2 ± 0.2	2.7 ± 0.2	2.9 ± 0.1	2.0 ± 0.2	0.3 ± 0.1	19.7	19.3	8.5	7.5	14.2	7.1	10.9	1.9
	VI	2.9 ± 0.2	2.1 ± 0.1	1.9 ± 0.0	2.8 ± 0.1	3.0 ± 0.1	0 ± 0	>99	5.5	9.6	23	10.9	19.1	1.9
	VII	2.6 ± 0.2	1.7 ± 0.1	1.1 ± 0.0	2.3 ± 0.1	2.9 ± 0.1	1.7 ± 0.0	0 ± 0	8.5	5.6	23.7	7.7	12.9	1.9
	VIII	3.0 ± 0.2	1.8 ± 0.2	1.6 ± 0.1	2.8 ± 0.2	3.0 ± 0.1	1.1 ± 0.1	1.7 ± 0.1	0.4 ± 0.2	4.3	14	5.5	7.5	1.7
	IX	2.5 ± 0.2	1.5 ± 0.2	0.8 ± 0.2	2.1 ± 0.2	2.6 ± 0.2	1.9 ± 0.2	1.1 ± 0.2	1.7 ± 0.2	0.4 ± 0.2	13.6	3.7	5.5	0.7
	X	6.3 ± 0.2	6.7 ± 0.9	6.6 ± 0.1	6.7 ± 0.2	6.4 ± 0.1	6.9 ± 0.0	7.1 ± 0.1	7.0 ± 0.1	6.8 ± 0.2	0.6 ± 0.0	12.7	16.2	3.8
	XI	2.6 ± 0.3	2.0 ± 0.3	1.9 ± 0.2	2.8 ± 0.2	2.9 ± 0.2	2.7 ± 0.2	1.9 ± 0.2	2.5 ± 0.3	1.7 ± 0.3	7.0 ± 0.1	0.5 ± 0.2	3.2	1.8
	XII	2.7 ± 0.2	2.0 ± 0.4	2.1 ± 0.1	2.6 ± 0.2	3.3 ± 0.2	2.9 ± 0.1	1.9 ± 0.1	2.6 ± 0.2	1.9 ± 0.2	7.3 ± 0.2	1.3 ± 0.3	0.3 ± 0.2	1.9
	xiii	2.6 ± 0.4	2.2 ± 0.2	2.7 ± 0.7	2.4 ± 0.4	2.9 ± 0.3	2.7 ± 0.6	2.6 ± 0.8	2.8 ± 0.6	2.4 ± 0.7	6.6 ± 0.5	2.9 ± 0.7	3.0 ± 0.7	2.8 ± 0.8

[a] Data in the left lower portions indicate mean percent sequence divergence [arithmetic mean ± standard deviation of percent sequence divergence (100 × pairwise nucleotide distances divided by total distance) within and between sequence clusters based on all pairwise comparisons of strains from the respective clusters]. Data in the right upper portions indicate the *k* parameter [ratio of the between-group divergence to the mean of the within-group divergence]. A ratio of more than 2 indicates that the groups can be considered separate sequence similarity clusters (26).

0.1) with the exceptions of cluster pairs VI-VII, VI-VIII, and VII-VIII.

Some clusters showed specific amino acid alterations in the 60-kDa heat shock protein coded by the *hsp60* gene (Table 1). For example, cluster V had a specific amino acid alteration at position 465, clusters IV and VI at position 468, cluster XI, including the *E. cloacae* type strain, at positions 430 and 466, and cluster XII, including the *E. dissolvens* type strain, at position 430 [*E. coli* amino acid numbering system (M11294)]. Interestingly, the *E. asburiae* type strain differed from the other strains of cluster I by an alteration at position 466.

Summarizing, *hsp60* analysis suggested the existence of at least 12 genetic clusters and one heterogeneous sequence crowd within the *E. cloacae* complex. Eleven clusters (II to XII) were supported by bootstraps, and eight of them had strong bootstrap support. Clusters VII and VIII were well supported at a higher node.

Molecular phylogeny based on *rpoB* sequence analysis. The sequences of a second housekeeping gene were investigated in order to reconfirm the clustering observed after *hsp60* analysis. For this purpose, a representative subset of 78 strains was chosen, including all type and reference strains of the *E. clo-acae* complex and the type strain of *E. gergoviae*. Sequences of a portion of 1,008 nucleotides of the *rpoB* gene were determined for these strains by bidirectional sequencing. About 700 nucleotides overlapped from both directions, leaving about 150 bp at each end sequenced unidirectionally. Multiple alignment by Clustal V revealed a total of 218 (22%) variable characters. The neighbor-joining relationships of the sequences are presented in Fig. 2 (left side). With the only exception being sequence crowd xiii, the *rpoB* tree was in strong agreement with the *hsp60* tree. All strains were assigned to the same clusters. Moreover, much sharper delineations were observed, and bootstrap values were higher for clusters I, VIII, and IX. All *k* parameters but those of sequence crowd xiii were well above 2.0 (2.8 to 99), indicating that the *rpoB* sequence clusters could be considered distinct genetic groups (Table 2). However, in contrast to the *hsp60* results, sequence crowd xiii was split into two groups (xiii.a and xiii.b). Correspondingly, its mean *k* parameter was well below 2.0 (1.6 ± 0.4). Recapitulating, the same genetic clusters observed for *hsp60* appeared for *rpoB*. Sequence crowd xiii was unstable and fell apart.

