JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2010, p. 4177–4185 Vol. 48, No. 11
0095-1137/10/$12.00 doi:10.1128/JCM.00274-10

Multilocus Sequence Typing Identifies Evidence for Recombination and Two Distinct Lineages of *Corynebacterium diphtheriae*▽

Frances Bolt,[1] Pamela Cassiday,[2] Maria Lucia Tondella,[2] Aruni DeZoysa,[3] Androulla Efstratiou,[3] Andreas Sing,[4] Aleksandra Zasada,[5] Kathryn Bernard,[6] Nicole Guiso,[7] Edgar Badell,[7] Marie-Laure Rosso,[7] Adam Baldwin,[1] and Christopher Dowson[1]*

Biological Sciences, Warwick University, Coventry, United Kingdom[1]; Division of Bacterial Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia[2]; Health Protection Agency, London, United Kingdom[3]; the National Consiliary Laboratory for Diphtheria (NCLD), Oberschleissheim, Germany[4]; the National Institute of Public Health (NIPH), Warsaw, Poland[5]; National Microbiology Laboratory, PHAC, Winnipeg, Canada[6]; and Institut Pasteur, Molecular Prevention and Therapy of Human Diseases Unit. Paris, France[7]

Received 10 February 2010/Returned for modification 29 March 2010/Accepted 24 August 2010

We describe the development of a multilocus sequence typing (MLST) scheme for *Corynebacterium diphtheriae*, the causative agent of the potentially fatal upper respiratory disease diphtheria. Global changes in diphtheria epidemiology are highlighted by the recent epidemic in the former Soviet Union (FSU) and also by the emergence of nontoxigenic strains causing atypical disease. Although numerous techniques have been developed to characterize *C. diphtheriae*, their use is hindered by limited portability and, in some instances, poor reproducibility. One hundred fifty isolates from 18 countries and encompassing a period of 50 years were analyzed by multilocus sequence typing (MLST). Strain discrimination was in accordance with previous ribotyping data, and clonal complexes associated with disease outbreaks were clearly identified by MLST. The data produced are portable, reproducible, and unambiguous. The MLST scheme described provides a valuable tool for monitoring and characterizing endemic and epidemic *C. diphtheriae* strains. Furthermore, multilocus sequence analysis of the nucleotide data reveals two distinct lineages within the population of *C. diphtheriae* examined, one of which is composed exclusively of biotype belfanti isolates and the other of multiple biotypes.

Diphtheria has historically evoked fear and terror due to its slow suffocating death and previously unknown origin, but socioeconomic improvement and the introduction of mass immunization in the 1940s and 1950s led its near-elimination in the developed world. However, diphtheria remains a global disease and is endemic in many countries. The World Health Organization (WHO) has recorded outbreaks throughout the world, including Afghanistan, Algeria, Iraq, Lao People's Republic, Mongolia, Papua New Guinea, Sudan, and Thailand (1). It is also a potentially resurgent infectious disease, exemplified in the 1990s by a notable epidemic in the newly independent states of the former Soviet Union (NIS), where vaccination had been employed since 1958 (33). At least 20 cases were reported beyond these countries, highlighting the potential threat of introduced strains from countries in which it is endemic and epidemic (29). Furthermore, according to serological surveillance studies, the proportion of susceptible individuals in vaccinated populations remains high (7, 10). Edmunds et al. estimated that there are inadequate protection levels in the United Kingdom for 70 to 75% of those aged 50 to 60 years old (7). Similar observations were recently reported for individuals who had followed the French vaccine recommendations (17).

It is therefore apparent that typing tools enabling global *Corynebacterium diphtheriae* surveillance are of great importance. Based upon their biochemical and morphological properties, four *C. diphtheriae* biotypes have been identified: mitis, gravis, intermedius, and belfanti (8). Several typing techniques for *C. diphtheriae* have been developed. Traditionally these techniques were based upon serologic, phage, and biotyping methods. However, since the methods provide limited resolution, molecular typing techniques, including amplified fragment length polymorphisms (AFLP) (4), random amplified polymorphic DNA (RAPD) (3, 24), multilocus enzyme electrophoresis (MEE) (28), spoligotyping (21), and pulsed-field gel electrophoresis (PFGE) (5), have been developed and show significant intraspecies genetic diversity. Recently a comparison of the different typing techniques was performed (6). It was shown that the most discriminative was ribotyping, the current "gold standard" typing method for *C. diphtheriae*. This method has identified 86 distinct ribotype patterns and clusters isolates associated with the former Soviet Union (FSU) outbreak (5, 11, 28). However, ribotyping is very dependent upon the use of a rigid standardized method, and without this, there are clearly difficulties in reproducibility (25). Additionally, typing methods based upon band matching do not clearly reveal the population structure or underlying evolutionary mechanisms of a given species.

Proposed in 1998, multilocus sequence typing (MLST) overcomes the problems encountered with ribotyping by directly indexing nucleotide variation within several core metabolic genes, thereby providing portable, reproducible, and high-resolution data appropriate for the evolutionary and epidemiological investigation of diphtheria (19). We describe the development of an MLST scheme to examine the genetic

* Corresponding author. Mailing address: Department of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, United Kingdom. Phone: 44 2476 523534. Fax: 44 2476 52356. E-mail: c.g.dowson@warwick.ac.uk.

▽ Published ahead of print on 15 September 2010.

relationship between a temporally and geographically diverse collection of *C. diphtheriae* isolates.

MATERIALS AND METHODS

Bacterial strain collection. As shown in Table 1, a total of 150 *C. diphtheriae* isolates were examined and obtained from the Centers for Disease Control and Prevention (CDC) (Atlanta, GA), the Health Protection Agency (HPA) (London, United Kingdom), the National Consiliary Laboratory for Diphtheria (Germany), and the National Institute of Public Health (Poland). The MLST scheme was validated by comparison to 29 previously ribotyped isolates encompassing 20 different ribotypes (5, 28). The collection is both temporally and geographically diverse, with isolates from 18 countries, covering a time period from 1957 to 2006. Of the 45 human cases with available clinical information, 28.9% were associated with carriage ($n = 13$), 42.2% were isolated from diphtheria ($n = 19$), 11.1% were isolated from cutaneous lesions ($n = 5$), 13.3% were isolated from patients with upper respiratory conditions, including pharyngitis ($n = 1$), tonsillitis ($n = 4$), and a sore throat ($n = 1$), and 4.3% ($n = 2$) were obtained from patients with both osteomyelitis and cutaneous lesions. Both toxigenic ($n = 96$) and nontoxigenic ($n = 52$) (toxigenic data were unavailable for two isolates) biotype gravis ($n = 43$), intermedius ($n = 6$), mitis ($n = 85$), and belfanti ($n = 16$) strains were examined.

Biotyping and toxigenicity testing. Toxigenicity was determined by the Elek immunoprecipitation method (Elek test) in accordance with the WHO guidelines (9). All isolates were biotyped using the API Coryne kit (bioMérieux, Lyon, France) according to the manufacturer's instructions.

Locus selection and amplification. Potential housekeeping genes were identified by comparing the *C. diphtheriae* (1), *Corynebacterium glutamicum* (*C. glutamicum*) (15), and *Corynebacterium efficiens* (*C. efficiens*) (26) genome sequences using the Artemis Comparison Tool (ACT) and the Double ACT program, available at http://www.sanger.ac.uk/Software/ACT/ and http://www.hpa-bioinfotools.org.uk/pise/double_act.html, respectively. Amplification and nested sequencing primers were designed for the loci *atpA*, *dnaE*, *dnaK*, *fusA*, *leuA*, *odhA*, and *rpoB* (Table 2) using Primer3 (30). The sequencing primers for *rpoB* were previously described by Khamis and colleagues (16).

