表3　平均同居世帯人員数の比率（インターネット調査／郵送調査）

年齢階級	犬飼育なし		犬飼育あり	
	男性	女性	男性	女性
20-29	0.80	0.72	1.01	1.03
30-39	0.80	0.77	0.60	0.85
40-49	0.79	0.72	0.91	0.74
50-59	0.87	0.97	1.01	1.16
60-69	0.66	0.81	0.75	0.78

表4　ロジスティック回帰分析結果

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.6508	0.3273	1.99	0.0469
sex	-0.0836	0.1064	-0.79	0.4324
Q4_1_1	-0.8344	0.4370	-1.91	0.0563
Q4_1_2	0.1289	0.2640	0.49	0.6254
Q4_1_3	0.3385	0.4066	0.83	0.4052
Q4_1_4	-0.2758	0.2580	-1.07	0.2852
Q4_1_5	-0.2031	0.7240	-0.28	0.7791
Q4_1_6	-0.2770	0.3763	-0.74	0.4617
Q4_1_7	0.1735	1.3192	0.13	0.8954
Q4_1_8	0.1597	0.3160	0.51	0.6134
Q4_1_9	0.7416	0.3884	1.91	0.0564
Q4_1_10	1.1082	0.5516	2.01	0.0447
Q4_1_11	-2.2220	0.9564	-2.32	0.0202
Q4_1_12	-0.3066	0.5403	-0.57	0.5705
Q4_1_13	0.6930	1.2166	0.57	0.5690
Q4_1_14	0.1982	0.3738	0.53	0.5960
Q4_1_15	0.2825	0.2257	1.25	0.2108
Q4_2_1	-0.2997	0.2016	-1.49	0.1373
Q4_2_2	-0.0798	0.2055	-0.39	0.6977
Q4_2_3	0.2887	0.2141	1.35	0.1777
Q4_2_4	-0.1604	0.1694	-0.95	0.3440
Q4_2_5	0.1637	0.5069	0.32	0.7468
Q4_2_6	0.2296	0.2753	0.83	0.4043
Q4_2_7	0.6026	0.6730	0.90	0.3707
Q4_2_8	-0.3801	0.2433	-1.56	0.1185
Q4_2_9	-0.6597	0.2887	-2.29	0.0224
Q4_2_10	-0.7031	0.4053	-1.73	0.0829
Q4_2_11	0.9139	0.5898	1.55	0.1214
Q4_2_12	0.0242	0.3782	0.06	0.9490
Q4_2_13	0.0689	0.6356	0.11	0.9136
Q4_2_14	-0.0143	0.2194	-0.07	0.9479
Q4_2_15	0.3294	0.1822	1.81	0.0707
Q4_3_1	-0.4098	0.1751	-2.34	0.0193
Q4_3_2	0.1975	0.1845	1.07	0.2846
Q4_3_3	-0.3577	0.2929	-1.22	0.2221
Q4_3_4	0.6125	0.3027	2.02	0.0431
Q4_3_5	-1.4291	0.6731	-2.12	0.0338
Q4_3_6	-0.3137	0.2727	-1.15	0.2501
Q4_3_8	-0.0336	0.3303	-0.10	0.9190
Q4_3_9	0.6062	0.4798	1.26	0.2066
Q4_3_10	-0.2038	0.6166	-0.33	0.7410
Q4_3_11	0.7201	0.6194	1.16	0.2451
Q4_3_12	0.5476	0.3063	1.79	0.0740
Q4_3_13	1.0519	0.8309	1.27	0.2056
Q4_3_14	0.3207	0.3276	0.98	0.3276
Q4_3_15	-0.8449	0.1911	-4.42	0.0000
Q7_1	1.0542	0.3813	2.76	0.0057
Q7_2	0.1877	0.2946	0.64	0.5240
Q7_3	0.3349	0.3335	1.00	0.3154
Q7_4	0.0333	0.2561	0.13	0.8966
Q7_5	0.6598	0.3682	1.79	0.0732
Q7_6	-0.1131	0.3681	-0.31	0.7587
Q7_7	-0.4676	0.5873	-0.80	0.4260
Q9	0.0152	0.0906	0.17	0.8670
age2	0.0277	0.1685	0.16	0.8694
age3	-0.0505	0.1653	-0.31	0.7599
age4	0.0316	0.1674	0.19	0.8505
age5	0.0687	0.1627	0.42	0.6730
area2	-0.3653	0.3067	-1.19	0.2338
area3	-0.8292	0.3249	-2.55	0.0108
area4	-0.6315	0.3150	-2.00	0.0451
area5	-0.3812	0.2426	-1.57	0.1162
area6	0.0442	0.4256	0.10	0.9173
area7	-0.5202	0.2658	-1.96	0.0504
area8	-0.3891	0.2569	-1.51	0.1300
area9	-0.1190	0.3098	-0.38	0.7009
area10	0.0558	0.3863	0.14	0.8853
area11	-0.1080	0.2753	-0.39	0.6948

表5　犬を飼育している世帯数割合 (%)

	JFPA	SPBI	SPBP
調整なし (人口ウエイトのみ)	17.8	17.5	23.9
世帯人員ウエイト	14.5	14.5	21.2
傾向スコアウエイト	18.6	18.5	·

表6　1世帯あたり平均飼育頭数 (犬を飼育している世帯)

	JFPA	SPBI	SPBP
調整なし (人口ウエイトのみ)	1.26	1.20	1.32
世帯人員ウエイト	1.25	1.20	1.32
傾向スコアウエイト	1.21	1.17	·