Molecular phylogeny based on *hemB* sequence analysis. The sequences of a third protein-coding gene were analyzed in

FIG. 2. *rpoB* tree and *ampC* PCR-RFLP. Left side: Neighbor-joining tree based on the analysis of 1,008 nucleotides (142 nucleotides variable) of the *rpoB* gene of 78 study strains. The tree was rooted with the *rpoB* sequence of the *E. gergoviae* type strain. Bootstrap values after 100 replicates are indicated at the nodes of the tree. Cluster denominations, labeling, and abbreviations correspond to those of Fig. 1. Sequence crowd xiii was split into groups xiiia and xiiib. Right side: For PCR-RFLP analysis. PCR products of partial *ampC* genes were restricted with *Hae*III, electrophoresed in 3% agarose, and strained with ethidium bromide. Resulting clusters were designated a to k.
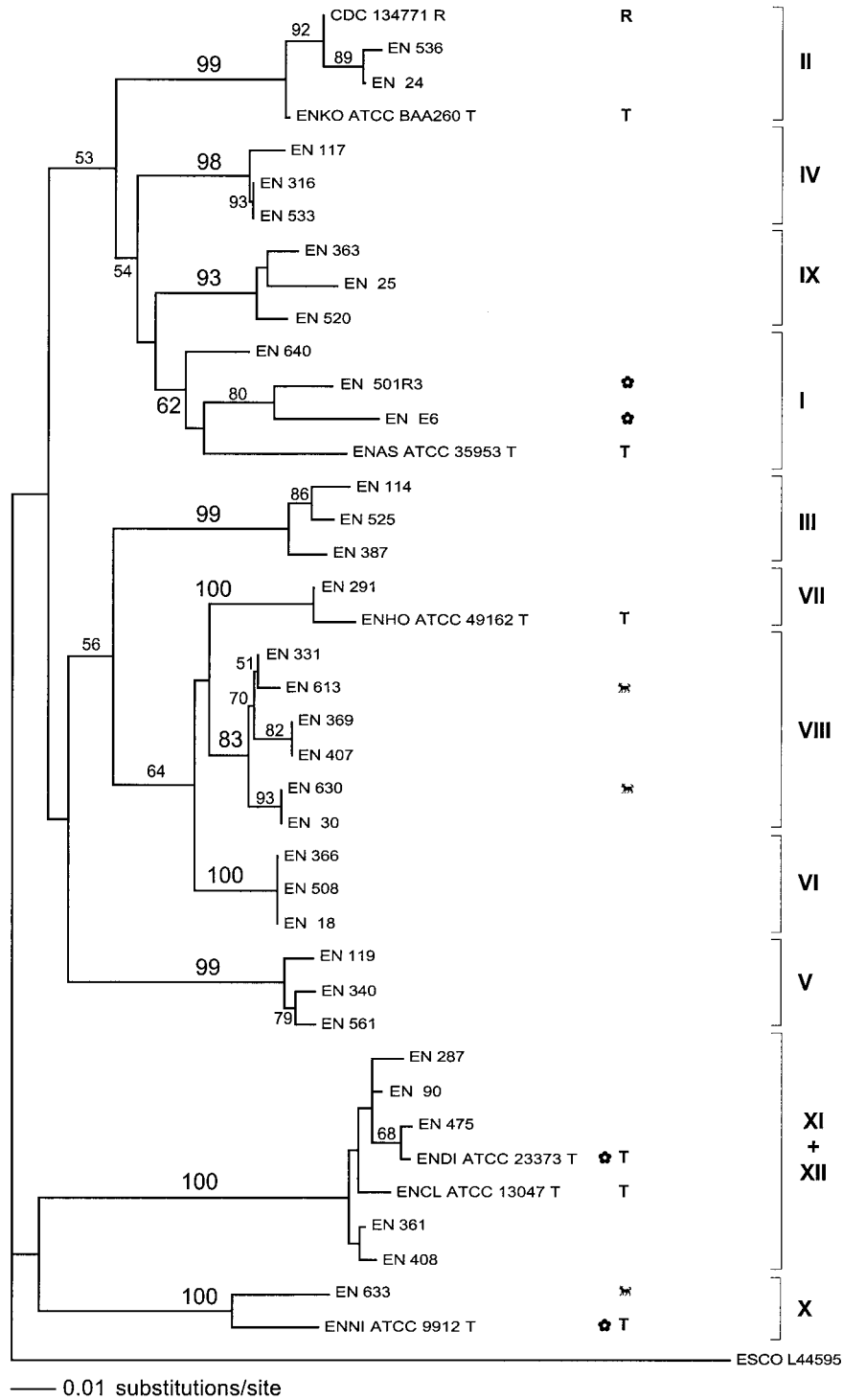
ampC-PCR-RFLP
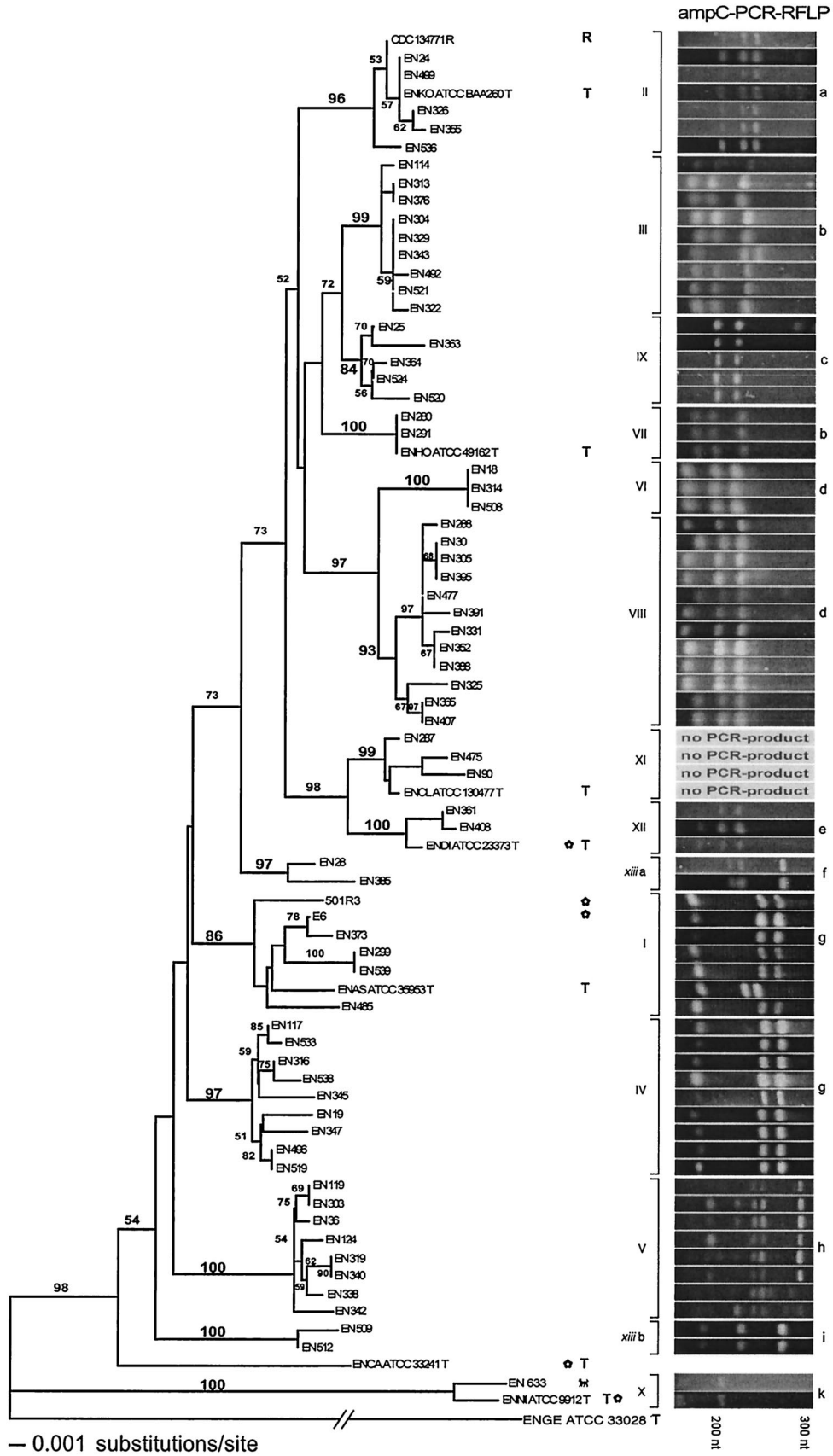
FIG. 3. Neighbor-joining tree based on the analysis of 214 nucleotides (68 nucleotides variable) of the *hemB* gene of 40 study strains. Only the genetic clusters (not the sequence crowd) were considered. The tree was rooted with the *hemB* sequence of *Escherichia coli* (GenBank accession number L44595). Cluster denominations, labeling, and abbreviations correspond to those of Fig. 1.