DNA was extracted primarily as described by Mothershed et al. (23). Each 25-μl PCR was carried out using 10 ng of chromosomal DNA, 5 μl Q solution (Qiagen, United Kingdom), 4.0 μl chromosomal DNA (5 to 20 ng/μl), 1.0 μl forward primer (10 pmol/μl), 1.0 μl reverse primer (10 pmol/μl), 2.5 μl 10× PCR buffer (Qiagen) (containing 15 mM $MgCl_2$), 0.5 μl deoxynucleoside triphosphate (dNTP) solution (Qiagen) (10 mM [each] dNTP), 0.125 μl *Taq* polymerase (Qiagen, 5 U/μl), 0.5 μl $MgCl_2$ (Qiagen) (25 mM), and PCR-grade water. All primer sets were designed to ensure they had similar melting temperatures, and reaction conditions were as follows: initial denaturation at 94°C for 1 min; 35 cycles of denaturation at 94°C for 1 min; and primer annealing at 58°C for 1 min and extension at 72°C for 2 min, followed by a final extension step of 72°C for 5 min. Amplicons were purified using the MiniElute UF plates (Qiagen, United Kingdom) according to the manufacturer's instructions and stored at −20°C.

Amplicon nucleotide sequences were determined by nested sequencing using the BigDye Terminator ready reaction mix, v3.1 (Perkin-Elmer Applied Biosystems, Foster City), following the manufacturer's protocol. The forward and reverse sequences of a given locus were edited, aligned, and trimmed to the desired length using the SeqManII software program (DNASTAR, Madison, WI).

Allele and sequence type designation. Allelic numbers were assigned to each unique allele for a given locus. For each isolate, the allelic profile was generated by combining the allele numbers for each locus in the order *atpA*, *dnaE*, *dnaK*, *fusA*, *leuA*, *odhA*, and *rpoB*. A novel sequence type (ST) designation was given to all unique allelic profiles, while isolates with identical profiles belonged to the same ST.

Tree congruence. Tree concordance was assessed using the method developed by Holmes and colleagues; however, here tree congruence data were increased to compare 200 rather than 100 randomly generated maximum-likelihood (ML) trees (14).

Phylogenetic analysis. The allelic sequences for each isolate were concatenated (2,545 bp), and phylogenetic trees, with 1,500 bootstrap replicates, were generated by the neighbor-joining method using the Jukes Cantor algorithm within the MEGA software program, v4 (32). Isolates were grouped based upon the MLST definition of a clonal complex (or eBURST group) being a cluster of isolates sharing at least six of seven alleles, using the eBURST program, available at www.mlst.net. To visualize clustering within the population and to detect recombination between STs, Splits decomposition analysis was performed using the SplitsTree software program, v4. Neighbor-joining phylogenetic analysis was done (www.mlst.net), and the index of association (I_A) was calculated using the LIAN software program, v3.5 (www.pubmlst.org). Significant I_A values were determined using the Monte-Carlo method with 1,000 resamplings. To ensure sampling bias did not affect the value, one representative of each ST was used. The allelic frequencies, GC content, number of polymorphic sites, and ratio of nonsynonymous substitutions to synonymous substitutions (d_N/d_S ratio) for all seven loci were calculated using the START software program, v2. Nucleotide identities were calculated using the maximum composite likelihood model within MEGA, v4.

RESULTS

Allelic variation. In order to validate the MLST scheme, a collection of temporally and geographically diverse isolates were analyzed, including two equine isolates previously described by Henricson et al. (13). Cultures from both epidemiologically linked and unrelated cases were examined to assess the scheme's performance. Isolates previously designated as belonging to one of the four *C. diphtheriae* biotypes, gravis, mitis, intermedius, and belfanti, were typed by MLST to better understand their genetic relationships to each other and to determine their epidemiological value.

As shown in Table 3, among the 150 isolates investigated, the mean average allele length for each locus was 363 bp and ranged from 342 bp (*rpoB*) to 384 bp (*leuA*). All alleles for a given locus were of equal lengths and, to aid further analysis, were in the correct reading frame. The proportion of variable sites at each locus varied from 5.1% (*dnaE*) to 10.3% (*fusA*) (mean average = 7%).

To determine the degree of selective pressure upon each locus, the ratio of nonsynonymous to synonymous substitutions (d_N/d_S) was determined. Since the ratios were significantly less than 1, it is clear that the genes chosen were not under purifying selection and were therefore suitable for MLST analysis (Table 3).

Assignment of allele and sequence types. A total of 73 STs were assigned to the 150 *C. diphtheriae* isolates investigated and divided into 11 clonal complexes designated by eBURST groups (Table 1). For this study, isolates were assigned as members of an eBURST group when six of seven MLST alleles were shared.

Strain identification. MLST identified two clonal complexes linked to diphtheria outbreaks. eBURST group 2 (composed of ST-8, ST-12, ST-52, and ST-66 strains) was associated with the FSU epidemic. Six isolates identified as having a Sankt-Peterburg ($n = 3$) or Rossija ($n = 3$) ribotype, the two clonally derived ribotypes linked with the FSU outbreak, clustered within this group, as did three epidemic strains identified by Skogen and colleagues in cultures obtained prior to the outbreak (31). Interestingly, the first nontoxigenic gravis strain to cause septicemia and endocarditis in Poland also belonged to eBURST group 2. This strain (493/K/04) was isolated in a region where no diphtheria cases had been reported for 10 years (34).

Twenty-eight of 31 isolates from Haiti ($n = 14$) and the Dominican Republic ($n = 17$) were collected during a diphtheria epidemic (2004 to 2006). Eighty-nine percent ($n = 25$) of the outbreak isolates were ST-31, and another (isolate 158) was ST-4, a single locus variant (SLV) of ST-31 at the *fusA* locus (98.06% allelic nucleotide similarity). The SLV is likely to have arisen by recombination, since multiple nucleotide

TABLE 1. Details of *C. diphtheriae* strains used[a]

