Genotypes of *Klebsiella oxytoca* Isolates from Patients with Nosocomial Pneumonia Are Distinct from Those of Isolates from Patients with Antibiotic-Associated Hemorrhagic Colitis

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Klebsiella oxytoca acts as a pathobiont in the dysbiotic human intestinal microbiota, causing antibiotic-associated hemorrhagic colitis (AAHC), but it also infects other organs, resulting in pneumonia and urinary tract and skin infections. The virulence of *K. oxytoca* is still poorly understood. The production of a specific cytotoxin has been linked to AAHC pathogenesis. To investigate the clonal relationships of *K. oxytoca* with regard to clinical origin and virulence attributes, we established a multilocus sequence typing (MLST) method and analyzed 74 clinical *K. oxytoca* isolates from asymptomatic carriers and patients with AAHC, respiratory infections, and other infections. The isolates were phenotypically characterized, typed, and compared phylogenetically based on the sequences of seven housekeeping genes. MLST analysis yielded 60 sequence types, 12 of which were represented by more than one isolate. The phylogenetic tree distinguished clusters of *K. oxytoca* isolates between patients with AAHC and those with respiratory infections. Toxin-positive and -negative strains were observed within one sequence type. Our findings indicate that AAHC isolates share a genetic background. Interestingly, *K. oxytoca* isolates from nosocomial pneumonia showed a different genetic clustering, suggesting that these strains do not originate from the intestines or that they are specialized for respiratory tract colonization. Our results further indicate a polyphyletic origin and possible horizontal transfer of the genes involved in *K. oxytoca* cytotoxin production. This work provides evidence that *K. oxytoca* isolates colonizing the two main clinically relevant habitats (lower gastrointestinal [GI] tract and respiratory tract) of the human host are genetically distinct. Applications of this MLST analysis should help clarify the sources of nosocomial infections.

Klebsiella oxytoca is a Gram-negative member of the human microbiota. It can be detected in the intestines of about 2 to 10% of healthy subjects, and until recently, *K. oxytoca* was considered to be a commensal member of the enteric microflora (1–3). However, we have shown that *K. oxytoca* is in fact an intestinal pathobiont and the causative agent of antibiotic-associated hemorrhagic colitis (AAHC) (2). Under conditions of intestinal dysbiosis, a state of microbial imbalance, *K. oxytoca* unleashes its pathogenic potential. Several factors can perturb the intestinal microbiota during the life span of an individual, including immune deficiency, infections, dietary changes, and drugs, like antibiotics (4, 5). The consequences of antibiotic-induced intestinal dysbiosis range from diarrheal symptoms to intestinal inflammation and infection. The characteristics of AAHC are sudden onset of bloody diarrhea and abdominal cramps during penicillin or cephalosporin therapy. The antibiotic penicillin is considered critical for triggering dysbiosis, as *K. oxytoca* exhibits a natural resistance to penicillins. Rapid colonic overgrowth of *K. oxytoca* follows during the acute phases of AAHC (3). The pathogenicity of *K. oxytoca* in colitis is not understood, but a correlation has been observed between isolates originating from AAHC patients and the secretion of cytotoxin(s) (1, 2, 6). Besides the potential to induce colitis under certain circumstances, enteric carriage of *K. oxytoca* may be important for the transmission of antibiotic resistance genes to other bacteria and as a source of nosocomial infections (7, 8). Indeed, this bacterium and the closely related species *Klebsiella pneumoniae* are important human pathogens causing hepatobiliary in-

fections and infections of the urinary tract and soft tissue, in addition to nosocomial pneumonia (9–11). In recent years, multidrug-resistant strains of both species have emerged as an important problem in the health care system (7, 12).

So far, no typing method has successfully identified a clonal relationship between *K. oxytoca* isolates with respect to the particular infections they cause, their isolation source, or their toxicity (6, 13). Here, we established a multilocus sequence typing (MLST) protocol to assess the genetic relatedness and population structure of clinical *K. oxytoca* isolates from patients with AAHC compared to those of isolates from patients with nosocomial (respiratory and urinary tract) and other infections. We further analyzed whether distinct MLST sequence types (STs) are associated with particular infections or with the production of a bacterial cyto-

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TABLE 1 Clinical and phenotypical attributes of *K. oxytoca* isolates^a

Isolation site	Diagnosis ^b	N and/or O ^c	Toxin ^d	Country of isolation ^e
Stool (40)	AAHC (16), diarrhea/colitis of other causes (11), IBD (3), asymptomatic carrier (7), follow-up/AAHC (1), asymptomatic carrier/UTI (1), NA (1)	N (9), O (25), NA (6)	Positive (31), Negative (9)	JPN (1), USA (1), NED (2), AUT (30), ESP (1), HKG (3), GER (2)
Respiratory tract (21)	Nosocomial pneumonia/VAP (13), COPD (2), cystic fibrosis (1), pneumonia (2), pharyngitis (2), pneumothorax (1)	N (15), O (6)	Positive (3), Negative (18)	AUT (21)
Urinary tract (4)	UTI (4)	N (2), O (2)	Negative (4)	AUT (4)
Blood (2)	AAHC with bacteremia (1), bacteremia (1)	O (2)	Positive (1), Negative (1)	AUT (2)
Skin/mucous membranes (7)	DFS (4), CSSTI (2), oral abscess (1)	O (7)	Positive (4), Negative (3)	AUT (7)

^a The number of isolates within a given category is shown in parentheses.

^b AAHC, antibiotic-associated hemorrhagic colitis; IBD, inflammatory bowel disease; UTI, urinary tract infection; VAP, ventilator-associated pneumonia; COPD, chronic obstructive pulmonary disease; DFS, diabetic foot syndrome; CSSTI, complicated skin and skin structure infection.

^c Isolates were classified as nosocomial (N) when infection occurred 48 h after hospitalization. O, outpatient; NA, information not available.

^d Cytotoxicity was assessed via an MTT-based cell culture assay (6).