order to substantiate the robustness of the genetic clustering observed. Therefore, the partial *hemB* sequences of a subset of 40 study strains were analyzed. Only the stable genetic clusters I to XII were considered. Of the 214 characters determined, 68

(32%) were variable. The neighbor-joining relationships are presented in Fig. 3. Genetic clusters I to X reemerged as in the *hsp60* and *rpoB* trees. Bootstrap supports were strong for all these clusters except for cluster I, containing the *E. asburiae*

type strain (62%). Similar to the *hsp60* tree, clusters VI, VII, and VIII formed a metacluster, which was strongly (98%) supported at a higher node. In contrast to the *hsp60* and *rpoB* analyses, clusters XI and XII, containing the type strains of *E. cloacae* and *E. dissolvens*, were not separable. Strains of both clusters were mixed in a common cluster (XI plus XII), which however had strong bootstrap support (100%).

PCR-RFLP analysis of *ampC* and susceptibility testing. The gene products of housekeeping genes interact at best weakly with the environment (32). In order to include a gene coding for a protein specialized on the interaction with the environment, the *ampC* gene was subjected to PCR-RFLP with *Hae*III. Based on antibiotic susceptibility testing, AmpC was expressed in 203 of the 206 (98%) study strains examined. Strains EN304, EN313, and EN633, which did not express AmpC, belonged to clusters III, IV, and X, respectively. However, *ampC* PCR was positive for all of them.

The *ampC* gene was amplified with one of the two PCR assays presented and restricted with *Hae*III for all strains included in the *rpoB* analysis (Fig. 2). By visual inspection of the resulting PCR-RFLP patterns, 10 different clusters were delineable, designated a to k. Three pairs of sequence clusters showed similar restriction patterns (I and IV, III and VII, and VI and VIII). Four strains of cluster VIII showed patterns aberrant from the others, demonstrating a little within-group variability. The similarity of the restriction patterns of clusters III and VII was striking: whereas the distance of cluster VII to cluster III was much higher than that to clusters VI and VIII in the *hsp60* and *hemB* trees, the distances were just the other way around in the *ampC* PCR-RFLP and *rpoB* tree. The *E. asburiae* type strain differed from the other strains of cluster I by an additional restriction site in the *ampC* pattern, underlining the relatively high within-group divergences of cluster I due to the distance of the *E. asburiae* type strain from the other members of the group (Fig. 1, Fig. 2, Fig. 3, and Table 3).

Whereas the PCR-RFLP patterns were similar for all strains of cluster XII, no PCR products were obtained for the strains of sequence cluster XI, including the *E. cloacae* type strain, with both PCR assays used. This supported the clustering of the *hsp60* and *rpoB* trees, in which *E. cloacae* and *E. dissolvens* represented two distinct phylogenetic lineages, whereas they were mixed in the *hemB* tree.

Do different genetic clusters show specific distributions to clinical materials? To some extent, genetic clusters were not randomly distributed to the different groups of clinical materials from which the strains were recovered. Some features should be outlined. First, *E. nimipressuralis* (cluster X) was not isolated from a human specimen. Second, 15% of strains of cluster VIII (59 strains) were recovered from blood cultures representing more than a third of all blood culture isolates. In contrast, only 3% of strains of the closely related cluster VI (28 strains) were recovered from blood cultures. Third, whereas none of the 58 strains of cluster III were recovered from abdominal specimens, 40% of studied strains of cluster II were recovered from abdominal specimens, representing almost half of all abdominal isolates. Fourth, whereas about 20% of strains of all clusters derived from superficial skin or wound swabs, none of the strains of clusters I and II did so. These data suggest that the different clusters could have different virulence-associated properties. However, since only strains with *E. cloacae* phenotypes were considered in this study, the other species might have been underrepresented. This might have distorted the picture. More appropriately designed studies are needed in order to evaluate the different clinical relevances of the clusters.

API20E analysis. Twenty-one different API20E codes were generated by the 206 study strains included. The predominating codes were 3.305.573 (58%) and 3.305.773 (19%). Only a few group-specific characteristics could be drawn out of the biochemical information from the API20E analysis. First, cluster IX was 100% myoinositol positive, whereas clusters VI and VII were 100% negative. Second, cluster VII was 100% sorbitol negative, but sorbitol-negative strains were also found in clusters I, III, VI, and VIII. Third, cluster IV was 100% rhamnose negative, which was otherwise found only in the *E. asburiae* type strain. This short analysis suggested that at least some of the clusters could have specific biochemical properties.

DISCUSSION

In spite of the considerable clinical relevance and the genetic heterogeneity of the nomenspecies *E. cloacae*, its population genetics have not attracted much attention. Previous studies demonstrated the genetic diversity of *E. cloacae* (21, 25, 46). However, none of them gave good insight into the phylogenetic relationships between the genetic groups delineated. The present study focused on this issue. We examined 220 strains out of a collection of 241 isolates with *E. cloacae* phenotypes, originating from 23 regions in 11 countries, plus all type strains and a reference strain of the genus *Enterobacter*; 12 robust genetic clusters and an unstable sequence crowd were delineated by the use of sequence and PCR-RFLP analysis of three housekeeping genes and the chromosomal *ampC* gene, coding for an Ambler class C β-lactamase.

Six species (*E. asburiae*, *E. cancerogenus*, *E. dissolvens*, *E. hormaechei*, *E. kobei*, and *E. nimipressuralis*) which are closely related to *E. cloacae* are subsumed in the so-called *E. cloacae* complex; 13% of our study strains clustered around their type strains, whereas 84% formed genetic clusters which were not associated with any type or reference strain. Only 3% of our study strains clustered with the type strain of *E. cloacae*. In DNA-DNA hybridization studies, Grimont and Grimont (21), who examined 49 clinical isolates, and Lindh and Ursing (25), who examined 123 clinical isolates, reported similarly low frequencies of true *E. cloacae* strains. It has been known for years that the choice of the type strain of *E. cloacae* was unfortunate, since its genotype does not represent the majority of what are generally considered *E. cloacae* strains (21).