表7　総飼育犬頭数推計値 (千頭)

	JFPA	SPBI	SPBP
調整なし (人口ウエイトのみ)	10067	9384	14130
世帯人員ウエイト	8171	7810	12511
傾向スコアウエイト	10044	9705	

図 1　飼育犬頭数分布 (SPBI 世帯人員ウエイト, SPBP 世帯人員ウエイト, SPBI 傾向スコアウエイト)

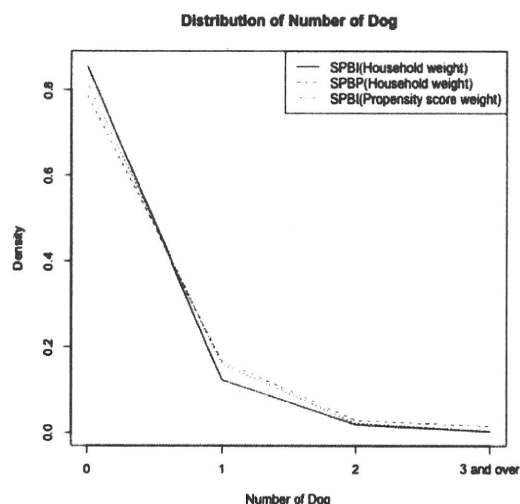
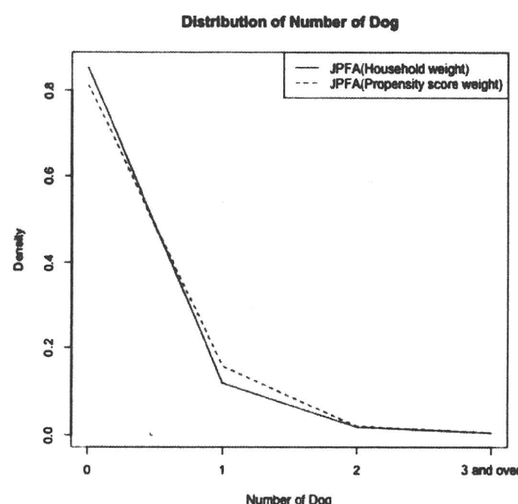


図 2　飼育犬頭数分布 (JFPA 世帯人員ウエイト, JFPA 傾向スコアウエイト)



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Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
高橋元秀、小宮貴子	ジフテリア	木村哲、喜田宏	改訂版　人獣共通感染症	医薬ジャーナル社	日本	2011	230-235

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Komiya T, Seto Y, et al.	Two Japanese *Corynebacterium ulcerans* isolates from the same hospital: ribotype, toxigenicity and serum antitoxin titre.	J Med Microbiol.	59(Pt 12)	1497-504.	2010
Iwaki M, Komiya T, Yamamoto A, et al.	Genome organization and pathogenicity of *Corynebacterium diphtheriae* C7(-) and PW8 strains.	Infect Immun.	78(9)	3791-800.	2010
Hall AJ, Iwaki M,Komiya T, et al.	Novel *Corynebacterium diphtheriae* in domestic cats.	Emerg Infect Dis.	16(4)	688-91.	2010
高橋元秀	ジフテリア毒素原性 *Corynebacterium ulcerans*の感染症	日本獣医師会雑誌	63	p813-818	2010
畑中章生、山本明彦、小宮貴子、他	茨城県で初めて確認されたコリネバクテリウム・ウルセランスによるジフテリア症例	国立感染症研究所　病原微生物検出情報	Vol.32	19-20	2011

吉村幸浩、山本明彦、小宮貴子	飼い猫の排膿に伴って、経皮的に腋窩リンパ節に膿瘍を生じたことが強く疑われる *C.ulcerans*感染症の例	国立感染症研究所　病原微生物検出情報	Vol.31	331	2010
高橋元秀	イヌ・ネコにおけるジフテリア毒素産生 ***Corynebacterium ulcerans*** の保菌調査状況	国立感染症研究所　病原微生物検出情報	Vol. 31	203-204	2010
若松正人、小河正雄、小宮貴子　他	大分県におけるイヌ・ネコの*C.* ***ulcerans*** 保菌状況	国立感染症研究所　病原微生物検出情報	Vol. 31	204-205	2010
島谷竜哉、浅野由紀子、小宮貴子他	愛媛県におけるイヌ・ネコの*C.* ***ulcerans*** 保菌状況	国立感染症研究所　病原微生物検出情報	Vol. 31	205-206	2010
中嶋　洋、大畠律子、小宮貴子他	岡山県におけるイヌ・ネコの*C.* ***ulcerans*** 保菌状況	国立感染症研究所　病原微生物検出情報	Vol. 31	206-207	2010
Nomura,A., Imaoka,K., Imanishi,H., Shimizu,H., Nagura,F., Maeda,K., Tomino,T..	Human *Brucella canis* infection diagnosed by blood culture.	Emerging Infectious Diseases	16(7)	1183-1185	2009
今岡浩一，木村昌伸.	ラットとストレプトバチルス感染症.	アニテックス	22(3)	47-49	2010
今岡浩一	犬を飼うときに気をつけたい感染症.	チャイルドヘルス	13(8)	567-570	2010

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Two Japanese *Corynebacterium ulcerans* isolates from the same hospital: ribotype, toxigenicity and serum antitoxin titre

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Two toxigenic *Corynebacterium ulcerans* isolates recovered from pharyngeal swabs of two patients from the same hospital in Japan during 2001–2002 were characterized by PFGE and ribotyping. Toxin production in different culture media was examined and serological analysis of patient sera was performed. The two isolates could not be distinguished by PFGE; however, their ribotypes were distinguishable. One of the isolates could represent a novel ribotype. Analysis of toxin production in different culture media demonstrated that the two isolates produced varying amounts of the diphtheria toxin. Serological analysis showed a greater than sevenfold increase in the serum antitoxin titre during the course of infection in one patient.