^e JPN, Japan; NED, Netherlands; AUT, Austria; ESP, Spain; HKG, Hong Kong; GER, Germany.

toxin that is thought to contribute to virulence in colitis (2, 14, 15). Tools to assess the genotype-virulence relationships of *K. oxytoca* isolates will be useful for obtaining insights into the epidemiological patterns and evolution of the pathogenicity of this important opportunistic human pathogen (16).

MATERIALS AND METHODS

Bacterial isolates and their characterization. The study (approved by the institutional review board of the Medical University of Graz, Austria) analyzed 74 *K. oxytoca* strains and 1 *K. pneumoniae* strain isolated from patients or healthy subjects. The details of the patient diagnoses and isolation sources are provided in Table 1 and Fig. 1; see also Table S1 in the supplemental material. The antibiotic resistance profiles were determined according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

Cytotoxin testing. Bacterial cytotoxicity toward cultured Hep2 cells was measured with an MTT [(3-(4,5-dimethyl-2-thiazolyl)-25-diphenyl-2H-tetrazolium bromide] assay using supernatants of bacterial culture medium (6). The isolates were designated toxin positive when Hep2 viability was <50% compared to that of phosphate-buffered saline (PBS)-treated cells.

MLST scheme. Seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) analyzed in a published MLST protocol for *K. pneumoniae* (17) were selected as targets for *K. oxytoca* (Table 2). To develop the MLST analysis for this species, the available genomic data in public databases were compared (GenBank accession no. CP003683, CP003218, AGDI000000000.1, AGDJ000000000.1, AGDL000000000.1, AKCF000000000.1, and AGDP000000000.1). Neighboring genes were ruled out to be under selective pressure. The primer sequences for PCR amplification and sequencing primers (Tables 2 and 3) were adapted from the *K. pneumoniae* MLST primers (17). The primer annealing sites were chosen within highly conserved regions of the target genes of the *K. oxytoca* reference strains to maximize the likelihood of amplification in all *K. oxytoca* strains. All allelic primer sequences (except for *rpoB* reverse) therefore differ from *K. pneumoniae* primers either in binding position within the gene or in the exact nucleotide sequence. The discriminatory index (D) was calculated as described by Hunter and Gaston (18) to verify the typing ability of the developed MLST scheme. The allele sequences and sequence types are available on <http://pubmlst.org/koxytoca/> (19).

PCR methods and sequencing. The 7 housekeeping genes were amplified by PCR using the primers listed in Table 2. For amplification,

Phusion high-fidelity DNA polymerase (Thermo Scientific, USA) was used in a total reaction volume of 30 μ l. The PCR template was generated by boiling a suspension of a single bacterial colony in H₂O for 10 min, followed by centrifugation at 13,000 rpm for 30 s. PCRs were performed, starting with 30 s of initial denaturation at 98°C, followed by 35 cycles of 10 s of denaturation (98°C), 30 s of primer annealing (54°C), and 1 min of extension at 72°C, with a final extension of 5 min (72°C). The PCR products were analyzed for fragment length and quality on an agarose gel stained with ethidium bromide.

Sanger cycle sequencing was performed using universal primers annealing to nonhomologous 5' ends of the PCR primers (Table 3), except for the *phoE* target, for which nested primers were used. Each nucleotide sequence was supported by a forward and reverse chromatogram.

Bioinformatics and phylogenetic analysis. Sequence chromatograms were edited using CLC Main Workbench 6, including the CLC MLST module (CLC bio, Denmark). Using the same software, an MLST scheme was set up, and the obtained sequences were assembled and compared to already existing allelic sequences of a given locus. A new allele number was assigned to each distinct sequence of a locus. The distinct combination of the seven allele numbers, one for each locus, determined the sequence type (ST).

For phylogenetic and nucleotide diversity analyses, the sequences of the 7 loci were concatenated. DnaSP 5.10.1 (20) was used to calculate polymorphism statistics from the sequence alignments. Phylogenetic trees were drawn using MEGA5 (21) and CLC Main Workbench 6, based on the Tamura-Nei parameter with gamma distribution and invariable sites (TN93 + G + I), according to Model test distributed in MEGA5. One thousand random bootstrapping replicates were performed to assess the stability of the phylogenetic tree. One clinical *K. pneumoniae* isolate of the local strain collection was typed to be used as the outgroup in phylogenetic analyses.

The likelihood of the outcome of two groups was determined using the odds ratio (OR) tool in Prism5 (GraphPad Software, USA). Statistical significance was assessed using the Fisher's exact test. The discriminatory index (D) was calculated as described by Hunter and Gaston (18) to quantify the typing ability of the developed MLST scheme. eBURST V3 (22) analysis was done to assess the presence of clonal complexes (CCs) that share 6 out of 7 alleles. SplitsTree version 4.12.6 (23) was used to draw split decomposition trees of the concatenated sequences of the STs to detect possible recombination-based network structures. As an additional recombination parameter, the index of

FIG 1 Neighbor-joining tree showing the genetic relatedness of 74 clinical *K. oxytoca* isolates combined with clinical and phenotypic information. Stool isolates are indicated by green squares and respiratory isolates by orange squares, shown to the right of the isolate number. Bootstrap values, denoting the reliability of the given branches, are shown next to the tree nodes. Only values of $\geq 60\%$ are shown. Clusters/subclusters are indicated in large letters on the tree. All the isolates were resistant to ampicillin. The scale bar represents 0.01 substitutions per site. CC, clonal complex; r.t., respiratory tract; u.t., urinary tract; AAHC, antibiotic-associated hemorrhagic colitis; COPD, chronic obstructive pulmonary disease; CSSTI, complicated skin and skin structure infection; DFS, diabetic foot syndrome; IBD, inflammatory bowel disease; UTI, urinary tract infection; VAP, ventilator-associated pneumonia; AUT, Austria; ESP, Spain; GER, Germany; HKG, Hong Kong; JPN, Japan; NED, Netherlands; ESBL, extended spectrum β -lactamase; CRE, carbapenem-resistant *Enterobacteriaceae*; N, nosocomial; O, outpatient; n/a, information not available.