Strain identification	eBURST group	Previous designation	Referral laboratory	Sequence type	Allelic profile	Yr isolated	Country/state/city of isolation	Disease	Site of isolation	Biotype	DT status
27	1	(MS) 7/97	NIPH	32	3-1-18-4-13-3-5	1997	Poland	NK	NK	G	NK
64	1	CD293	CDC	32	3-1-18-4-13-3-5	1996	Kazakhstan	NK	NK	M	+
66	1	CD295	CDC	32	3-1-18-4-13-3-5	1996	Kazakhstan	NK	Throat	M	−
78	1	CD373	CDC	36	3-7-18-4-13-3-5	2005	USA, California	Sore throat	Throat	G	−
96	1	722	CDC	43	15-1-18-4-13-3-5	1986	Russia, Kovrov	Carrier	Throat	G	+
127	1	CD286	CDC	58	3-1-26-4-13-3-5	1996	Kazakhstan	NK	NK	G	+
129	1	CD303	CDC	70	3-1-18-4-20-3-5	1997	USA, Michigan	NK	Blood	G	−
8	2	95-135	HPA	8	3-5-6-5-3-3-6	1993	Finland	NK	NK	G	+
25	2	493/K/04	NIPH	8	3-5-6-5-3-3-6	2004	Poland	NK	NK	G	-
36	2	G4174	CDC	8	3-5-6-5-3-3-6	1993	Russia, Leningrad	Diphtheria	NK	G	+
45	2	CD126	CDC	8	3-5-6-5-3-3-6	1985	Russia, Moscow	Diphtheria	NK	G	+
46	2	CD130	CDC	8	3-5-6-5-3-3-6	1987	Russia, Sverdlovsk	Tonsillitis	NK	G	+
47	2	CD131	CDC	8	3-5-6-5-3-3-6	1987	Russia, Sverdlovsk	Carrier	NK	G	+
62	2	CD290	CDC	8	3-5-6-5-3-3-6	1996	Kazakhstan	NK	NK	G	+
63	2	CD291	CDC	8	3-5-6-5-3-3-6	1996	Kazakhstan	NK	NK	G	+
65	2	CD294	CDC	8	3-5-6-5-3-3-6	1996	Kazakhstan	NK	Nose	M	-
126	2	CD283	CDC	8	3-5-6-5-3-3-6	1996	Kazakhstan	NK	NK	G	+
13	2	93-146	HPA	12	1-5-6-5-3-3-6	1993	Russia, Kaliningrad	Carrier	NK	G	+
124	2	CD267	CDC	52	3-5-6-5-3-10-6	1996	Kazakhstan	NK	NK	G	+
69	3	CD306	CDC	42	6-7-10-8-9-12-10	1997	USA, Michigan	NK	Sputum	B	-
95	2	749	CDC	66	3-5-6-5-11-3-6	1987	Russia, Kovrov	NK	Throat	G	+
116	3	CD198	CDC	51	6-7-10-8-9-19-10	1999	USA, California	NK	Nose	B	-
133	3	CD310	CDC	54	6-7-10-22-9-12-10	1998	USA, Ohio	NK	Sputum	B	-
10	4	93-32	HPA	10	5-2-7-1-3-5-8	1993	UK	NK	NK	G	+
108	4	CD170	CDC	49	2-6-7-1-3-5-8	1973	South Dakota	NK	NK	G	+
115	4	CD196	CDC	63	5-6-7-1-3-5-8	1978	USA, Colorado	NK	Throat	G	+
19	5	95-115	HPA	18	8-6-7-6-6-3-8	1995	Russia, Vladimir region	Diphtheria	NK	G	+
20	5	93-181	HPA	19	2-6-7-6-6-3-8	1966	Russia, Moscow	Carrier	NK	G	+
12	5	93-69	HPA	25	5-6-7-6-6-3-8	NK	Russia, Murmansk	Diphtheria	NK	G	+
39	5	CD085	CDC	25	5-6-7-6-6-3-8	1957	Russia, Vladivostok	Diphtheria	NK	G	+
40	5	CD087	CDC	25	5-6-7-6-6-3-8	1957	Russia, Vladivostok	Carrier	NK	G	+
41	5	CD092	CDC	25	5-6-7-6-6-3-8	1964	Russia, Moscow	Carrier	NK	G	+
42	5	CD094	CDC	25	5-6-7-6-6-3-8	1965	Russia, Moscow	Carrier	NK	G	+
43	5	CD097	CDC	25	5-6-7-6-6-3-8	1971	Russia, Moscow	Carrier	NK	G	+
51	5	PR79	CDC	25	5-6-7-6-6-3-8	1996	USA, South Dakota	NK	NK	G	+
125	5	CD268	CDC	25	5-6-7-6-6-3-8	1996	Kazakhstan	NK	NK	G	+
97	6	G4212	CDC	45	2-10-24-1-3-15-2	1992	Russia, Murmansk	Diphtheria	NK	M	+
128	6	CD301	CDC	53	2-10-24-1-3-3-2	1997	USA, Oregon	NK	Throat	M	-
52	7	1507	CDC	28	2-9-3-13-3-3-3	1995	Kyrgyzstan	NK	NK	M	+
53	7	1512	CDC	41	2-9-22-13-3-3-3	1995	Kyrgyzstan	NK	NK	M	+
21	8	90-39	HPA	20	8-3-3-2-7-3-3	1990	UK, London	Tonsillitis	NK	M	+
67	8	CD304	CDC	40	8-3-19-2-7-3-3	1997	Guatemala	NK	NK	M	-
103	8	CD081	CDC	40	8-3-19-2-7-3-3	1997	Russia, Moscow	NK	NK	M	-
118	8	CD209	CDC	40	8-3-19-2-7-3-3	1997	Russia	NK	NK	M	-
4	9	95-385	HPA	4	3-2-4-3-4-4-4	1995	Dominican Republic	NK	NK	M	+
158	9	CD333	CDC	4	3-2-4-3-4-4-4	2004	Dominican Republic	NK	NK	M	+
59	9	CD216	CDC	31	3-2-4-15-4-4-4	2000	Dominican Republic	NK	NK	M	+
74	9	CD340	CDC	31	3-2-4-15-4-4-4	2004	Haiti	Diphtheria	Throat	M	+
149	9	CD324	CDC	31	3-2-4-15-4-4-4	2004	Dominican Republic	NK	NK	M	+
150	9	CD325	CDC	31	3-2-4-15-4-4-4	2004	Dominican Republic	NK	NK	M	+
151	9	CD326	CDC	31	3-2-4-15-4-4-4	2004	Dominican Republic	NK	NK	M	+
152	9	CD327	CDC	31	3-2-4-15-4-4-4	2004	Dominican Republic	NK	NK	M	+
153	9	CD328	CDC	31	3-2-4-15-4-4-4	2004	Dominican Republic	NK	NK	M	+
155	9	CD330	CDC	31	3-2-4-15-4-4-4	2004	Dominican Republic	NK	NK	M	+
156	9	CD331	CDC	31	3-2-4-15-4-4-4	2004	Dominican Republic	NK	NK	M	+
157	9	CD332	CDC	31	3-2-4-15-4-4-4	2004	Dominican Republic	NK	NK	M	+
159	9	CD334	CDC	31	3-2-4-15-4-4-4	2004	Dominican Republic	NK	NK	M	+
161	9	CD336	CDC	31	3-2-4-15-4-4-4	2004	Dominican Republic	NK	NK	M	+
162	9	CD337	CDC	31	3-2-4-15-4-4-4	2004	Dominican Republic	NK	NK	M	+
164	9	CD339	CDC	31	3-2-4-15-4-4-4	2004	Dominican Republic	NK	NK	M	+
167	9	CD342	CDC	31	3-2-4-15-4-4-4	2004	Haiti	Diphtheria	NK	M	+
169	9	CD344	CDC	31	3-2-4-15-4-4-4	2004	Haiti	NK	Nose	M	+
170	9	CD345	CDC	31	3-2-4-15-4-4-4	2004	Haiti	Diphtheria	NK	M	+
171	9	CD346	CDC	31	3-2-4-15-4-4-4	2004	Haiti	Diphtheria	NK	M	+
172	9	CD347	CDC	31	3-2-4-15-4-4-4	2004	Haiti	Diphtheria	NK	M	+
173	9	CD350	CDC	31	3-2-4-15-4-4-4	2004	Haiti	NK	Throat	M	+
174	9	CD352	CDC	31	3-2-4-15-4-4-4	2004	Haiti	Diphtheria	NK	M	+
176	9	CD354	CDC	31	3-2-4-15-4-4-4	2005	Haiti	Diphtheria	NK	M	+
177	9	CD359	CDC	31	3-2-4-15-4-4-4	2005	Haiti	Diphtheria	NK	M	+
178	9	CD360	CDC	31	3-2-4-15-4-4-4	2005	Haiti	Diphtheria	NK	M	+
179	9	CD371	CDC	31	3-2-4-15-4-4-4	2005	Haiti	Diphtheria	NK	M	+
181	9	CD400	CDC	31	3-2-4-15-4-4-4	2006	Haiti	Diphtheria	NK	M	+
55	10	CD204	CDC	29	10-8-16-14-10-3-5	1990	Russia, Selivavovo	NK	Throat	M	−
57	10	CD211	CDC	30	10-8-16-14-10-3-9	1997	Russia	NK	NK	M	−
102	10	CD080	CDC	30	10-8-16-14-10-3-9	1997	Russia, Moscow	NK	NK	M	−
104	10	CD082	CDC	30	10-8-16-14-10-3-9	1997	Russia, Moscow	NK	NK	M	−
117	10	CD208	CDC	30	10-8-16-14-10-3-9	1997	Russia	NK	NK	M	−
5	11	93-186	HPA	5	2-4-4-1-3-3-5	1989	Russia, St. Petersburg	Carrier	NK	M	−
22	11	93-218	HPA	5	2-4-4-1-3-3-5	1993	Russia, St. Petersburg	Carrier	NK	M	−
56	11	CD207	CDC	5	2-4-4-1-3-3-5	1997	Russia	NK	NK	M	−
68	11	CD305	CDC	5	2-4-4-1-3-3-5	1997	USA, Massachusetts	NK	Sputum	M	−