For this reason, many authors preferred to use reference strain CDC 1347-71[R] as representative of *E. cloacae* for their DNA-DNA hybridization experiments (4, 31, 37); 5% of our study strains grouped around CDC 1347-71[R] which complied with the rate reported by Lindh and Ursing (25). In our study, the type strain of *E. kobei* fell into the same cluster. *E. kobei* was described based on a small group of clinical isolates selected as being Voges-Proskauer negative (24). The species showed close relatedness to CDC enteric group 69 (16). Data on the relatedness of strain CDC 1347-71[R] and CDC enteric group 69 are not available in the literature. CDC 1347-71[R] and

our study strains clustering with the *E. kobei* type strain were all Voges-Proskauer positive. The following two problems are raised by these findings and need to be addressed in further DNA-DNA hybridization studies. First, if reference strain CDC 1347-71^R in fact represents *E. kobei* and not *E. cloacae*, the relatedness of the species *E. hormaechei*, *E. asburiae*, *E. dissolvens*, and *E. nimipressuralis* to *E. cloacae* needs to be reevaluated. Second, if CDC1347-71^R, CDC enteric group 69, and *E. kobei* represent the same relatedness group, the species description of *E. kobei* needs to be emended. Forty percent of our strains of the *E. kobei* cluster were isolated from intra-abdominal sites, and 45% of all intra-abdominal isolates belonged to the *E. kobei* cluster. Whether this is just an odd coincidence needs to be investigated in an appropriately designed epidemiological study including more clinical strains.

We found that 2% of our study strains clustered with the *E. dissolvens* type strain. The species *E. dissolvens* was reassigned from the genus *Erwinia* to the genus *Enterobacter* with the reservation that it might in fact be identical to *E. cloacae* (4). Strains of *E. dissolvens* and *E. cloacae* were 60 to 82% related and had ΔT_m values far below 5°C in reciprocal DNA. hybridization studies (21, 25). In our study, *E. cloacae* and *E. dissolvens* strains were closely related to each other but still represented two distinct phylogenetic lineages in three of the four genes analyzed. Extended multilocus sequencing and DNA-DNA hybridization studies with larger sets of strains are needed in order to finally decide whether both species denominations should be maintained or whether one of them should be abandoned.

We found that 3% of our study strains clustered around the *E. hormaechei* type strain. The description of the species *E. hormaechei* was based on 23 strains which were selected on the criterions that they were similar to *E. cloacae* but did not produce acid from D-sorbitol and melibiose. They formed a distinct DNA-DNA hybridization group and had 63% DNA relatedness to reference strain CDC 1347-71^R (31). In the hybridization study of Gimont and Grimont (21), the largest cluster formed around the *E. hormaechei* type strain and consisted of seven biotypes with only one (3a, one strain) matching the original description of the species. Later, Davin-Regli et al. (11) reported outbreaks with *E. hormaechei* strains that were D-sorbitol and melibiose positive, suggesting that additional biotypes might exist.

In our study, two study strains together with the *E. hormaechei* type strain formed the small genetic cluster VII. As far as possible with the information from the API20E system, the biochemical properties of this cluster largely met the species definition of *E. hormaechei* (31). These data suggest that the species *E. hormaechei* is actually a valid description (31) and represents only a small phylogenetic lineage within a larger genogroup (21). Our clusters VI and VIII were closely related to *E. hormaechei* cluster VII. DNA-DNA hybridization studies are needed to verify whether these clusters form a common DNA relatedness group allowing emending and broadening of the species description of *E. hormaechei*.

We found that 4% of our strains clustered with the type strain of *E. asburiae*. The species *E. asburiae* was deduced from the former enteric group 17, which formed a single DNA relatedness group and consisted of *Enterobacter* strains which were Voges-Proskauer, melibiose, and L-rhamnose negative (4). In previous studies, this biotype represented only one of at least three different biotypes found in the DNA-DNA hybridization group around the *E. asburiae* type strain (21). In our *hsp60* analysis, cluster I was only poorly supported by bootstraps due to the *E. asburiae* type strain, which grouped slightly apart the cluster. In the *ampC* PCR-RFLP, the *E. asburiae* type strain had an additional restriction site. None of the strains of cluster I except the *E. asburiae* type strain was Voges-Proskauer, melibiose, or L-rhamnose negative. These data reflect the geno- and phenotypic heterogeneity of *E. asburiae*. In former studies, it played only a minor role as a clinical pathogen (9, 12, 40). In our study, one strain of the *E. asburiae* cluster (EN373) was isolated from the blood culture of a neonate (18). Many more strains need to be analyzed in order to get a better understanding of the species *E. asburiae*.

E. nimipressuralis was isolated from elm trees with a disease called wetwood. The species was transferred from the genus *Erwinia* to the genus *Enterobacter* and was 52 to 67% related to *E. cloacae* in reciprocal DNA hybridization reactions (4). Until now, it has never been described as a human pathogen. In our study, only one isolate from a dog's footpad clustered with the *E. nimipressuralis* type strain, reconfirming the suggested low clinical relevance of this species. Little is known about the species *E. cancerogenus*. First ascribed to the genus *Erwinia*, it has recently been transferred to the genus *Enterobacter* (13) as a senior synonym of *Enterobacter taylorae*, which was 61% related to *E. cloacae* reference strain CDC 1371-71^R in reciprocal DNA hybridization reactions (17). Human infections with *E. cancerogenus* are incidental (1), which was also reflected by the fact that none of our strains clustered with the *E. cancerogenus* type strain.