TABLE 2 Nucleotide polymorphisms among *K. oxytoca* isolates and MLST target and PCR primer information

Gene	Putative gene function	Oligonucleotide	Oligonucleotide sequence (5' to 3') ^a	Size of analyzed fragment (bp)	No. of alleles ^b	No. of polymorphic sites ^b	Mean % G+C content	Variation indices ^b	
								π^c	dN/dS^d
<i>gapA</i>	Glyceraldehyde-3-phosphate dehydrogenase	gapA_fwd gapA_rev	<u>GTTTTC</u> CCAGTCACGACGTTGTATG AAGTATGACTCCACTCACGG TTGTGAGCGGATAACAATTTCAA CGCCTTTCATTGCGCCTTCGGAA	450	8 (9)	15 (16)	53.7	0.01340 (0.01335)	0.000 (0.000)
<i>infB</i>	Translation initiation factor 2	infB_fwd infB_rev	<u>GTTTTC</u> CCAGTCACGACGTTGTACTCT CTGCTGGACTACATTCG TTGTGAGCGGATAACAATTTCCGCT TTCAGCTCAGAACTTC	318	15 (17)	48 (50)	59.2	0.05615 (0.05625)	0.139 (0.137)
<i>mdh</i>	Malate dehydrogenase	mdh_fwd mdh_rev	<u>GTTTTC</u> CCAGTCACGACGTTGTATCCC AACTGCCCTTCAGGTTTCAG TTGTGAGCGGATAACAATTTCCCTTC CACGTAGGGCGCATTC	477	25 (25)	86 (86)	52.1	0.05222 (0.05233)	0.032 (0.031)
<i>pgi</i>	Phosphoglucose isomerase	pgi_fwd pgi_rev	<u>GTTTTC</u> CCAGTCACGACGTTGTAGA GAAAAACCTGCGGGTGCTGCTG TTGTGAGCGGATAACAATTTCCGGT TAATCAGGCGGTAGTGAGC	432	27 (29)	45 (45)	56.2	0.03008 (0.03023)	0.008(0.008)
<i>phoE</i>	Phosphoporphine E	phoE_fwd phoE_rev	<u>GTTTTC</u> CCAGTCACGACGTTGTAACC TGGCGCAACACCGGATTCCTC TTGTGAGCGGATAACAATTTCTTC AGCTGGTTGATTTTGTAAATCCAC	420	26 (28)	52 (54)	53.7	0.03315 (0.03337)	0.020 (0.019)
<i>rpoB</i>	RNA polymerase subunit β	rpoB_fwd rpoB_rev	<u>GTTTTC</u> CCAGTCACGACGTTGTAGGC GAAATGGCGGAAAAACCA TTGTGAGCGGATAACAATTTCCGAG TCTTCGAAGTTGTAACC	501	19 (21)	40 (42)	54.3	0.01777 (0.01812)	0.001 (0.001)
<i>tonB</i>	Periplasmic energy transducer	tonB_fwd tonB_rev	<u>GTTTTC</u> CCAGTCACGACGTTGTACTCT ATACTTCGGTACATCAGGTT TTGTGAGCGGATAACAATTTCCCTG TTTGGGGGCCAGCACCTGGT	405	25 (27)	78 (79)	61.9	0.05920 (0.06001)	0.092 (0.095)
Concatenated sequence				3,003			55.6	0.03607 (0.03631)	

^a Specific oligonucleotides: bold-type sequence binds target gene, underlined sequence (overhang) serves as annealing site for sequencing primer (*phoE* was sequenced with distinct nested primers).
^b Publicly available *K. oxytoca* NCBI sequence data were combined with the Sanger sequences from clinical isolates to generate a separate alignment which yielded the values given in brackets.
^c Diversity index (π) is equal to the average number of nucleotide differences per site.
^d dN/dS , ratio of nonsynonymous to synonymous substitutions.

TABLE 3 Primers used for Sanger sequencing in this study

Primer name	Sequence (5' to 3')
MLST_seq_fwd	GTTTCCCAGTCACGACGTTGTA
MLST_seq_rev	TTGTGAGCGGATAACAATTTC
phoE_seq_fwd	TTTCTTCGGCGTGGTAGATCC
phoE_seq_rev	GTAATCCACAAAGGCATTC

association (I_A^S) value was computed using LIAN 3.6 with 1,000 random resamplings to provide a quantitative analysis of the recombination and linkage disequilibrium rates within the *K. oxytoca* population analyzed. *P* values from the parametric and Monte Carlo methods were assessed (24). For all statistical methods, a *P* value of <0.05 was considered statistically significant.

RESULTS

Sequence types and genetic diversity. All genes included in the MLST scheme were affected by sequence variation. A range of 8 (*gapA*) to 27 (*pgi*) distinct alleles was detected (Table 2). The isolates comprised 60 distinct sequence types (STs). All STs are shown in Table S1 in the supplemental material, along with their isolation sources and details regarding patient diagnoses. Twelve of the STs are represented by more than one isolate (Table 4). The differences between the STs were as small as a single nucleotide polymorphism within the entire concatenated sequence (3,003 bp). An alignment of the seven gene sequences for insertions/deletions identified solely isolate 2 in the *tonB* locus, which was affected by an insertion of four codons plus two downstream deletion events involving two codons each. Synonymous substitutions were 1,000 (for *rpoB*) to 7 (for *infB*) times more frequent than nonsynonymous substitutions. The clinical *K. pneumoniae* isolate included in the typing scheme did not share any alleles with the typed *K. oxytoca* isolates. The discriminatory index of the MLST scheme was calculated, using the method of Hunter and Gaston (18), to be 0.9858.