Continued on following page

TABLE 1—*Continued*

Strain identification	eBURST group	Previous designation	Referral laboratory	Sequence type	Allelic profile	Yr isolated	Country/state/city of isolation	Disease	Site of isolation	Biotype	DT status
9	11	93-266	HPA	9	2-4-4-1-3-3-7	1993	Russia, St. Petersburg	Diphtheria	NK	G	+
1		94-94	HPA	1	1-1-1-1-1-1-1	NK	Sweden	NK	NK	M	+
113		CD187	CDC	1	1-1-1-1-1-1-1	1984–1986	Sweden	NK	NK	M	+
2		94-249	HPA	2	2-2-2-1-2-2-2	NK	Romania	NK	NK	M	−
3		85-29	HPA	3	3-3-3-2-3-3-3	1985	UK, Swansea	NK	NK	M	+
23		93-211	HPA	3	3-3-3-2-3-3-3	1993	UK	NK	NK	M	+
6		93-154	HPA	6	4-2-5-2-3-3-5	1993	UK	NK	NK	M	+
7		94-282	HPA	7	2-5-6-4-5-5-2	1994	Italy	Pharyngitis	NK	M	−
11		95-387	HPA	11	3-4-3-1-4-3-2	1995	Dominican Republic	NK	NK	M	+
14		94-263	HPA	13	6-4-8-1-7-3-9	1994	Thailand	Skin lesion	NK	M	+
15		92-48	HPA	14	3-2-9-7-8-3-2	1992	UK, Bristol	NK	NK	M	+
16		94-260	HPA	15	7-7-10-8-9-6-10	1993	Germany	NK	NK	B	−
17		93-121	HPA	16	5-8-11-9-3-3-5	1993	UK	NK	NK	M	+
18		93-132	HPA	17	5-8-12-10-10-6-4	1993	Russia, Kaliningrad	Carrier	NK	M	+
24		85-2	HPA	21	9-4-13-11-3-3-9	1985	UK, Swansea	Tonsillitis	NK	M	+
34		CD027	CDC	22	2-2-14-4-11-3-4	1958	Canada, Toronto	Ulcer	NK	M	+
35		C78	CDC	23	6-7-10-12-16-7-11	1986	Canada, E. Ontario	NK	Throat	B	−
37		711	CDC	24	2-4-15-6-7-8-9	1985	Russia, Vladimir	NK	Throat	M	+
38		G4182	CDC	24	2-4-15-6-7-8-9	1992	Russia, Vladimir	NK	Throat	M	+
44		CD113	CDC	24	2-4-15-6-7-8-9	1976	Russia, Ivanobsaya	Tonsillitis	NK	M	+
54		CD203	CDC	24	2-4-15-6-7-8-9	1990	Russia, Vladimir	NK	Throat	M	−
48		C75	CDC	26	8-2-16-1-3-3-12	1996	Canada, James Bay	NK	Throat	B	+
193		CD149	CDC	26	8-2-16-1-3-3-12	1998	Canada, James Bay	NK	Throat	B	−
194		CD150	CDC	26	8-2-16-1-3-3-12	1998	Canada, James Bay	NK	Throat	B	+
195		CD162	CDC	26	8-2-16-1-3-3-12	1995	Canada, James Bay	NK	Ear	B	+
196		CD163	CDC	26	8-2-16-1-3-3-12	1995	Canada, James Bay	NK	Throat	B	+
49		CD164	CDC	27	2-4-17-4-7-5-4	1995	Argentina	Diphtheria	NK	M	+
70		CD314	CDC	33	11-1-20-16-3-9-14	1998	USA, Massachusetts	NK	Blood	G	−
71		CD315	CDC	33	11-1-20-16-3-9-14	1998	USA, Massachusetts	NK	Blood	G	−
72		CD317	CDC	34	12-7-10-17-9-7-10	1999	USA, Maine	NK	Sputum	B	−
76		CD372	CDC	35	6-7-21-12-15-7-11	2005	USA, Pennsylvania	NK	Bronchial	B	−
79		CD376	CDC	37	6-7-10-18-9-13-2	2006	USA, Colorado	NK	Sputum	B	−
61		CD222	CDC	38	2-10-29-1-12-10-13	1971	USA, Arizona	NK	NK	I	−
107		CD169	CDC	38	2-10-29-1-12-10-13	1979	USA, South Dakota	NK	Throat	I	+
120		CD230	CDC	38	2-10-29-1-12-10-13	1971	USA, Washington	NK	NK	I	−
122		CD233	CDC	38	2-10-29-1-12-10-13	1971	USA, Washington	NK	NK	I	−
123		CD235	CDC	38	2-10-29-1-12-10-13	1971	USA, Washington	NK	NK	I	−
73		CD318	CDC	39	13-1-8-4-14-11-2	1999	USA, Maine	NK	Sputum	G	−
80		CD383	CDC	39	13-1-8-4-14-11-2	1996	USA, California	NK	NK	G	−
131		CD308	CDC	39	13-1-8-4-14-11-2	1998	USA, California	NK	Blood	G	−
137		CD322	CDC	39	13-1-8-4-14-11-2	1999	USA, California	NK	Synovial	G	−
138		CD323	CDC	39	13-1-8-4-14-11-2	1999	USA, California	NK	Blood	G	−
29		(MZ) 113/G	NIPH	44	14-2-23-4-2-14-2	1990s	Poland	Skin ulcer	Throat	M	+
30		(MZ) 115/S	NIPH	44	14-2-23-4-2-14-2	1990s	Poland	Skin ulcer	Skin	M	+
31		(MM) 4/G	NIPH	44	14-2-23-4-2-14-2	1990s	Poland	NK	Throat	I	+
32		(AW) 6/G	NIPH	44	14-2-23-4-2-14-2	1990s	Poland	NK	Throat	M	+
98		724	CDC	46	16-4-8-1-7-16-9	1987	Russia, Vladimir	NK	NK	M	+
99		760	CDC	47	2-11-9-19-17-17-9	1989	Russia, Kovrov	NK	Throat	M	−
100		1709	CDC	48	4-4-25-6-3-5-9	1988	Russia, Penza	NK	Throat	M	+
109		CD177	CDC	50	2-2-4-1-3-3-2	1981	South Dakota	NK	NK	M	−
110		CD178	CDC	50	2-2-4-1-3-3-2	1981	South Dakota	NK	NK	M	−
111		CD179	CDC	50	2-2-4-1-3-3-2	1982	South Dakota	NK	NK	M	−
139		CD364	CDC	55	2-13-28-4-4-4-4	2004	USA, New York	NK	Blood	M	−
112		CD186	CDC	56	19-12-11-9-18-18-2	1984	Sweden	NK	NK	G	+
114		CD188	CDC	57	17-10-30-23-20-3-16	1985	Sweden	NK	NK	G	+
130*		CD307	CDC	59	18-1-27-21-20-5-16	1998	USA, Virginia	Chest wound	Thoracic exudate	G	+
132*		CD309	CDC	59	18-1-27-21-20-5-16	1998	USA, Virginia	NK	Placenta	G	−
101		750	CDC	60	2-5-3-1-3-3-9	1988	Russia, Kovrov	NK	NK	M	+
106		CD167	CDC	61	3-4-8-1-7-3-4	1973	South Dakota	NK	NK	M	−
105		CD089	CDC	62	4-1-18-4-3-16-2	1957	Russia, Vladivostok	Carrier	NK	G	−
140		CD365	CDC	64	2-10-17-13-3-2-4	2005	USA, New York	NK	Ankle	M	−
26		1034	NIPH	65	6-7-10-12-9-12-15	2001	Poland	NK	Nose	B	NK
75		CD349	CDC	67	3-2-3-6-3-3-2	2004	USA, Massachusetts	Carrier	Throat	M	+
154		CD329	CDC	67	3-2-3-6-3-3-2	2004	Dominican Republic	NK	NK	M	+
166		CD341	CDC	68	3-4-3-4-4-3-4	2004	Haiti	Skin lesion	NK	M	+
28		(PJ)891/N/00	NIPH	69	6-7-21-17-9-7-11	2000	Poland	NK	Nose	B	−
183		CD402	CDC	71	21-7-21-12-21-12-11	2006	USA, Minnesota	NK	Nasal discharge	B	−
186		53	NCLD	72	2-8-4-1-3-4-2	NK	Angola	Osteomyelitis, cutaneous	Thigh	M	+
185		52	NCLD	73	2-8-9-27-18-3-18	NK	Angola	Osteomyelitis, cutaneous	Foot	M	+