INTRODUCTION

Disease caused by *Corynebacterium ulcerans* is now an emerging threat to human health (CDC, 1997; DeWinter *et al.*, 2005; Kisely *et al.*, 1994; Lartigue *et al.*, 2005; PHLS, 2000; Sing *et al.*, 2005; Von Hunolstein *et al.*, 2003), and has recently been classified as diphtheria by the European Centre for Disease Prevention and Control (http://ecdc.europa.eu/en/healthtopics/pages/diphtheria.aspx). In Japan, the Ministry of Health, Labour and Welfare released notifications concerning this disease twice in 2002 and 2009, although the disease is not classified as diphtheria in this country.

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Abbreviations: CD_{50}, 50 % cytopathic dose; CDC, Centers for Disease Control and Prevention; MRD, minimal reactive dose; PHLS, Public Health Laboratory Service; PLD, phospholipase D; SCIEH, Scottish Centre for Infection and Environmental Health.

A table of primer details and figures of PCR identification and sequence alignment data are available as supplementary material with the online version of this paper.

C. ulcerans is known as a major cause of mastitis in cows. A significant portion of human *C. ulcerans* infections in the UK has been associated with ingestion of raw dairy products (Galbraith *et al.*, 1982; Hart, 1984; Kisely *et al.*, 1994). This organism has also been recently recognized as a human pathogen associated with companion animals such as dogs and cats (De Zoysa *et al.*, 2005; Dias *et al.*, 2010; Lartigue *et al.*, 2005; SCIEH, 2002; Taylor & Efstratiou, 2002) and pigs (Schuhegger *et al.*, 2009).

The first case of human infection with toxigenic *C. ulcerans* was described in 1970 (Fakes & Downham, 1970) in the UK. In early 2001, 30 years after the first report, the organism was identified as a human pathogen in Japan in a patient who had been feeding 20 cats in and around her home (Hatanaka *et al.*, 2003). The second case in Japan occurred around late 2002, and the two cases occurred in locations about 5 km apart. Both cases were reported from the same hospital. The first case has already been briefly reported (Hatanaka *et al.*, 2003), but there have been no reports of the second case. No detailed characterization of the isolates or analysis of patient sera has been reported for either of the cases.

Diphtheria caused by *Corynebacterium diphtheriae* results in a detectable rise in the serum antitoxin titre against diphtheria toxin during the course of infection (Danilova

et al., 2006). We were interested to know whether toxigenic *C. ulcerans* would be capable of inducing a similar reaction. In this study, we present the detailed molecular characteristics of the *C. ulcerans* isolates from the two Japanese cases, and show that a marked increase in serum antitoxin titre occurred during the course of infection in one patient.

METHODS

Bacterial strains, culture media and standard materials. Bacterial strains used in this study are listed in Table 1. Identification of the strains was performed using an API Coryne kit (bioMérieux). The bacterial strains were cultivated on Loeffler medium (Kyokuto Pharmaceutical Industrial), sheep blood agar plates (Nissui Pharmaceutical), in iron-depleted Pope liquid medium (Tasman & Van Ramhorst, 1951; Tchorbanov *et al.*, 2004) or brain heart infusion broth (Difco; Becton Dickinson). Japanese national reference diphtheria test toxin lot M59 was used as a control toxin. Japanese national standard diphtheria antitoxin lot 10, which was calibrated against the World Health Organization international standard antitoxin, was used as a reference material for antitoxin unit definition.

Gram staining and Neisser staining. Gram staining of the bacterial strains was performed using a Gram staining kit (Nissui Pharmaceutical). Neisser staining was performed according to the method originally described by Neisser (Hendrickson & Krenz, 1991).

PFGE typing. PFGE typing of the bacterial strains was performed essentially according to De Zoysa *et al.* (1995). The bacterial strains to be analysed were briefly cultured on sheep blood agar plates overnight at 37 °C. The bacterial cells were then collected, embedded in agarose plugs and lysed overnight at 37 °C using freshly prepared lysis solution (Murray *et al.*, 1990) containing 1 mg lysozyme ml^{-1}. The cells were then treated with proteolysis buffer (Murray *et al.*, 1990) for 48 h at 37 °C. DNA from the cells embedded in the plugs was subsequently digested with *Sfi*I (New England Biolabs) overnight at 37 °C and the digested samples were applied to an analytical agarose gel. PFGE was performed in a CHEF DRII apparatus (Bio-Rad) at 11 °C with a pulse time of 5 to 20 s for the first 20 h, and then 1 to 5 s for the following 18 h.

Ribotyping. Ribotyping of bacterial strains was performed as described by De Zoysa *et al.* (1995) and Regnault *et al.* (1997). Isolated genomic DNA was digested with *Bst*EII (Roche Diagnostics), electrophoresed in an agarose gel and transferred to HyBond Plus nylon membrane (Amersham Biosciences). The transferred DNA was hybridized with a DIG-labelled OligoMix 5 probe mixture (Grimont *et al.*, 2004; Regnault *et al.*, 1997), and signals were detected with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics). Ribotype profiles were analysed using the BioNumerics software program (version 3.0; Applied Maths).