Phylogenetic relationship of *K. oxytoca* isolates. The concatenated sequences of all seven loci were used to draw a phylogenetic tree with *K. pneumoniae* as the outgroup. The resulting neighbor-joining phylogeny (Fig. 1) comprises two major clusters, A and B, with the latter divided into subclusters B1 and B2. Cluster A shows overall closer genetic relatedness (sum of branch lengths [SBL], 0.019) than cluster B (SBL, 0.132) and subclusters B1 (SBL, 0.065) and B2 (SBL, 0.043). The majority of the AAHC isolates (13 of 16; OR, 5.7; *P* < 0.05) belong to cluster A. In contrast, respiratory isolates are almost exclusively found in subcluster B1 (17 of 21; OR, 23.9; *P* < 0.0001). Accordingly, isolates originating in nosocomial pneumonia are also more abundant in subcluster B1 (11 of 13; OR, 18.5; *P* < 0.0001). The predominance of AAHC isolates in cluster A correlates with the overrepresentation of stool isolates in this group (28 of 38; OR, 5.6; *P* < 0.005). Stool isolates were also overrepresented in subcluster B2. The strains of other isolation sources (urine, skin, and blood) were evenly distributed between the clusters. Geographically diverse isolates were found to be closely related (Fig. 1; see also Fig. S1 in the supplemental material) and even share the same ST (Table 4). *K. oxytoca* reference strains with published sequences were found in all clusters (see Fig. S1 and Table S1 in the supplemental material). STs that show high genetic similarity were grouped into clonal complexes (CCs) by eBURST (22) analysis (Fig. 2; see also Table S1 in the supplemental material). Each CC is made up of STs that differ from each

other by only one allele. CC1 includes ST21, ST33, ST46, and putative founder ST51. CC2 includes ST2 (putative founder), ST18, ST19, and ST61. CC3 includes ST109 (putative founder), ST41, and ST13. Clonal complexes 4 to 8 each contain two different STs. Thirty-nine STs are singletons that are not related to any other ST.

Distribution of cytotoxicity and antibiotic resistance in the *K. oxytoca* population. The MLST-based phylogeny indicates that toxin-producing isolates are present in cluster A as well as in cluster B, although their prevalence is higher in cluster A (27/38 [71%]) and B2 (9/11 [82%]) than in subcluster B1 (3/25 [12%]). This association was strengthened when strains with published sequences were included in the analysis (see Fig. S1 in the supplemental material). The larger number of stool isolates in cluster A and B2 and the high frequency of toxin production in stool isolates (6) are consistent with this clustering. It is also interesting to note that two of three toxin-positive isolates in subcluster B1 originated in AAHC patients.

Our analysis also indicates that strains with identical sequence types can have different toxicity phenotypes. Twelve of the 60 STs are represented by more than one isolate (Table 4). The six isolates of ST 4 include 4 toxin-negative and 2 toxin-positive isolates. Also, ST18, ST33, ST36, and ST38 each include a mixture of one toxin-negative and one toxin-positive isolate. The remaining sequence types comprising multiple isolates each contain strains with the same toxin phenotype: ST1 (2 isolates), ST2 (2 isolates), ST9 (7 isolates), ST11 (2 isolates), ST40 (2 isolates), ST41 (2 isolates), and ST44 (2 isolates). Isolates within the same ST also differed in isolation date, body site, geographic origin, and antibiotic resistance pattern.

Most (9 of 11) isolates producing extended-spectrum β -lactamases (ESBL) are located in cluster A (Fig. 1; see also Fig. S1 in the supplemental material). Generally, antibiotic resistance did not coincide with toxin production or any other parameter, such as geographic origin, isolation date, or diagnosis.

***K. oxytoca* diversity within one patient.** The isolation of multiple *K. oxytoca* strains from the same patient allowed us to assess genetic heterogeneity within individual human colonization or infection cases. Isolates 180 and 180-1 were both cultured from the same stool sample from a patient with AAHC. While isolate 180 does not produce the cytotoxin and is an ST33 strain of cluster B1, isolate 180-1 belongs to ST45 of cluster A and produces toxin. Isolates 195 and 195-H were also obtained at the same time from the same patient but from different body sources (stool versus urine). While these isolates share the same ST, only the stool isolate is toxin positive. A case of temporal carriage of the identical *K. oxytoca* strain in a patient during acute AAHC as well as in remission (follow-up isolate) is displayed by isolates 34 and 45. They are identical in ST and toxicity and were obtained at a 1-month interval (Table 4).

Clonal diversity and relationships within the *K. oxytoca* population. The calculated split graph (23) (Fig. 3) shows low levels of recombination, indicated by minor interconnected networks, involving the STs of cluster B. The branching displayed in the split graph correlates with the major clusters of the *K. oxytoca* MLST neighbor-joining phylogeny. The index of association (I_A^S) was calculated to assess the amount of recombination within the population and to detect possible associations between alleles. The resulting I_A^S of 0.2600 (*P* < 0.001) indicates significant linkage disequilibrium within the *K. oxytoca* population. Intragenic recombination affecting the separate MLST loci was then compared.

TABLE 4 Clinical, genetic, and phenotypic information of sequence types represented by more than one *K. oxytoca* isolate