All our study strains were identified as *E. cloacae* by their phenotypic characteristics. Nonetheless, 13% of them belonged to other species of the genus. The species of the *E. cloacae* complex were without exception described based on phenotypically selected strain collections, of which the genetic relatedness was proven in a second step. From our findings, it follows that the species descriptions of presumably all species of the *E. cloacae* complex need to be revised and potentially emended.

The value of sequence data in population genetic studies has been more and more acknowledged (27). Since its advent, the outstanding role of the analysis of 16S rDNA sequences for the determination of phylogenetic relationships between prokaryotes is indisputable. However, for closely related species such as *Enterobacteriaceae*, the role of the 16S rDNA sequence analysis in population genetic studies has been questioned (29, 32). For the *E. cloacae* complex in particular, its limitations have been demonstrated (44). We analyzed the genetic relationships of 16S rDNA sequences of *E. cloacae* strains in a limited prestudy (data not shown). Only vague genetic clusters were generated. In view of this restricted discriminatory power of the 16S rDNA, we decided to focus on analysis of protein coding genes (45).

The analyses of four protein coding genes, *hsp60*, *rpoB*, *hemB*, and *ampC*, were included in the study. Sequencing of partial *hsp60*, also known as *groEL* or *cpn60*, has been successfully applied for the classification of many bacteria and turned out to be useful for the phylogenetic analysis of *Enterobacter* in the present study (19, 20, 28, 33, 45). Mollet et al. (29) dem-

onstrated that phylogenetic trees generated on the bases of *rpoB* sequences were well compatible with the currently accepted classification of *Enterobacteriaceae*. In this aspect, it was far superior to 16S rDNA, a fact that we could reproduce in our study. Anyway, housekeeping genes—as most of the genes typically sequenced by bacterial systematists—are not likely to be involved in the ecological delimitation of bacterial populations (32). β-Lactamases evolved as defense mechanisms against competitors. They directly affect the competitive superiority of the bacterial strains in their ecological niches (8). *ampC*, coding for an Ambler class C cephalosporinase, is coded on the chromosome and is universally present in the genus *Enterobacter*. The clonality of *ampC* genes on the species level (including *E. asburiae* and *E. hormaechei*) has recently been shown (36). Following our PCR-RFLP results, *ampC* genes seemed to be clonal even on the level of genetic groups.

The population genetics presented provide a framework of the genetic structure of the *E. cloacae* complex. This may be of value for further studies examining the epidemiological, phenotypic, and virulence-associated properties of clones of the *E. cloacae* complex. In *Escherichia coli*, it has been demonstrated that factors associated with virulence are not randomly distributed among genetic groups (3, 7). Some virulence-associated properties have been described for strains of the *E. cloacae* complex (23). Clonality of virulence factors might cause the unequal distribution of the genetic clusters to the clinical materials which we noticed in our study. DNA-DNA hybridization experiments and biotyping of our strain collection will help determine to what extent our sequence clusters correspond to distinct species. Additionally, this will allow us to correlate sequence data for protein coding genes with DNA reassociation data, as exemplified in previous studies for 16S rDNA (41).

REFERENCES

1. **Abbott, S. L., and J. M. Janda.** 1997. *Enterobacter cancerogenus* (*Enterobacter taylorae*) infections associated with severe trauma or crush injuries. Am. J. Clin. Pathol. **107:**359–361.
2. **Araujo, W. L., J. Marcon, W. Maccheroni, Jr., J. D. Van Elsas, J. W. Van Vuurde, and J. L. Azevedo.** 2002. Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Appl. Environ. Microbiol. **68:**4906–4914.
3. **Boyd, E. F., and D. L. Hartl.** 1998. Chromosomal regions specific to pathogenic isolates of *Escherichia coli* have a phylogenetically clustered distribution. J. Bacteriol. **180:**1159–1165.
4. **Brenner, D. J., A. C. McWhorter, A. Kai, A. G. Steigerwalt, and J. J. Farmer, 3rd.** 1986. *Enterobacter asburiae* sp. nov., a new species found in clinical