PCR and nucleotide sequence determination. Genomic DNA was isolated from the bacterial strains using a blood mini kit (Qiagen) according to the kit instructions. PCR detection of the diphtheria toxin (*tox*) gene was performed by amplifying a 248 bp fragment located in the middle of the catalytic domain (A subunit) of the gene using oligonucleotide primers Tox 1 and Tox 2 (Supplementary Table S1 available with the online journal) (Mikhailovich *et al.*, 1995). The phospholipase D (PLD)-encoding gene was detected using primers CorynePLD-F and CorynePLD-R1 (Supplementary Table S1 available with the online journal).

The nucleotide sequence of the *tox* gene was determined by amplifying five fragments covering the entire ORF (spanning a region upstream as well as downstream of the *tox* gene) using the primer pairs of DT-1 and Tox 1, Tox 2 and DT-2, DT-3 and Dipht 6R, Dipht 6F and DT-4, and DT-5 and DT-6 (Supplementary Table S1 available with the online journal) (Mikhailovich *et al.*, 1995; Nakao *et al.*, 1996). Nucleotide sequences of the amplified fragments were determined by cycle sequencing using a BigDye terminator kit (Applied Biosystems) and analysed using an Applied Biosystems model 310 genetic analyser.

Toxigenicity assays. Immunological detection of diphtheria toxin was performed using a further modification of the modified Elek method (Reinhardt *et al.*, 1998). A 9 mm well was made aseptically on an agar plate containing 20 g proteose peptone l^{-1} (Becton Dickinson), 1 g Bacto yeast extract l^{-1} (Becton Dickinson), 2.5 g NaCl l^{-1}, 20 % (v/v) newborn calf serum (Gibco; Life Technologies) and 15 g Bacto agar l^{-1} (Becton Dickinson). The bacterial strains to be tested were then inoculated at a distance of 10 mm from the edge of the well, which was then filled with an aqueous solution containing 4.5 units of standard diphtheria antitoxin. The precipitin lines formed were observed after 1 to 2 days of cultivation at 37 °C.

For Vero cell and rabbit skin toxigenicity tests (Miyamura *et al.*, 1974a, b), the liquid condensed at the base of the Loeffler slant ('Loeffler condensation') and the culture supernatant of the Pope liquid medium ('Pope culture supernatant') were sterile filtered and used as specimens. Serially diluted specimens were added to Vero cells seeded in 96-well culture plates, and the neutralization end points were determined by observing cytotoxic effects 4 days post-incubation, as described by Miyamura *et al.* (1974a, b). Standard antitoxin

Table 1. Bacterial isolates and strains used in this study

Isolate and strain no.	Patient source			Clinical symptom	Reference
	Sex	Age	Site of isolation		
Corynebacterium ulcerans 0102	F	52	Throat (pseudomembrane)	Dyspnoea, fever	Hatanaka *et al.* (2003)
Corynebacterium ulcerans 0211	M	54	Throat (pseudomembrane)	Sore throat, fever	This study
Corynebacterium ulcerans ATCC 51799	–	–	–	–	ATCC
Corynebacterium diphtheriae PW8	–	–	–	–	Laboratory stock
Corynebacterium diphtheriae ATCC 700971	–	–	–	–	ATCC (NCTC 13129)
Rhodococcus equi ATCC 6939	–	–	–	–	ATCC
Citrobacter koseri CIP 105177	–	–	–	–	CIP

ATCC, American Type Culture Collection; CIP, Collection de l'Institut Pasteur; F, female; M, male; NCTC, National Collection of Type Cultures; PW8, Park–Williams no. 8.

(40 IU ml^{-1}) was used to confirm that the toxicity was due to diphtheria toxin.

For the rabbit skin toxigenicity test, specimens were appropriately diluted in PBS containing 0.2 % gelatin, and 100 μl of this solution were intradermally injected into the shaved back of a female Japanese white rabbit weighing 3.5 kg. The diameter of local erythema formed was measured on day 2 post-injection. Standard antitoxin (1 IU ml^{-1}) was used to confirm that the toxicity was due to diphtheria toxin. Animal experiments were performed with the approval of the Animal Experiment Committee of the National Institute of Infectious Diseases. PLD activity was visualized by enhanced haemolysis on sheep blood agar plates when *C. ulcerans* isolates and *Rhodococcus equi* ATCC 6939 were streaked close to each other on the plates.

Serum antitoxin titre determination. Anti-diphtheria toxin neutralization titre was determined by the Vero cell cytotoxicity assay described above. Serially diluted patient serum or standard antitoxin was mixed with 16 CD_{50} ml^{-1} [i.e. (16 times the CD_{50}) ml^{-1}] of the reference diphtheria test toxin and added to the Vero cell cultures (Miyamura *et al.*, 1974a). The antitoxin titre of patient sera was determined by comparing the neutralization end points of the patient sera and standard antitoxin, and was expressed in IU.

RESULTS

Cases

The first case has been reported previously (Hatanaka *et al.*, 2003). Briefly, the patient was a 52-year-old woman who presented to the Asahi General Hospital in Chiba Prefecture, Japan, on 16 February 2001, with dyspnoea and a sore throat. *C. ulcerans* was isolated from a pharyngeal pseudomembrane. She recovered without any serious sequelae following erythromycin treatment. The strain 0102 was isolated from this patient.