[a] "NK" is used where clinical data are not known. Toxigenicity information is provided by "+" for toxin positive and "−" for toxin negative under the diphtheria toxin status (DT) column. Biotypes are indicated as follows: B, biotype belfanti; I, intermedius; G, gravis; and M, mitis. All isolates were cultured from humans with the exception of those highlighted by an asterisk (*), which were obtained from horses. Strains were obtained from the Health Protection Agency (HPA), Centers for Disease Control (CDC), the National Consiliary Laboratory for Diphtheria (NCLD), and the National Institute of Public Health (NIPH).

substitutions were detected within the *fusA* variant and both alleles were identified in other isolates within the data set. Two distinct strains that also circulated during the epidemic, 154 and 166, were not closely related to the outbreak strains, sharing only two and three of the seven loci, respectively. The epidemic strain was present prior to the suspected outbreak since two of the three isolates obtained in the preepidemic period (1995 to 2000) also belonged to the outbreak clonal

TABLE 2. *C. diphtheriae* housekeeping genes, gene functions, and amplification and sequencing primers used in the MLST scheme[a]

Gene	Gene function	Amplification primer, 5′–3′		Amplicon size (bp)	Sequencing primer, 5′–3′		Allele size (bp)
		Fwd	Rev		Fwd	Rev	
atpA	ATP synthase alpha chain	GCGATTGCGAAC TACACC	CTCGAGGAAT ACCTRACC	1,029	AGAAGGCGACGA AGTMAAGC	CRGAATCAGAA GCTGGWGCA	378
dnaE	DNA polymerase III alpha subunit	TGCGTCATCTGA TTGAAA	CGGTCCAATA AGACACCA	858	GTGCGACAAGCT GGTGTG	GGCTTWCGGCC ATTYTTG	354
dnaK	Chaperone protein DnaK	ACTTGGGTGGCG GTACTT	TGGTGAACGT CTCGGAAC	696	AGATGGCTATGC AGCGTCT	GATGAGCTTGG TCATCACG	345
fusA	Elongation factor G	TACCGCGAGAAG CTCGTT	GAAGGTTGG GTCCTCTTC	683	CGTAAGCTGACC GTTAACTC	CCATGGACTCR AGGATGA	360
leuA	2-Isopropylmalate synthase	CGTGCACTTCTA CAACTC	ACCGTGATCG GTCTTCAT	865	CCYATCATCATCA AYCTGCC	CAGCTGGTTGC AGTAYTC	384
odha	2-Oxoglutarate dehydrogenase	CGGCAAGGAAAS CATGAC	GTTGTCGCCR AACATCTG	505	TBCAAGATCGCA TYGARRC	TWGGCTCGATG TGKCCTTC	382
rpoB	RNA polymerase beta chain	AAGCGCAAGATC CAGGAC	TCGAACTCGT CGTCATCC	845	CGWATGAACATY GGBCAGGT	TCCATYTCRCC RAARCGCTG	342

[a] Fwd, forward; Rev, reverse.

complex. The remaining isolate (isolate 11) was distinct from the epidemic cluster but was a double locus variant (DLV) of isolate 166 obtained in 2004.

MLST analysis indicates that some strains examined in this study are geographically dispersed while others are associated with specific geographical regions. For example, eBURST group 1 is composed of isolates from Poland, Russia, Kazakhstan, and the United States, while only isolates from the Caribbean region belong to eBURST group 9.

C. diphtheriae has been isolated from animals (2, 12, 18, 13, 27). Zoonotic infections with *C. diphtheriae*, although currently rare, may act as a reservoir for human infection. It is therefore important to characterize the isolates to understand their relationship to human strains. Two biotype gravis equine isolates, previously described by Henricson (13), were identical by MLST, and although comprising a unique ST, they clearly cluster within the typical human *C. diphtheriae* population, as shown by Fig. 1.

Relationship between MLST and ribotyping. In total, 86 validated ribotypes have been assigned to the *C. diphtheriae* ribotype database (11). As shown by Fig. 1, the examination of 29 previously ribotyped isolates by MLST was in concordance with the ribotyping data. This study of 20 of the total 86 ribotype patterns by MLST represents a preliminary comparison but is sufficient to help validate the MLST scheme in terms of correctly assigning strains that were identical and part of an outbreak and those believed to be clearly distinct strains.

TABLE 3. Characteristics of the seven loci used in the *C. diphtheriae* MLST scheme

Housekeeping gene	Allele length (bp)	No. of distinct alleles	No. of polymorphic sites	Proportion of polymorphic sites (%)	Mean G+C content (%)	d_N/d_S
atpA	378	20	21	5.5	57.49	0.0394
dnaE	354	13	18	5.1	50.96	0.0100
dnaK	345	30	35	10.1	57.94	0.0024
fusA	360	23	37	10.3	56.22	0.0026
leuA	384	20	25	6.5	56.82	0.0201
odhA	381	19	23	6.0	57.96	0.0145
rpoB	342	17	18	5.3	57.37	0.0081
Mean avg	363	20.3	25.3	7.0	56.4	0.0140

In two instances, MLST provided greater strain discrimination than ribotyping. First, the Lyon ribotype isolates, 19 and 20, were SLVs of each other, differing at the *atpA* locus, and were identified as ST-18 and ST-19. This clonal expansion is likely to have arisen by recombination rather than point mutation, since four base pair changes (4/378) were identified and both alleles were frequently detected in other STs. De Zoysa was also able to differentiate between the two isolates using AFLP, where a single band difference was observed (4). Second, one of three Sankt-Peterburg ribotype isolates (isolate 13) was an SLV at the *atpA* locus, where two base pairs substitutions were identified.

Previous studies identified two predominant ribotypes associated with the FSU outbreak: Sankt-Peterburg and Rossija. Three isolates of each ribotype were analyzed by MLST. With the exception of isolate 13, discussed previously, all were indistinguishable by MLST (ST-8). AFLP, RAPD, and PFGE typing studies were also unable to differentiate between the two ribotypes, and only a single band difference was detected by ribotyping (3, 4, 5). Therefore, the preliminary MLST data further support the suggestion (4, 5) that Sankt-Peterburg and Rossija ribotyped isolates are part of a shared clonal complex, along with ST-12 and ST-52, here forming eBURST group 2.

Likewise, isolates belonging to ribotypes Vladimir (ST-25) and Lyon (ST-18 and ST-19) are clonally derived (eBURST group 5), as are isolates belonging to ribotypes Cluj (ST-5) and Gatchina (ST-9), closely related to each other (eBURST group 11).

Relationship between sequence type, biotype, and toxin status. There was some linkage between ST, biotype, and toxin status. However, of the 73 STs containing more than one isolate, 21% were associated with multiple biotypes and 32% with variable toxin status. There was no clear association between disease status and ST, and carriage isolates could not be differentiated from disease-causing strains.

Importantly, while some of the clusters identified by MLST contained isolates of only one biotype, isolates with either the gravis, intermedius, or mitis biotype were not found within