Sequence type	Isolate	Toxin ^a	Geographic origin ^b	Isolation site	Diagnosis ^c	Isolation date (mo/yr)	Antibiotic resistance type ^d	Nosocomial (N)/outpatient (O) ^e	Clonal complex	Cluster
1	34	+	AUT (Graz)	Stool	AAHC	3/2004		O		A
1	45	+	AUT (Graz)	Stool	Follow-up/AAHC	4/2004		O		A
2	56	+	AUT (Graz)	Stool	AAHC	4/2004		O	2	A
2	379	+	ESP	Stool/rectal swab	NA	5/2009	CRE	N	2	A
4	33	—	AUT (Styria)	Skin	CSSTI	5/2004		O	8	A
4	73	—	AUT (Graz)	Stool	Asymptomatic carrier	4/2004	ESBL	N	8	A
4	81	+	AUT (Graz)	Stool	Asymptomatic carrier	6/2005		O	8	A
4	204	+	AUT (Graz)	Stool	AAHC	8/2008	ESBL	O	8	A
4	231	—	AUT (Graz)	Respiratory tract	Nosocomial pneumonia	10/2010	ESBL + CRE	N	8	A
4	402	—	AUT (Graz)	Respiratory tract	Pneumonia	6/2013		O	8	A
9	37	+	AUT (Graz)	Stool	Asymptomatic carrier	4/2004	ESBL	N		A
9	128	+	AUT (Styria)	Stool	IBD	3/2007		O		A
9	188	+	AUT (Vienna)	Stool	AAHC	1/2008		NA		A
9	222	+	AUT (Graz)	Stool	AAHC	11/2009	ESBL	O		A
9	232	+	AUT (Graz)	Blood	AAHC with bacteremia	8/2010		O		A
9	382	+	AUT (Graz)	Stool	IBD	8/2013		O		A
9	425	+	GER	Stool	Diarrhea	2013		O		A
11	75	—	AUT (Graz)	skin	DFS	4/2004		O	4	B1
11	400	—	AUT (Graz)	Respiratory tract	VAP	6/2013		N	4	B1
18	113	—	AUT (Burgenland)	Stool	Asymptomatic carrier	6/2005		O	2	A
18	336	+	HKG	Stool	Diarrhea	NA		NA	2	A
33	180	—	AUT (Graz)	Stool	AAHC	12/2007		O	1	A
33	227	+	AUT (Vienna)	Stool	AAHC	6/2010		O	1	A
36	195	+	AUT (Graz)	Stool	UTI	1/2008		N		A
36	195-H	—	AUT (Graz)	Urinary tract	UTI	1/2008		N		A
38	131	—	AUT (Styria)	Stool	Asymptomatic carrier	4/2007		O		B2
38	333	+	AUT (Salzburg)	Stool	Colitis	3/2014		O		B2
40	21	—	AUT (Graz)	Respiratory tract	VAP	11/2003		N		B1
40	284	—	AUT (Graz)	Respiratory tract	Nosocomial pneumonia	2/2012		N		B1
41	23	—	AUT (Graz)	Respiratory tract	VAP	11/2003		N	3	B1
41	389	—	AUT (Graz)	Respiratory tract	Pneumothorax	9/2013		O	3	B1
44	40	—	AUT (Graz)	Respiratory tract	VAP	5/2004	ESBL	N		B1
44	179	—	AUT (Styria)	Urinary tract	UTI	11/2007	ESBL	O		B1

^a Cytotoxicity was assessed via an MTT-based cell culture assay (6).

^b AUT, Austria; ESP, Spain; GER, Germany; HKG, Hong Kong.

^c AAHC, antibiotic-associated hemorrhagic colitis; NA, information not available; CSSTI, complicated skin and skin structure infection; IBD, inflammatory bowel disease; DFS, diabetic foot syndrome; VAP, ventilator-associated pneumonia; UTI, urinary tract infection.

^d All isolates were resistant to ampicillin. ESBL, extended spectrum β -lactamase; CRE, carbapenem-resistant *Enterobacteriaceae*.

^e Isolates were classified as nosocomial when infection occurred after 48 h of hospitalization.

Our analysis showed congruency for *gapA*, *infB*, *mdh*, *pgi*, and *tonB*: all display two main branches, each comprising half of the isolates (see Fig. S2 in the supplemental material). The trees of *phoE* and *rpoB* differed slightly from the others. Due to the observed congruency of the single-locus trees, we conclude that the phylogenetic signal is consistent between the loci. This in turn indicates a mutational evolutionary background within this population of *K. oxytoca* isolates.

DISCUSSION

Previous attempts to type *K. oxytoca* strains using *gyrA* and *parC*, genes that are subject to antibiotic selection pressure (25), and

pulsed-field gel electrophoresis (PFGE) (6, 13) were unable to define a clonal relationship for particular *K. oxytoca* pathotypes. We therefore established an MLST protocol specifically for *K. oxytoca* to enable the phylogenetic-virulence relationships of clinical *K. oxytoca* isolates to be analyzed. MLST tools decipher bacterial population structures based on gene sequence comparison. An additional assessment of the distribution of virulence factors across the bacterial population provides insights into the possible presence of pathogenicity-associated subgroups (16, 26).

The discriminatory index (0.9858) determined for the MLST scheme developed in this study is comparable to established

FIG 2 Clonal diversity of *K. oxytoca* isolates. eBURST (22) was used to calculate clonal complexes (CC) that contain single-locus variants (SLVs) that share 6 of 7 MLST alleles (STs connected by lines). The single-locus difference between SLVs is indicated by the gene name next to the connecting line. The relative positions and spacing between the STs are not related to genetic distance. Each ST is represented by a dot, the size of which varies directly with the frequency of the ST in the population.

MLST schemes for *Enterobacteriaceae* (27, 28) and thus is appropriate for evolutionary population genetics. Additionally, whole-genome comparison of the eight publicly available *K. oxytoca* genome sequences was done using genomic BLAST, followed by dendrogram construction based on genetic distance. The resulting dendrogram matches the phylogenetic clustering of these same reference *K. oxytoca* sequences achieved using this MLST method (see Fig. S3 in the supplemental material). Therefore, the subset of concatenated MLST sequences compared in this study provides related results similar to those of whole-genome-based comparisons.

The observed congruency of the single-locus trees (see Fig. S2 in the supplemental material) and the significant linkage disequilibrium (I_A^S , 0.2600) provide evidence for a consistent phylogenetic signal of the separate loci and for clonality within the *K. oxytoca* population analyzed (29). Evidence for only minor recombination levels can also be seen in the split tree (Fig. 3). It appears that the nucleotide diversity in this population can be attributed mainly to a mutational process. This consistency suggests a predominantly clonal long-term evolution of *K. oxytoca*, which makes phylogenetic and epidemiological interpretations valid (16).