The second case was a 54-year-old man who presented to the same hospital with a sore throat and fever on 28 October 2002. *C. ulcerans* was isolated from a yellow-white pseudomembrane observed in his pharynx and was designated 0211. He recovered without any serious sequelae following clarithromycin treatment. No contact with dairy livestock, raw dairy products or domestic animals was reported.

Identification of the isolates

Bacteriological characteristics of the two *C. ulcerans* isolates are summarized in Table 2. Both strains exhibited Gram-positive short rod morphology on Gram staining. Clearly distinguishable metachromatic granules were observed at the ends of the cells by Neisser staining (Hendrickson & Krenz, 1991). Both strains were biochemically characterized as *C. ulcerans* with a probability of 99.7 % using the API Coryne kit (code 0111326).

Genotyping of the isolates

PFGE patterns are shown in Fig. 1(a). The PFGE patterns of isolates 0102 and 0211 are indistinguishable, whereas the pattern for *C. ulcerans* ATCC 51799 is clearly different from that for the Japanese isolates. The ribotype profiles of the two isolates are shown in Fig. 1(b), and comparison by cluster analysis of these profiles with that of the *C. ulcerans* ribotypes in an in-house database (De Zoysa *et al.*, 2005) revealed that the ribotype profile of isolate 0102 showed 100 % similarity with ribotype U4, which is a predominant profile seen among human clinical *C. ulcerans* isolates in the UK. The ribotype profile of isolate 0211 did not match with any of the known *C. ulcerans* ribotypes (U1–U9), suggesting that it could potentially be a new ribotype (Fig. 1c).

Toxigenicity tests

The presence of the *tox* gene in both isolates was demonstrated by PCR using oligonucleotide primers Tox 1 and Tox 2 (Supplementary Table S1 available with the online journal) (Mikhailovich *et al.*, 1995) to detect a 248 bp amplified fragment corresponding to the middle of the catalytic domain (A subunit) of the diphtheria toxin gene. The *tox* gene was detected in both strains (Table 2).

Table 2. Bacteriological characteristics of the isolates

Test	Isolate	
	0102	0211
Gram staining	Gram positive	Gram positive
Metachromatic granules by Neisser staining	+	+
Identification by API Coryne kit	*C. ulcerans* (99.7 %; code 0111326)	*C. ulcerans* (99.7 %; code 0111326)
Toxigenicity		
tox gene PCR	+	+
Elek assay	+	ND
PLD		
pld gene PCR	+	+
R. equi-stimulated haemolysis	+	+

ND, Not detectable.

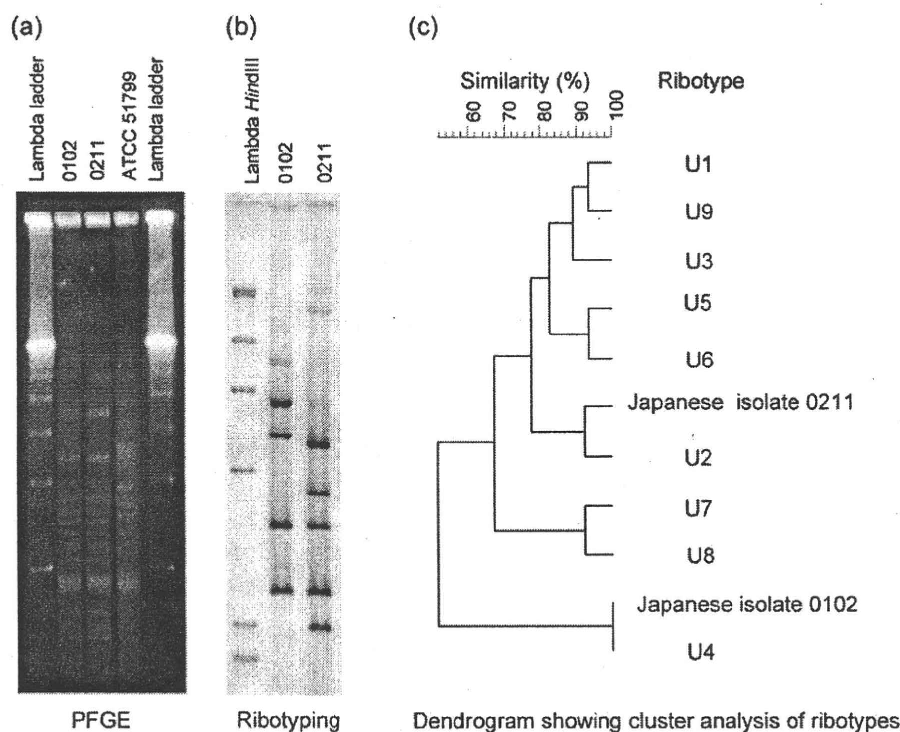

Fig. 1. PFGE and ribotype patterns of the isolates. (a) PFGE patterns. Genomic DNA of *C. ulcerans* clinical isolates 0102 and 0211 and of *C. ulcerans* ATCC 51799 was digested with *Sfi*I and subjected to PFGE as described in Methods. Lambda phage DNA ladders were used as molecular mass markers. (b) Ribotype patterns of the clinical isolates. Genomic DNA of *C. ulcerans* clinical isolates 0102 and 0211 were digested with *Bst*EII and subjected to agarose gel electrophoresis and Southern blotting as described in Methods. Lambda *Hin*dIII-digested DNA was used as a molecular mass marker. (c) Cluster analysis of ribotypes. Similarity between ribotyping band patterns of the clinical isolates were compared with those of nine known ribotypes (U1–U9), which were calculated according to the UPGMA method.