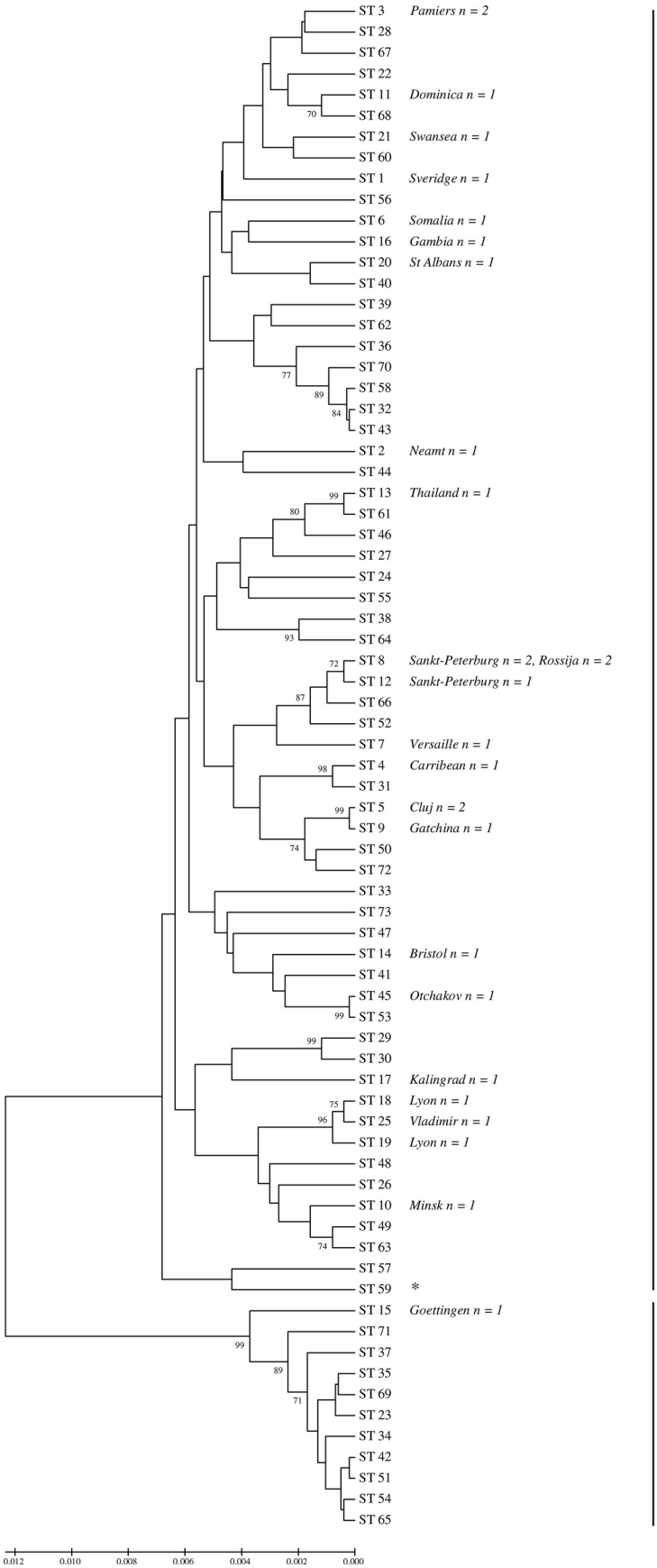

FIG. 1. Genetic relationships between the 73 sequence types identified and their relationship to ribotyping results. Where available, ribotyping data and the number of each ribotyped isolate are shown in italics to the right of the ST to which the ribotype corresponded. ST-59 (identified by an asterisk) refers to equine isolates 130 and 132. Lineage I and lineage II are highlighted by the line to the right of the phylogenetic tree.

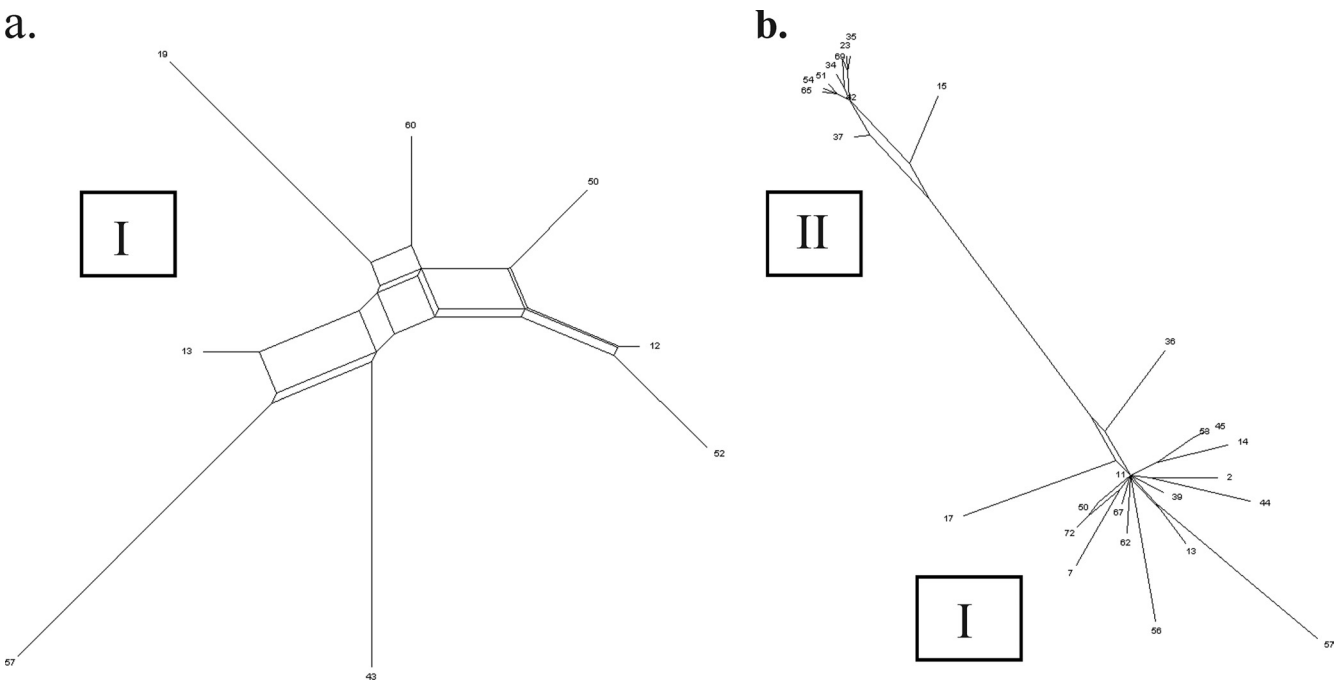

FIG. 2. Splits decomposition analysis illustrating the population structure within *C. diphtheriae*. (a) Splits within 8 STs from lineage I. (b) Relationship between representative STs from lineages I and II.

genetically distinct subgroups. However, as illustrated by Fig. 1 and 2, two distinct lineages (I and II) were identified within our *C. diphtheriae* collection. Lineage I contained the largest proportion of isolates and exclusively all of the isolates biotyped as mitis, gravis, and intermedius. Five "belfanti" isolates were also found within lineage I; however, these "belfanti" isolates were all of ST-26, from James Bay, Canada, and all bar one produced diphtheria toxin (DT), which is atypical for the biotype (20). By comparison, lineage II was composed exclusively of all 11 remaining biotype belfanti isolates, representing 11 distinct STs. These lineage II belfanti isolates are of typical phenotype, being nontoxigenic. Furthermore, based upon concatenated MLST nucleotide data, lineage I strains were, on average, 2.5% divergent from those in lineage II, each forming distinct subgroups when represented by neighbor-joining trees (Fig. 1).

Recombination. The balance between recombination and mutation has a significant impact upon the population biology of bacteria and their ability to evolve under strong selective pressures. As previously discussed, many SLVs within a clonal complex are likely to have arisen by recombination since multiple substitutions were observed and the allele was identified in other strains within the data set. The MLST data were further analyzed to determine the potential for genetic exchange within isolates of *C. diphtheriae*.

The degree of recombination within a bacterial population can be determined using the index of association (I_A), which measures the level of linkage between alleles at different loci. An I_A not significantly greater than zero after 1,000 computer randomizations suggests the organism is in linkage equilibrium and is therefore freely recombining, while a population with an I_A significantly greater than zero is considered to be clonal. Overall, the I_A value was 0.1176 ($P = <0.01$). To minimize any distortions created when analyzing two relatively distinct populations, the I_A value was also individually calculated for both lineages I (0.0667; $P = <0.01$) and II (0.0395; $P = 0.14$).

To further examine the impact of recombination upon the *C. diphtheriae* collection, the congruence observed between gene tree topologies was determined using the method described by Holmes (14). In clonal populations, the genetic relationships at all loci will be the same, while for recombinogenic species or subpopulations, the phylogenetic trees will appear incongruent. As shown by Table 4, pairwise comparisons between the gene tree topologies revealed significant discordance for all loci. However, only the association between the *atpA* ML tree and the *dnaK*, *odhA*, and *rpoB* trees and that between the *dnaK* tree and *atpA* and *odhA* were deemed to have no more similarity than that with trees of random topology. It is therefore apparent that while recombination plays a role in the evolution of *C. diphtheriae*, it has not obscured all phylogenetic signals.