5. **Brisse, S., and J. Verhoef.** 2001. Phylogenetic diversity of *Klebsiella pneumoniae* and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, *gyrA* and *parC* gene sequencing, and automated ribotyping. Int. J. Syst. Evol. Microbiol. **51:**915–924.
6. **Clementino, M. M., I. de Filippis, C. R. Nascimento, R. Branquinho, C. L. Rocha, and O. B. Martins.** 2001. PCR analyses of tRNA intergenic spacer, 16S–23S internal transcribed spacer, and randomly amplified polymorphic DNA reveal inter- and intraspecific relationships of *Enterobacter cloacae* strains. J. Clin. Microbiol. **39:**3865–3870.
7. **Clermont, O., S. Bonacorsi, and E. Bingen.** 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl. Environ. Microbiol. **66:**4555–4558.
8. **Cohan, F. M.** 1994. Genetic exchange and evolutionary divergence in procaryotes. Am. Nat. **143:**965–986.
9. **Cosgrove, S. E., K. S. Kaye, G. M. Eliopoulous, and Y. Carmeli.** 2002. Health and economic outcomes of the emergence of third-generation cephalosporin resistance in *Enterobacter* species. Arch. Intern. Med. **162:**185–190.
10. **Dauga, C.** 2002. Evolution of the gyrB gene and the molecular phylogeny of Enterobacteriaceae: a model molecule for molecular systematic studies. Int. J. Syst. Evol. Microbiol. **52:**531–547.
11. **Davin-Regli, A., C. Bosi, R. Charrel, E. Ageron, L. Papazian, P. A. Grimont, A. Cremieux, and C. Bollet.** 1997. A nosocomial outbreak due to *Enterobacter cloacae* strains with the *E. hormaechei* genotype in patients treated with fluoroquinolones. J. Clin. Microbiol. **35:**1008–1010.
12. **de Man, P., E. van Der Veeke, M. Leemreijze, W. van Leeuwen, G. Vos, J. van Den Anker, H. Verbrugh, and A. van Belkum.** 2001. *Enterobacter* species in a pediatric hospital: horizontal transfer or selection in individual patients? J. Infect. Dis. **184:**211–214.
13. **Dickey, R. S., and C. H. Zumoff.** 1988. Emended description of *Enterobacter cancerogenus* comb. nov. (formerly *Erwinia cancerogena*). Int. J. Syst. Bacteriol. **38:**371–374.
14. **Dolina, M., and R. Peduzzi.** 1993. Population genetics of human, animal, and environmental *Yersinia* strains. Appl. Environ. Microbiol. **59:**442–450.
15. **Dushay, M. S., B. Asling, and D. Hultmark.** 1996. Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defense of *Drosophila*. Proc. Natl. Acad. Sci. USA **93:**10343–10347.
16. **Farmer, J. J., 3rd, B. R. Davis, F. W. Hickman-Brenner, A. McWhorter, G. P. Huntley-Carter, M. A. Asbury, C. Riddle, H. G. Wathen-Grady, C. Elias, G. R. Fanning, et al.** 1985. Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. J. Clin. Microbiol. **21:**46–76.
17. **Farmer, J. J., 3rd, G. R. Fanning, B. R. Davis, C. M. O'Hara, C. Riddle, F. W. Hickman-Brenner, M. A. Asbury, V. A. Lowery 3rd, and D. J. Brenner.** 1985. *Escherichia fergusonii* and *Enterobacter taylorae*, two new species of Enterobacteriaceae isolated from clinical specimens. J. Clin. Microbiol. **21:**77–81.
18. **Fernandez-Baca, V., F. Ballesteros, J. A. Hervas, P. Villalon, M. A. Dominguez, V. J. Benedi, and S. Alberti.** 2001. Molecular epidemiological typing of *Enterobacter cloacae* isolates from a neonatal intensive care unit: three-year prospective study. J. Hosp. Infect. **49:**173–182.
19. **Goh, S. H., R. R. Facklam, M. Chang, J. E. Hill, G. J. Tyrrell, E. C. Burns, D. Chan, C. He, T. Rahim, C. Shaw, and S. M. Hemmingsen.** 2000. Identification of *Enterococcus* species and phenotypically similar *Lactococcus* and *Vagococcus* species by reverse checkerboard hybridization to chaperonin 60 gene sequences. J. Clin. Microbiol. **38:**3953–3959.
20. **Goh, S. H., S. Potter, J. O. Wood, S. M. Hemmingsen, R. P. Reynolds, and A. W. Chow.** 1996. *HSP60* gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. J. Clin. Microbiol. **34:**818–823.
21. **Grimont, F., and P. A. Grimont.** 1992. The genus *Enterobacter*, p. 2797–2815. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The procaryotes, 2nd ed. Springer Verlag, New York, N.Y.
22. **Halda-Alija, L., S. P. Hendricks, and T. C. Johnston.** 2001. Spatial and temporal variation of *Enterobacter* genotypes in sediments and the underlying hyporheic zone of an agricultural stream. Microb. Ecol. **42:**286–294.
23. **Keller, R., M. Z. Pedroso, R. Ritchmann, and R. M. Silva.** 1998. Occurrence of virulence-associated properties in *Enterobacter cloacae*. Infect. Immun. **66:**645–649.
24. **Kosako, Y., K. Tamura, R. Sakazaki, and K. Miki.** 1996. *Enterobacter kobei* sp. nov., a new species of the family Enterobacteriaceae resembling *Enterobacter cloacae*. Curr. Microbiol. **33:**261–265.
25. **Lindh, E., and J. Ursing.** 1991. Genomic groups and biochemical profiles of clinical isolates of *Enterobacter cloacae*. APMIS **99:**507–514.
26. **Lobkovsky, E., P. C. Moews, H. Liu, H. Zhao, J. M. Frere, and J. R. Knox.** 1993. Evolution of an enzyme activity: crystallographic structure at 2-A resolution of cephalosporinase from the *ampC* gene of *Enterobacter cloacae* P99 and comparison with a class A penicillinase. Proc. Natl. Acad. Sci. USA **90:**11257–11261.
27. **Maiden, M. C., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and

B. G. Spratt. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. USA 95:3140–3145.

28. Marston, E. L., J. W. Sumner, and R. L. Regnery. 1999. Evaluation of intraspecies genetic variation within the 60-kDa heat-shock protein gene (groEL) of Bartonella species. Int. J. Syst. Bacteriol. 49:1015–1023.