The sizes of the amplified fragments were indistinguishable from each other and from a DNA fragment amplified from genomic DNA of *C. diphtheriae* vaccine strain PW8 by agarose gel electrophoresis (data not shown).

Immunological detection of diphtheria toxin was performed using the modified Elek method (Reinhardt *et al.*, 1998). A precipitin line was observed for isolate 0102 but isolate 0211 did not exhibit a clearly observable precipitin line on the Elek plate, suggesting that the amount of toxin produced by isolate 0211 in this culture medium was less than that produced by isolate 0102.

The activity of the toxin produced by the isolates was then measured using Vero cell and rabbit skin toxigenicity tests according to Miyamura *et al.* (1974a). Loeffler condensation of isolate 0102 exhibited 513 CD_{50} ml^{-1} of cytotoxicity based on the Vero cell test, and the toxicity was completely neutralized by 40 IU ml^{-1} (1 IU per culture well) of diphtheria antitoxin (Table 3). In contrast, Loeffler condensation of isolate 0211 showed approximately twofold less cytotoxicity (208 CD_{50} ml^{-1}) but neutralization by the same concentration of antitoxin was only partial, retaining 114 CD_{50} ml^{-1} (Table 3). Because *C. diphtheriae* vaccine strain PW8 showed 2×10^4 CD_{50} ml^{-1} and this activity was completely neutralized by the same concentration of antitoxin, the partial neutralization observed for 0211 was not thought to be caused by insufficient serum antitoxin titre.

Toxin activity was also detectable by the rabbit skin toxigenicity test (Miyamura *et al.*, 1974a). Loeffler condensation of isolate 0102 contained 80 MRD ml^{-1} [i.e. (80 times the MRD) ml^{-1}] of activity. In contrast to the results from the Vero cell assay, 20 MRD ml^{-1} of this activity remained unneutralized after treatment with 1 IU standard diphtheria antitoxin ml^{-1} (Table 3). The condensation of isolate 0211 showed less activity (20 MRD ml^{-1}), which was neutralized incompletely by the antitoxin (Table 3).

C. ulcerans strains are known to produce another toxic factor PLD. The presence of the PLD-encoding gene and secretion of the active enzyme (visualized by cross-streaking with *R. equi* ATCC 6939) occurred in both isolates (Supplementary Fig. S1 available with the online journal). PLD may be an additional factor contributing to

Table 3. Toxicity of *C. ulcerans* assessed by Loeffler condensation and Pope culture supernatant

Assays were done in duplicate and repeated three times (Vero cytotoxicity) or once (rabbit erythema).

	Loeffler condensation			Pope culture supernatant		
	C. ulcerans		*C. diphtheriae*	*C. ulcerans*		*C. diphtheriae*
	0102	0211	PW8	0102	0211	PW8
Vero cytotoxicity (CD_{50} ml^{-1})						
Before neutralization	513 ± 238*	208 ± 50*	2.0×10^4†	640†	$1.4 \pm 0.006 \times 10^4$*	$3.9 \pm 0.02 \times 10^5$*
After neutralization‡	ND	114 ± 17*	ND	ND	ND	ND
Rabbit erythema assay (MRD)						
Before neutralization	80 (40, 160)§	20ǁ	2.0×10^4ǁ	640ǁ	1.6×10^4 (8.0×10^3, 3.2×10^4)§	2.0×10^4ǁ
After neutralizationǁ	20¶	10¶	ND	ND	ND	ND

ND, Not detectable.
*Mean ± SE.
†The same results were obtained in three repeats.
‡Neutralization with 40 IU diphtheria antitoxin ml^{-1}.
§Geometric mean of a duplicate assay. Lower and higher values are indicated in parentheses.
ǁThe same results were obtained in a duplicate assay.
¶Neutralization with 1 IU diphtheria antitoxin ml^{-1}.

unneutralized cytotoxicity of Vero cells and to erythema formation on rabbit skin.

Nucleotide sequences of the *tox* genes

We then determined the nucleotide sequence of five fragments covering the entire *tox* gene and its flanking regions by amplifying these with the primer pairs shown in Supplementary Table S1 (available with the online journal) (Mikhailovich *et al.*, 1995; Nakao *et al.*, 1996). The alignment of the nucleotide sequences of the *tox* genes in *C. ulcerans* isolates 0102 and 0211, and *C. diphtheriae* PW8, is shown in Supplementary Fig. S2 (available with the online journal). The sequences from isolates 0102 (GenBank/EMBL/DDBJ accession no. AB304278) and 0211 were identical to each other and to that of the German *C. ulcerans* isolate A6361 (Sing *et al.*, 2003). In addition, the nucleotide sequence of the upstream region, as determined by sequencing of DNA fragments amplified with primers DT-1 and Tox 1 (Supplementary Table S1 available with the online journal), were identical to each other.

The *tox* gene of isolates 0102 and 0211showed a difference of 81 nt (corresponding to 27 amino acids) compared with that of the strain PW8 (Supplementary Fig. S2 available with the online journal). The major difference between the *C. ulcerans* toxin and PW8-derived toxin was observed in the receptor-binding domain in the B subunit of the toxin molecule.