DISCUSSION

Diphtheria is still endemic in many countries and, as exemplified by the FSU outbreak, is a potentially resurgent disease. Furthermore, given the low levels of protection within adult populations (in particular seniors), accurate and reproducible typing methods are required to monitor and characterize *C. diphtheriae*. Although numerous typing techniques for *C. diphtheriae* have been described, their use is often hindered by limited reproducibility and subjective analysis. MLST is able to circumvent these limitations by directly analyzing nucleotide information within selectively neutral housekeeping genes. The data produced are objective and, due to

TABLE 4. Congruence tests for each gene tree compared to other gene tree topologies and random tree data

Locus	−ln *L* of ML tree	Δ −ln *L* of competing ML trees	*P* value	Δ −ln *L* of random trees	99th percentile Δ −ln *L* in random trees	Loci outside 99th percentile of random trees
atpA	769.22826	213.8152–248.929	<0.003	232.16–283.69	236.16	*dnaE*, *fusA*, *leuA*
dnaE	738.1112	228.804–329.4	<0.002	388.24–523.46	422.61	*atpA*, *dnaK*, *fusA*, *leuA*, *odhA*, *rpoB*
dnaK	936.21459	530.28–680.82	0.00	627.12–798.26	677.52	*dnaE*, *fusA*, *leuA*, *rpoB*
fusA	814.1686	259.33–382.6	0.00	470.99–582.30	473.91	*atpA*, *dnaE*, *dnaK*, *leuA*, *odhA*, *rpoB*
leuA	840.8003	254.82–310.98	<0.002	352.30–482.90	373.51	*atpA*, *dnaE*, *dnaK*, *fusA*, *odhA*, *rpoB*
odhA	803.9006	183.05–226.76	<0.003	254.17–466.62	267.98	*atpA*, *dnaE*, *dnaK*, *fusA*, *leuA*, *rpoB*
rpoB	656.8732	224.42–321.82	<0.003	340.90–405.42	345.88	*atpA*, *dnaE*, *dnaK*, *fusA*, *leuA*, *odhA*

their portability, amenable to international collaborations. The *C. diphtheriae* MLST database can be accessed at http://pubmlst.org/cdiphtheriae.

This is the first use of MLST to characterize isolates of *C. diphtheriae*. MLST effectively typed 150 diverse *C. diphtheriae* isolates and confirmed findings of previous studies indicating that there is significant intraspecies genetic diversity. The data presented demonstrate that recombination has played a role in the evolution of *C. diphtheriae*. This was made evident by splits decomposition analysis and in the significant discordance observed between all MLST gene trees. However, since the congruence was deemed to show no greater similarity than that to trees of random topology within only two MLST loci, genetic exchange does not obscure all phylogenetic signals. Analysis of the complete genome sequence of *C. diphtheriae* reveals recent acquisition of pathogenicity factors. The observed recombination in this study highlights an obvious opportunity for these and other determinants to move across the population of *C. diphtheriae*.

To validate the accuracy and discriminatory power of the MLST scheme, the data were compared to an available subset of strains typed by the current gold standard, ribotyping. The MLST data were generally in concordance with the ribotyping findings. However, MLST provided greater strain resolution in two instances and ribotyping in one. MLST identified an SLV within three Sankt-Peterburg ribotype isolates and was able to distinguish between two ribotype Lyon isolates, which De Zoysa et al. had previously distinguished (5). As with AFLP, PFGE, and RAPD studies (3, 4, 5), MLST was unable to differentiate between the two predominant ribotypes associated with the FSU outbreak: Sankt-Peterburg and Rossija. Spoligotyping studies of this epidemic clonal group showed a clear divergence between these two ribotypes and suggested that the Rossija ribotype may have originated from one particular subpopulation of ribotype Sankt-Peterburg (21, 22). Likewise, it is clear by MLST that the Vladimir and Lyon ribotypes are clonally derived (eBURST group 5), as are the Cluj and Gatchina ribotypes (eBURST group 11).

MLST also identified a clonal complex (eBURST group 9) associated with an epidemiologically linked diphtheria outbreak in Haiti and the Dominican Republic, which both share the island of Hispaniola. A suspected diphtheria outbreak is believed to have originated in the Fond des Blancs region of Haiti in 2004 (CDC, personal communication). From 2001 to 2003, 13 diphtheria cases in Haiti and 120 in the Dominican Republic were reported (www.who.int/immunization_monitoring/data/en/). This increased significantly, to 253 cases in Haiti and 177 in the Dominican Republic, from 2004 to 2006 (see above URL). Of 28 isolates obtained during the outbreak, 93% belonged to a clonal complex comprising ST-31 and ST-4 isolates (eBURST group 9). Since two further isolates, collected in 2000 and 1995, were ST-31 and ST-4, respectively, it is evident that the outbreak strains were circulating in the preepidemic period and may belong to the regions of endemicity for a *C. diphtheriae* reservoir.

While members of some clonal complexes were globally distributed, other groups were associated with specific locations. Notably, members of eBURST group 8 were obtained from geographically disparate countries (Thailand, Russia, and Guatemala), whereas exclusively Russian isolates belonged to eBURST group 10. However, these findings may be the result of sampling limitations and require a wider sample analysis.

It was not possible to identify a definitive association between ST and toxigenicity for biotypes mitis, intermedius, and gravis. Likewise, there was not always a clear association between biotype and ST, indicating that biotypes are not necessarily stable epidemiological markers, which is wholly consistent with *C. diphtheriae* being identified in this work as having only a weakly clonal structure. However, isolates identified by biotype and nontoxigenic status as typical belfanti strains clustered together within lineage II.

MLST provides a valuable tool for monitoring and characterizing endemic and epidemic *C. diphtheriae* strains. The data produced are portable, reproducible, and unambiguous. Strain discrimination was in accordance with ribotyping data, and clonal complexes associated with disease outbreaks were identified.

ACKNOWLEDGMENTS

We thank Keith Jolley for his assistance with the gene tree congruence data and for setting up the MLST database.

We thank the BBSRC, Micropathology LTD, and Society for General Microbiology for financial support at Warwick University. We also thank the Centers for Disease Control and the Institut Pasteur Foundation, which currently curates the *C. diphtheriae* MLST database (http://pubmlst.org/cdiphtheriae/).

REFERENCES

1. **Cerdeño-Tárraga, A. M., A. Efstratiou, L. G. Dover, M. T. G. Holden, M. Pallen, S. D. Bentley, G. S. Besra, C. Churcher, K. D. James, A. De Zoysa, T. Chillingworth, A. Cronin, L. Dowd, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, S. Moule, M. A. Quail, E. Rabbinowitsch, K. M. Rutherford, N. R. Thomson, L. Unwin, S. Whitehead, B. G. Barrell, and J. Parkhill.** 2003. The complete genome sequence and analysis of *Corynebacterium diphtheriae* NCTC13129. Nucleic Acids Res. **31:**6516–6523.
2. **Corboz, L., R. Thoma, U. Braun, and R. Zbinden.** 1996. Isolation of *Corynebacterium diphtheriae subsp. belfanti* from a cow with chronic active dermatitis. Schweiz. Arch. Tierheilkd. **138:**596–599.
3. **De Zoysa, A. S., and A. Efstratiou.** 1999. PCR typing of *Corynebacterium diphtheriae* by random amplification of polymorphic DNA. J. Med. Microbiol. **48:**335–340.