29. Mollet, C., M. Drancourt, and D. Raoult. 1997. rpoB sequence analysis as a novel basis for bacterial identification. Mol. Microbiol. 26:1005–1011.

30. National Committee for Clinical Laboratory Standards. 2000. Performance standards for antimicrobial susceptibility testing; approved standard, 7th ed., vol. 20, no. 1. National Committee for Clinical Laboratory Standards, Villanova, Pa.

31. O'Hara, C. M., A. G. Steigerwalt, B. C. Hill, J. J. Farmer, 3rd, G. R. Fanning, and D. J. Brenner. 1989. Enterobacter hormaechei, a new species of the family Enterobacteriaceae formerly known as enteric group 75. J. Clin. Microbiol. 27:2046–2049.

32. Palys, T., L. K. Nakamura, and F. M. Cohan. 1997. Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. Int. J. Syst. Bacteriol. 47:1145–1156.

33. Rastogi, N., K. S. Goh, and M. Berchel. 1999. Species-specific identification of Mycobacterium leprae by PCR-restriction fragment length polymorphism analysis of the hsp65 gene. J. Clin. Microbiol. 37:2016–2019.

34. Roberts, D. P., P. D. Dery, I. Yucel, J. Buyer, M. A. Holtman, and D. Y. Kobayashi. 1999. Role of pfkA and general carbohydrate catabolism in seed colonization by Enterobacter cloacae. Appl. Environ. Microbiol. 65:2513–2519.

35. Roehrborn, A., L. Thomas, O. Potreck, C. Ebener, C. Ohmann, P. E. Goretzki, and H. D. Roher. 2001. The microbiology of postoperative peritonitis. Clin. Infect. Dis. 33:1513–1519.

36. Rottman, M., Y. Benzerara, B. Hanau-Bercot, C. Bizet, A. Philippon, and G. Arlet. 2002. Chromosomal ampC genes in Enterobacter species other than Enterobacter cloacae, and ancestral association of the ACT-1 plasmid-encoded cephalosporinase to Enterobacter asburiae. FEMS Microbiol Lett. 210:87–92.

37. Ryun Chung, Y., D. J. Brenner, A. G. Steigerwalt, B. Sup Kim, H. Tae Kim, and K. Yun Cho. 1993. Enterobacter pyrinus sp. nov., an organism associated with brown leaf spot disease of pear trees. Int. J. Syst. Bacteriol. 43:157–161.

38. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.

39. Sanders, W. E., Jr., and C. C. Sanders. 1997. Enterobacter spp.: pathogens poised to flourish at the turn of the century. Clin. Microbiol. Rev. 10:220–241.

40. Sanders, W. E., Jr., J. H. Tenney, and R. E. Kessler. 1996. Efficacy of cefepime in the treatment of infections due to multiply resistant Enterobacter species. Clin. Infect. Dis. 23:454–461.

41. Stackebrandt, E., and B. M. Goebel. 1994. Taxonomic note: A place for DNA-DNA reassociation and 16S-rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44:846–849.

42. Steigerwalt, A. G., G. R. Fanning, M. A. Fife-Asbury, and D. J. Brenner. 1976. DNA relatedness among species of Enterobacter and Serratia. Can. J. Microbiol. 22:121–137.

43. Swofford, D. L. 1998. PAUP: phylogenetic analysis with parsimony (and other methods), version 4.0b10. Sinauer Associates, Sunderland, Mass..

44. Tang, Y. W., N. M. Ellis, M. K. Hopkins, D. H. Smith, D. E. Dodge, and D. H. Persing. 1998. Comparison of phenotypic and genotypic techniques for identification of unusual aerobic pathogenic gram-negative bacilli. J. Clin. Microbiol. 36:3674–3679.

45. Teng, L. J., P. R. Hsueh, J. C. Tsai, P. W. Chen, J. C. Hsu, H. C. Lai, C. N. Lee, and S. W. Ho. 2002. groESL sequence determination, phylogenetic analysis, and species differentiation for viridans group streptococci. J. Clin. Microbiol. 40:3172–3178.

46. Weischer, M., and H. J. Kolmos. 1993. Ribotyping of selected isolates of Enterobacter cloacae and clinical data related to biotype, phage type, O-serotype, and ribotype. APMIS 101:879–886.

47. Weischer, M., H. J. Kolmos, M. E. Kaufmann, and V. T. Rosdahl. 1993. Biotyping, phage typing, and O-serotyping of clinical isolates of Enterobacter cloacae. APMIS 101:838–844.