A comparison of the SBL values of clusters A and B of the neighbor-joining phylogeny shows closer overall relatedness within cluster A. Cluster A harbors predominantly isolates from stool samples, suggesting specialization for the gastrointestinal (GI) tract. This finding was independent of the geographic origin of the isolates. Intrahospital spread and local epidemic dissemination can therefore be ruled out as possible causes of the observed narrow genetic distances in cluster A. Cluster B shows more diversity regarding isolation site and less relatedness among the strains.

Subcluster B1 harbors the majority of respiratory isolates, mainly derived from nosocomial pneumonia, while in subcluster B2, mainly fecal isolates are present. The niche adaptations of specific *Escherichia coli* genotypes were also revealed using MLST in previous studies. While *E. coli* strains causing intestinal disease belong to phylogenetic groups A, B1, and E, strains causing extraintestinal diseases are found mainly in the phylogenetic group B2 (30, 31). Future studies should therefore investigate the potential for respiratory tract colonization by different phylotypes of other facultative pathogenic enteric bacteria.

The gastropulmonary or rectopulmonary hypothesis (32) currently proposes that nosocomial pneumonia, especially ventilator-associated pneumonia (VAP), is caused by Gram-negative bacteria originating from the GI tract (33, 34). In contrast, our findings provide evidence that the colonization of the two main *K. oxytoca* habitats (lower GI tract and respiratory tract) requires distinct genetic backgrounds. The STs of the majority of the lower GI isolates in our analysis were not associated with respiratory infections, suggesting that bacterial translocation from the lower GI tract is very unlikely. However, it is possible that *K. oxytoca* isolates from the upper GI tract (pharynx and stomach) are genetically distinct from the lower GI tract isolates and may represent a source of respiratory colonization and nosocomial pneumonia. Selective decontamination of the digestive tract (SDD) has been investigated in several studies to reduce VAP and sepsis cases caused by enteric Gram-negative bacteria (35). In light of our findings, the concept of SDD should be reconsidered for avoiding bacterial translocation of Gram-negative rods from the lower GI tract, since the majority of *K. oxytoca* respiratory isolates in this population represent genotypes not associated with the large intestine. The findings of this study support strategies using selective

FIG 3 Split decomposition analysis for *K. oxytoca* strains. SplitsTree (23) was used for analysis of concatenated sequences of the seven MLST loci. Each *K. oxytoca* ST (included in Fig. 1), regardless of frequency within the population, was included once in the analysis.

oral decontamination rather than decontamination of the lower intestine for avoiding VAP. Moreover, the prophylactic use of antibiotics in SDD is subject to debate at present, since the increase in antibiotic resistance that would potentially result among Gram-negative bacteria is a major drawback of this method (34, 36). Applications of MLST analyses to hospital and environmental isolates will be important for better understanding the sources of nosocomial infections and thus improving prevention strategies. A public database has been established to facilitate epidemiological studies of *K. oxytoca* populations (<http://pubmlst.org/koxytoca/>).

Toxin-positive *K. oxytoca* were found in both clusters A and B, although proportionally, the numbers of toxin-producing isolates were higher in cluster A and subcluster B2. This finding correlates with the higher prevalence of toxin production within stool isolates (which are predominantly found in the same clusters) than for isolates from other isolation sites (6). Given that stool isolates

show a higher frequency of toxin-positive phenotypes, it is conceivable that toxin production confers a fitness advantage to isolates of the GI tract. Multiple independent occurrences of toxin-producing isolates in the tree nonetheless indicate a polyphyletic origin of the toxin. Thus, this finding implies a horizontal mode of dissemination of the genes involved in toxin biosynthesis rather than vertical or clonal transmission. The finding that isolates sharing the same ST exhibit different toxicity phenotypes supports this notion (Table 4). A study of *E. coli* toxin genotypes did not link toxin production to a specific genetic background. Instead, the acquisition of plasmid-carried genes for the heat-labile and heat-stable enterotoxins was observed in phylogenetically closely and distantly related strains (37). For *K. oxytoca*, this might be similarly explained, for example, by the localization of toxin genes on a mobile genetic element. However, our efforts to correlate the toxicity of *K. oxytoca* strains with plasmid carriage did not support this link (data not shown). An alternative explanation that is con-

sistent with a mechanism of horizontal gene transfer of the toxin genes might be their organization within a genomic island. Horizontal transfer on a mobile genomic island is known to drive dissemination of several bacterial virulence factors, like the iron uptake system encoded by the *E. coli* high-pathogenicity island, which is widely distributed among the strains of different phylogenetic groups (38, 39).

The relationships of genotypes and virulence attributes are even more important in light of the observation that asymptomatic carriers of *K. oxytoca* and AAHC patients can concurrently harbor genetically heterogeneous *K. oxytoca* strains (6). Our MLST analysis confirmed these previous PFGE typing results, that one AAHC patient can simultaneously carry diverse *K. oxytoca* strains (isolates 180 and 180-1), which differ not only in their genetic background but also in toxin production (Table 4). This finding makes it important to consider the possibility that multiple *K. oxytoca* strains exist in patient samples during clinical laboratory analysis. A diagnostic tool to check for the presence of *K. oxytoca* toxin biosynthesis genes would be useful for assessing the presence of toxin-producing *K. oxytoca* isolates. In addition to carriage of heterologous *K. oxytoca* genotypes, prolonged carriage of the same strain might also be observed during active AAHC (isolate 34) and later in remission (isolate 45). As shown previously, the abundance of *K. oxytoca* in feces is several-fold higher during active AAHC than in healthy carriers (4×10^6 CFU/ml compared to $<10^1$ CFU/ml for healthy carriers) (3). Therefore, overgrowth of toxin-producing *K. oxytoca* strains already present in the intestine at the start of antibiotic therapy might cause colitis in AAHC. The cessation of antibiotic therapy is usually sufficient to resolve AAHC. It follows that regrowth of the normal microbiota reduces the abundance of *K. oxytoca* to levels too low to cause disease. This assumption is also supported by the fact that most AAHC isolates were found in similar clusters (A and B2) as isolates from asymptomatic intestinal carriers. This pathophysiologic model is also consistent with known infection models in animals for AAHC (2); however, it is different from antibiotic colitis that is due to *Clostridium difficile*, as patients who develop colitis are newly infected with spores of the bacterium, whereas healthy carriers rarely develop *C. difficile* colitis (40).