4. **De Zoysa, A. S., and A. Efstratiou.** 2000. Use of amplified fragment length polymorphisms for typing *Corynebacterium diphtheriae*. J. Clin. Microbiol. **38:**3843–3845.
5. **De Zoysa, A., A. Efstratiou, R. C. George, M. Jahkola, J. Vuopio-Varkila, S. Deshevoi, G. Tseneva, and Y. Rikushin.** 1995. Molecular epidemiology of *Corynebacterium diphtheriae* from northwestern Russia and surrounding countries studied by using ribotyping and pulsed-field gel electrophoresis. J. Clin. Microbiol. **33:**1080–1083.
6. **De Zoysa, A., P. Hawkey, A. Charlett, and A. Efstratiou.** 2008. Comparison of four molecular typing methods for characterization of *Corynebacterium diphtheriae* and determination of transcontinental spread of *C. diphtheriae* based on BstEII rRNA gene profiles. J. Clin. Microbiol. **46:**3626–3635.
7. **Edmunds, W. J., R. G. Pebody, H. Aggerback, S. Baron, G. Berbers, M. A. Conyn-van Spaendonck, H. O. Hallander, R. Olander, P. A. Maple, H. E. Melker, P. Olin, F. Fievret-Groyne, C. Rota, S. Salmaso, A. Tischer, C. von-Hunolstein, and E. Miller.** 2000. The sero-epidemiology of diphtheria in Western Europe. ESEN Project. European Sero-Epidemiology Network. Epidemiol. Infect. **125:**113–125.
8. **Efstratiou, A., K. H. Engler, I. K. Mazurova, T. Glushkevich, J. Vuopio-Varkila, and T. Popovic.** 2000. Current approaches to the laboratory diagnosis of diphtheria. J. Infect. Dis. **181:**S138–S145.
9. **Engler, K. H., T. Glushkevich, I. K. Mazurova, R. C. George, and A. Efstratiou.** 1997. A modified Elek test for detection of toxigenic corynebacteria in the diagnostic laboratory. J. Clin. Microbiol. **35:**495–498.
10. **Gidding, H. F., J. L. Backhouse, M. A. Burgess, and G. L. Gilbert.** 2005. Immunity to diphtheria and tetanus in Australia: a national serosurvey. Med. J. Aust. **183:**301–304.
11. **Grimont, P. A. D., F. Grimont, A. Efstratiou, A. De Zoysa, I. Mazurova, C. Ruckly, M. Lejay-Collin, S. Martin-Delautre, B. Regnault and members of the European Laboratory Working Group on Diphtheria.** 2004. International nomenclature for *Corynebacterium diphtheriae* ribotypes. Res. Microbiol. **155:**162–166.
12. **Hall, A. J., P. K. Cassiday, K. A. Bernard, F. Bolt, A. G. Steigerwalt, D. Bixler, L. C. Pawloski, A. M. Whitney, M. Iwaki, A. Baldwin, C. G. Dowson, T. Komiya, M. Takahashi, H. P. Hinrikson, and M. L. Tondella.** 2010. Novel *Corynebacterium diphtheriae* in domestic cats. Emerg. Infect. Dis. **16:**688–691.
13. **Henricson, B., M. Segarra, J. Garvin, J. Burns, S. Jenkins, C. Kim, T. Popovic, A. Golaz, and B. Akey.** 2000. Toxigenic *Corynebacterium diphtheriae* associated with an equine wound infection. J. Vet. Diagn. Invest. **12:**253–257.
14. **Holmes, E. C., R. Urwin, and M. C. Maiden.** 1999. The influence of recombination on the population structure and evolution of the human pathogen *Neisseria meningitidis*. Mol. Biol. Evol. **16:**741–749.
15. **Kalinowski, J., B. Bathe, D. Bartels, N. Bischoff, M. Bott, A. Burkovski, N. Dusch, L. Eggeling, B. J. Eikmanns, L. Gaigalat, A. Goesmann, M. Hartmann, K. Huthmacher, R. Krämer, B. Linke, A. C. McHardy, F. Meyer, B. Möckel, W. Pfefferle, A. Pühler, D. A. Rey, C. Rückert, O. Rupp, H. Sahm, V. F. Wendisch, I. Wiegräbe, and A. Tauch.** 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J. Biotechnol. **104:**5–25.
16. **Khamis, A., D. Raoult, and B. La Scola.** 2004. *rpoB* gene sequencing for identification of *Corynebacterium* species. J. Clin. Microbiol. **42:**3925–3931.
17. **Launay, O., C. Toneatti, C. Bernède, E. Njamkepo, K. Petitprez, A. Leblond, S. Larnaudie, C. Goujon, M. N. Ungeheuer, F. Ajana, C. Raccurt, J. Beytout, C. Chidiac, D. Bouhour, D. Guillemot, and N. Guiso.** 2009. Antibodies to tetanus, diphtheria and pertussis among healthy adults vaccinated according to the French vaccination recommendations. Hum. Vaccin. **5:**341–346.
18. **Leggett, B. A., A. De Zoysa, Y. E. Abbott, N. Leonard, B. Markey, and A. Efstratiou.** 2010. Toxigenic *Corynebacterium diphtheriae* isolated from a wound in a horse. Vet. Rec. **166:**656–657.
19. **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. U. S. A. **95:**3140–3145.
20. **Marston, C. K., F. Jamieson, F. Cahoon, G. Lesiak, A. Golaz, M. Reeves, and T. Popovic.** 2001. Persistence of a distinct *Corynebacterium diphtheriae* clonal group within two communities in the United States and Canada where diphtheria is endemic. J. Clin. Microbiol. **39:**1586–1590.
21. **Mokrousov, I., O. Narvskaya, E. Limeschenko, and A. Vyazovaya.** 2005. Efficient discrimination within a *Corynebacterium diphtheriae* epidemic clonal group by a novel macroarray-based method. J. Clin. Microbiol. **43:**1662–1668.
22. **Mokrousov, I., A. Vyazovaya, V. Kolodkina, E. Limeschenko, L. Titov, and O. Narvskaya.** 2009. Novel macroarray-based method of *Corynebacterium diphtheriae* genotyping: evaluation in a field study in Belarus. Eur. J. Clin. Microbiol. Infect. Dis. **28:**701–703.
23. **Mothershed, E. A., P. K. Cassiday, K. Pierson, L. W. Mayer, and T. Popovic.** 2002. Development of a real-time PCR assay for rapid detection of the diphtheria toxin gene. J. Clin. Microbiol. **40:**4713–4719.
24. **Nakao, H., and T. Popovic.** 1998. Use of random amplified polymorphic DNA for rapid molecular subtyping of *Corynebacterium diphtheriae*. Diagn. Microbiol. Infect. Dis. **30:**167–172.
25. **Neal, S., and A. Efstratiou.** 2009. Diphtheria Surveillance Network: focus on microbiological diagnostics and molecular typing in Europe, abstr. S-11.2. Abstr. Eur. Sci. Conf. Appl. Infect. Dis. Epidemiol.
26. **Nishio, Y., Y. Nakamura, Y. Kawarabayasi, Y. Usuda, E. Kimura, S. Sugimoto, K. Matsui, A. Yamagishi, H. Kikuchi, K. Ikeo, and T. Gojobori.** 2003. Comparative complete genome sequence analysis of the amino acid replacements responsible for the thermostability of *Corynebacterium efficiens*. Genome Res. **13:**1572–1579.
27. **Parish, H. J., and C. C. Okell.** 1926. A note on the isolation of virulent diphtheria bacilli from wounds of horses. J. Exp. Pathol. **7:**173–174.
28. **Popovic, T., S. Y. Kombarova, M. W. Reeves, H. Nakao, I. K. Mazurova, M. Wharton, I. K. Wachsmuth, and J. D. Wenger.** 1996. Molecular epidemiology of diphtheria in Russia, 1985–1994. J. Infect. Dis. **174:**1064–1072.
29. **Popovic, T., I. K. Mazurova, A. Efstratiou, J. Vuopio-Varkila, M. W. Reeves, A. De Zoysa, T. Glushkevich, and P. Grimont.** 2000. Molecular epidemiology of diphtheria. J. Infect. Dis. **181:**S168–S177.
30. **Rozen, S., and H. J. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers, p. 365–386. *In* A. Krawetz and S. Misener (ed.), Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, NJ.
31. **Skogen, V., V. V. Cherkasova, N. Maksimova, C. K. Marston, H. Sjursen, M. V. Reeves, O. Olsvik, and T. Popovic.** 2002. Molecular characterization of *Corynebacterium diphtheriae* isolates, Russia, 1957–1987. Emerg. Infect. Dis. **8:**516–518.
32. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.
33. **Vitek, C. R., and M. Wharton.** 1998. Diphtheria in the Former Soviet Union: reemergence of a pandemic disease. Emerg. Infect. Dis. **4:**539–550.
34. **Zasada, A. A., M. Zaleska, R. B. Podlasin, and I. Seferyńska.** 2005. The first case of septicemia due to nontoxigenic *Corynebacterium diphtheriae* in Poland: case report. Ann. Clin. Microbiol. Antimicrob. **4:**8.