Dependence of toxin production on culture medium

In contrast to the identity in *tox* gene sequences, the two isolates differed in terms of the effect of culture conditions on toxin production. Toxin production by the two *C. ulcerans* isolates on the Loeffler medium and in iron-depleted Pope liquid medium, a medium frequently used for diphtheria toxin production (Tasman & Van Ramhorst, 1951; Tchorbanov *et al.*, 2004), were compared (Table 3). A large difference was observed between the two *C. ulcerans* isolates in their response to culture media (Table 3). Strain 0211 exhibited a more than 70-fold difference in Vero cell cytotoxicity when cultured in the different media (208 CD_{50} ml^{-1} for Loeffler condensation and 14 000 CD_{50} ml^{-1} for Pope culture supernatant). In contrast, strain 0102 showed a less than twofold difference in cytotoxic activity between the Loeffler medium and Pope medium (513 CD_{50} ml^{-1} for Loeffler condensation and 640 CD_{50} ml^{-1} for Pope culture supernatant), suggesting that the two strains do not share a common mechanism for regulating toxin activity. The strains also differed in the rabbit skin toxigenicity test. Strain 0211 exhibited an erythema forming activity at 20 MRD when cultured on Loeffler medium and 800-fold more activity (1.6×10^4 MRD ml^{-1}) in Pope medium; these results are comparable to that of *C. diphtheriae* vaccine strain PW8. In contrast, strain 0102 showed only an eightfold difference in its ability to induce erythema formation (80 vs 640 MRD ml^{-1} on Loeffler medium and Pope medium, respectively).

The product of the *dtxR* gene is an important factor regulating *C. diphtheriae* diphtheria toxin gene expression in response to the iron content in culture media (Boyd *et al.*, 1990). In the two *C. ulcerans* strains, the *dtxR* gene was detectable by Southern blotting (Fig. 2). In strain 0102, the nucleotide sequence of the *dtxR* gene (including its 5′- and 3′-flanking regions) amplified with the primers listed in Supplementary Table S1 (available with the online journal) was identical to reported sequences (data not

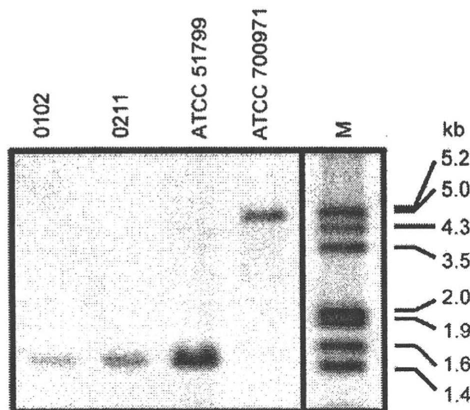

Fig. 2. Presence of the *dtxR* gene in the clinical isolates. Genomic DNA from *C. ulcerans* 0102 and 0211, ATCC 51799 and *C. diphtheriae* ATCC 700971 (equivalent to NCTC 13129) was digested with *Hin*dIII and was subjected to Southern blotting and detected using a DIG-labelled *dtxR* probe. M represents lambda *Eco*RI–*Hin*dIII digested DNA, which was used as a molecular mass marker.

shown) (Boyd *et al.*, 1990). However, for strain 0211, amplification of the region was not possible, suggesting a difference in nucleotide sequences in at least one of the primer regions flanking the *dtxR* gene, which could affect the isolate's toxin production in response to culture conditions.

Serum antitoxin titre

Respiratory infection with toxigenic *C. diphtheriae* is known to cause elevation of serum antitoxin during the course of infection (Danilova *et al.*, 2006); however, it is not yet clear if *C. ulcerans* respiratory infection can cause such an elevation. From the second case reported in our study (in which strain 0211 was isolated), patient serum was obtained periodically from the time of first presentation to the hospital on 28 October 2002 until 16 February 2005. The diphtheria antitoxin titre was measured by Vero cell neutralization assay (Miyamura *et al.*, 1974a) using Japanese national standard diphtheria antitoxin lot 10 as a reference (Table 4). The vaccination history of the patient was unclear. At first presentation to the hospital, the serum antitoxin titre was undetectable. However, the antitoxin titre started to increase 2 weeks thereafter (0.39 IU ml^{-1}) and rapidly rose to almost 2.5 IU ml^{-1} within a further 3 weeks. The high antibody titre was retained until at least the last sampling date (more than 2 years later). The fully protective level of antitoxin titre against diphtheria is believed to be 0.1 IU ml^{-1} (Galazka, 1993; Hasselhorn *et al.*, 1998). This result indicates that human respiratory *C. ulcerans* infection can induce an antibody response against diphtheria toxin, as well as in the reported cases of *C. diphtheriae* infection (Danilova *et al.*, 2006), and that the rise in antibody titre can be utilized as a marker of *C. ulcerans* infection.

Table 4. Serum antitoxin titre from the second case

All assays were done in triplicate and repeated three times, twice or once.

Date	Titre (IU ml^{-1})
28 October 2002	ND
11 November 2002	0.39 ± 0.06*
2 December 2002	2.47 ± 0.00*
14 April 2003	2.72 ± 0.05*
24 December 2003	1.18†
16 February 2005	0.92‡

ND, Not detectable.
*Values are the mean ± SE; assays were repeated three times.
†Repeated assays gave the same results; assays were repeated twice.
‡The same results were obtained in triplicate; assays were repeated once.