The MLST method established in this study revealed clustering of clinical *K. oxytoca* isolates according to habitat (stool versus respiratory tract) and to specific infections (AAHC and nosocomial pneumonia). Toxin production in *K. oxytoca*, however, was not associated with any genetic background. The results suggest specific niche adaptation of genetically distinct *K. oxytoca* strains that cause different types of human infections. Efforts to sequence whole genomes of *K. oxytoca* isolates should provide insight into the underlying basis for the association of distinct genotypes with body habitat adaptations.

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REFERENCES

1. Beaugerie L, Metz M, Barbut F, Bellaiche G, Bouhnik Y, Raskine L, Nicolas JC, Chatelet FP, Lehn N, Petit JC, Infectious Colitis Study Group. 2003. *Klebsiella oxytoca* as an agent of antibiotic-associated hemorrhagic colitis. Clin. Gastroenterol. Hepatol. 1:370–376. [http://dx.doi.org/10.1053/S1542-3565\(03\)00183-6](http://dx.doi.org/10.1053/S1542-3565(03)00183-6).
2. Högenauer C, Langner C, Beubler E, Lippe IT, Schicho R, Gorkiewicz G, Krause R, Gerstgrasser N, Krejs GJ, Hinterleitner TA. 2006. *Klebsiella oxytoca* as a causative organism of antibiotic-associated hemorrhagic colitis. N. Engl. J. Med. 355:2418–2426. <http://dx.doi.org/10.1056/NEJMoa054765>.
3. Zollner-Schwetz I, Högenauer C, Joainig M, Weberhofer P, Gorkiewicz G, Valentin T, Hinterleitner TA, Krause R. 2008. Role of *Klebsiella oxytoca* in antibiotic-associated diarrhea. Clin. Infect. Dis. 47:e74–e78. <http://dx.doi.org/10.1093/cid/cin107>.
4. Dethlefsen L, Ravel DA. 2011. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc. Natl. Acad. Sci. U. S. A. 108(Suppl 1):4554–4561. <http://dx.doi.org/10.1073/pnas.1000087107>.
5. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, Brown D, Stares MD, Scott P, Bergerat A, Louis P, McIntosh F, Johnstone AM, Lobley GE, Parkhill J, Flint HJ. 2011. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J. 5:220–230. <http://dx.doi.org/10.1038/ismej.2010.118>.
6. Joainig MM, Gorkiewicz G, Leitner E, Weberhofer P, Zollner-Schwetz I, Lippe I, Feierl G, Krause R, Hinterleitner T, Zechner EL, Högenauer C. 2010. Cytotoxic effects of *Klebsiella oxytoca* strains isolated from patients with antibiotic-associated hemorrhagic colitis or other diseases caused by infections and from healthy subjects. J. Clin. Microbiol. 48:817–824. <http://dx.doi.org/10.1128/JCM.01741-09>.
7. Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srinivasan A, Kallen A, Limbago B, Fridkin S, National Healthcare Safety Network (NHSN) Team and Participating NHSN Families. 2013. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009–2010. Infect. Control Hosp. Epidemiol. 34:1–14. <http://dx.doi.org/10.1086/668770>.
8. Molton JS, Tambyah PA, Ang BS, Ling ML, Fisher DA. 2013. The global spread of healthcare-associated multidrug-resistant bacteria: a perspective from Asia. Clin. Infect. Dis. 56:1310–1318. <http://dx.doi.org/10.1093/cid/cit020>.
9. Brisse S, Grimont F, Grimont PD. 2006. The genus *Klebsiella*, p. 159–196. In Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackebrandt E (ed), The prokaryotes. Springer, New York, NY.
10. Polage CR, Solnick JV, Cohen SH. 2012. Nosocomial diarrhea: evaluation and treatment of causes other than *Clostridium difficile*. Clin. Infect. Dis. 55:982–989. <http://dx.doi.org/10.1093/cid/cis551>.
11. Podschun R, Ullmann U. 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev. 11:589–603.
12. Sbrana F, Malacarne P, Viaggi B, Costanzo S, Leonetti P, Leonildi A, Casini B, Tascini C, Menichetti F. 2013. Carbapenem-sparing antibiotic regimens for infections caused by *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae* in intensive care unit. Clin. Infect. Dis. 56:697–700. <http://dx.doi.org/10.1093/cid/cis969>.
13. Cheng VC, Yam WC, Tsang LL, Yau MC, Siu GK, Wong SC, Chan JF, To KK, Tse H, Hung IF, Tai JW, Ho PL, Yuen KY. 2012. Epidemiology of *Klebsiella oxytoca*-associated diarrhea detected by Simmons citrate agar supplemented with inositol, tryptophan, and bile salts. J. Clin. Microbiol. 50:1571–1579. <http://dx.doi.org/10.1128/JCM.00163-12>.
14. Higaki M, Chida T, Takano H, Nakaya R. 1990. Cytotoxic component(s) of *Klebsiella oxytoca* on HEP-2 cells. Microbiol. Immunol. 34:147–151. <http://dx.doi.org/10.1111/j.1348-0421.1990.tb00999.x>.
15. Minami J, Katayama S, Matsushita O, Sakamoto H, Okabe A. 1994. Enterotoxin activity of *Klebsiella oxytoca* cytotoxin in rabbit intestinal loops. Infect. Immun. 62:172–177.
16. Urwin R, Maiden MC. 2003. Multi-locus sequence typing: a tool for global epidemiology. Trends Microbiol. 11:479–487. <http://dx.doi.org/10.1016/j.tim.2003.08.006>.

17. Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. 2005. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *J. Clin. Microbiol.* 43:4178–4182. <http://dx.doi.org/10.1128/JCM.43.8.4178-4182.2005>.
18. Hunter PR, Gaston MA. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* 26:2465–2466.
19. Jolley KA, Maiden MC. 2010. BIGSdb: scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* 11:595. <http://dx.doi.org/10.1186/1471-2105-11-595>.
20. Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25:1451–1452. <http://dx.doi.org/10.1093/bioinformatics/btp187>.
21. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28:2731–2739. <http://dx.doi.org/10.1093/molbev/msr121>.
22. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J. Bacteriol.* 186:1518–1530. <http://dx.doi.org/10.1128/JB.186.5.1518-1530.2004>.
23. Huson DH, Bryant D. 2006. Application of phylogenetic networks in evolutionary studies. *Mol. Biol. Evol.* 23:254–267. <http://dx.doi.org/10.1093/molbev/msj030>.
24. Haubold B, Hudson RR. 2000. LIAN 3.0: detecting linkage disequilibrium in multilocus data. *Bioinformatics* 16:847–849. <http://dx.doi.org/10.1093/bioinformatics/16.9.847>.
25. Deguchi T, Yasuda M, Kawamura T, Nakano M, Ozeki S, Kanematsu E, Nishino Y, Kawada Y. 1997. Improved antimicrobial activity of DU-6859a, a new fluoroquinolone, against quinolone-resistant *Klebsiella pneumoniae* and *Enterobacter cloacae* isolates with alterations in GyrA and ParC proteins. *Antimicrob. Agents Chemother.* 41:2544–2546.
26. Brisse S, Verhoef J. 2001. Phylogenetic diversity of *Klebsiella pneumoniae* and *Klebsiella oxytoca* clinical isolates revealed by randomly amplified polymorphic DNA, *gyrA* and *parC* genes sequencing and automated ribotyping. *Int. J. Syst. Evol. Microbiol.* 51:915–924. <http://dx.doi.org/10.1099/00207713-51-3-915>.
27. Nemoy LL, Kotetishvili M, Tigno J, Keefer-Norris A, Harris AD, Perencevich EN, Johnson JA, Torpey D, Sulakvelidze A, Morris JG, Jr, Stine OC. 2005. Multilocus sequence typing versus pulsed-field gel electrophoresis for characterization of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates. *J. Clin. Microbiol.* 43:1776–1781. <http://dx.doi.org/10.1128/JCM.43.4.1776-1781.2005>.
28. Liu WB, Liu B, Zhu XN, Yu SJ, Shi XM. 2011. Diversity of *Salmonella* isolates using serotyping and multilocus sequence typing. *Food Microbiol.* 28:1182–1189. <http://dx.doi.org/10.1016/j.fm.2011.04.001>.
29. Maiden MC. 2006. Multilocus sequence typing of bacteria. *Annu. Rev. Microbiol.* 60:561–588. <http://dx.doi.org/10.1146/annurev.micro.59.030804.121325>.
30. Clermont O, Olier M, Hoede C, Diancourt L, Brisse S, Keroudean M, Glodt J, Picard B, Oswald E, Denamur E. 2011. Animal and human pathogenic *Escherichia coli* strains share common genetic backgrounds. *Infect. Genet. Evol.* 11:654–662. <http://dx.doi.org/10.1016/j.meegid.2011.02.005>.
31. Le Gall T, Clermont O, Gouriou S, Picard B, Nassif X, Denamur E, Tenaillon O. 2007. Extraintestinal virulence is a coincidental by-product of commensalism in B2 phylogenetic group *Escherichia coli* strains. *Mol. Biol. Evol.* 24:2373–2384. <http://dx.doi.org/10.1093/molbev/msm172>.
32. Kallet RH, Quinn TE. 2005. The gastrointestinal tract and ventilator-associated pneumonia. *Respir. Care* 50:910–921; discussion 921–923.
33. Hurley JC. 1995. Prophylaxis with enteral antibiotics in ventilated patients: selective decontamination or selective cross-infection? *Antimicrob. Agents Chemother.* 39:941–947. <http://dx.doi.org/10.1128/AAC.39.4.941>.
34. Rotstein C, Evans G, Born A, Grossman R, Light RB, Magder S, McTaggart B, Weiss K, Zhanel GG. 2008. Clinical practice guidelines for hospital-acquired pneumonia and ventilator-associated pneumonia in adults. *Can. J. Infect. Dis. Med. Microbiol.* 19:19–53.
35. Ramirez P, Bassi GL, Torres A. 2012. Measures to prevent nosocomial infections during mechanical ventilation. *Curr. Opin. Crit. Care* 18:86–92. <http://dx.doi.org/10.1097/MCC.0b013e32834ef3ff>.
36. Bonten MJ, Krueger WA. 2006. Selective decontamination of the digestive tract: cumulating evidence, at last? *Semin. Respir. Crit. Care Med.* 27:18–22. <http://dx.doi.org/10.1055/s-2006-933669>.
37. Chaudhuri RR, Henderson IR. 2012. The evolution of the *Escherichia coli* phylogeny. *Infect. Genet. Evol.* 12:214–226. <http://dx.doi.org/10.1016/j.meegid.2012.01.005>.
38. Schubert S, Darlu P, Clermont O, Wieser A, Magistro G, Hoffmann C, Weinert K, Tenaillon O, Matic I, Denamur E. 2009. Role of intraspecies recombination in the spread of pathogenicity islands within the *Escherichia coli* species. *PLoS Pathog.* 5:e1000257. <http://dx.doi.org/10.1371/journal.ppat.1000257>.
39. Dobrindt U, Hochhut B, Hentschel U, Hacker J. 2004. Genomic islands in pathogenic and environmental microorganisms. *Nat. Rev. Microbiol.* 2:414–424. <http://dx.doi.org/10.1038/nrmicro884>.
40. Kyne L, Warny M, Qamar A, Kelly CP. 2000. Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. *N. Engl. J. Med.* 342:390–397. <http://dx.doi.org/10.1056/NEJM200002103420604>.