DISCUSSION

In the present study, two toxigenic *C. ulcerans* isolates recovered from pharyngeal swabs were characterized. The two isolates were indistinguishable by PFGE but could be distinguished by ribotyping. The isolate 0211, isolated in 2002, could potentially represent a new ribotype that has not been seen in Europe or North America. Geographically specific distribution has already been reported for some *C. ulcerans* ribotypes (De Zoysa *et al.*, 2005). Whether this new ribotype is specific to the Asian region or not will be clarified with further collection of clinical isolates from this region.

In the 2002 case, the patient showed a marked increase in serum antitoxin titre during the course of infection, which is, to our knowledge, the first evidence for such an increase in a *C. ulcerans* respiratory case. The patient showed a remarkable increase in titre within 2 weeks after he had first presented to the hospital, and the elevated titre remained for more than 2 years at least. Whether this rapid and lasting antibody response was based on previous vaccination is not clear, because insufficient information was available on the vaccination history of the patient. An increase in serum antitoxin titre during cutaneous infection caused by *C. ulcerans* has also been reported by Wagner *et al.* (2001).

Analysis in different culture media showed that the two isolates exhibited varying degrees of toxin activity (Table 3). First, for both isolates the toxin activities in Loeffler condensation were not completely neutralized by diphtheria antitoxin. The deduced amino acid sequence of their *tox* gene product was different from that of *C. diphtheriae* PW8 by 28 amino acid residues, mainly in the receptor-binding domain of the toxin molecule. However, it is not likely that the difference could account for the incomplete neutralization. When cultured in Pope liquid medium,

both isolates showed higher toxin activity than in Loeffler condensation, and the activity was completely neutralized by the same antitoxin. This suggests that, in addition to the *tox* gene product, on Loeffler medium the *C. ulcerans* isolates could secrete toxic substances immunologically discrete from the *tox* gene product, including PLD. In contrast, in Pope medium, the major part of toxicity could be attributed to their *tox* gene products.

Another feature varying between the two isolates was the response to culture conditions. The 0211 isolate responded well to the change of culture conditions. In contrast, the response of 0102 was not remarkable. This indicates that the isolates do not share the same mechanism for the regulation of toxin activity, even though the nucleotide sequence of the upstream region of the *tox* gene was identical in these isolates, as determined by the sequencing of DNA fragments obtained by PCR with primers DT-1 and Tox 1.

Concerning the regulatory gene *dtxR*, the presence of the gene was demonstrated in both isolates by the Southern hybridization experiment. However, for isolate 0211, the nucleotide sequence of the gene could not be determined because amplification of DNA fragments spanning from the upstream region to the N-terminal region of the ORF was impossible with primers dtxRprimerF#6 and dtxRprimerR#2 (Supplementary Table S1 available with the online journal) designed based on the reported nucleotide sequence (Boyd *et al.*, 1990). Within this region there might be some significant difference in nucleotide sequence that remains to be elucidated, which affects the regulation of *tox* gene regulation.

Mass immunization against diphtheria, as well as tetanus and pertussis, has been conducted in many countries for decades with diphtheria toxoid. With the increasing importance of *C. ulcerans* human infection, immunization with toxoid and therapy with diphtheria antitoxin are considered to be effective against the disease (CDC, 1997; De Zoysa *et al.*, 2005; Tiwari *et al.*, 2008; Von Hunolstein *et al.*, 2003). In the present paper we have shown that serum from a patient was able to neutralize diphtheria toxin at a high titre. Our data also suggest that cross protection between *C. diphtheriae* and *C. ulcerans* infections would be possible.

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REFERENCES

Boyd, J., Oza, M. N. & Murphy, J. R. (1990). Molecular cloning and DNA sequence analysis of a diphtheria *tox* iron-dependent regulatory element (*dtxR*) from *Corynebacterium diphtheriae*. *Proc Natl Acad Sci U S A* **87**, 5968–5972.

CDC (1997). Respiratory diphtheria caused by *Corynebacterium ulcerans* – Terre Haute, Indiana, 1996. *MMWR Morb Mortal Wkly Rep* **46**, 330–332.

Danilova, E., Jenum, P. A., Skogen, V., Pilinikov, V. F. & Sjursen, H. (2006). Antidiphtheria antibody responses in patients and carriers of *Corynebacterium diphtheriae* in the Arkhangelsk region of Russia. *Clin Vaccine Immunol* **13**, 627–632.

DeWinter, L. M., Bernard, K. A. & Romney, M. G. (2005). Human clinical isolates of *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* collected in Canada from 1999 to 2003 but not fitting reporting criteria for cases of diphtheria. *J Clin Microbiol* **43**, 3447–3449.

De Zoysa, A., Efstratiou, A., George, R. C., Jahkola, M., Vuopio-Varkila, J., Deshevoi, S., Tseneva, G. Y. & Rikushin, Y. (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.

De Zoysa, A., Hawkey, P. M., Engler, K., George, R., Mann, G., Reilly, W., Taylor, D. & Efstratiou, A. (2005). Characterization of toxigenic *Corynebacterium ulcerans* strains isolated from humans and domestic cats in the United Kingdom. *J Clin Microbiol* **43**, 4377–4381.

Dias, A. A. S. O., Silva, F. C., Pereira, G. A., Souza, M. C., Camello, T. C. F., Damasceno, J. A. L. D., Pacheco, L. G. C., Miyoshi, A., Azevedo, V. A. & other authors (2010). *Corynebacterium ulcerans* isolated from an asymptomatic dog kept in an animal shelter in the metropolitan area of Rio de Janeiro, Brazil. *Vector Borne Zoonotic Dis* (in press). doi: 10.1089/vbz.2009.0132.

Fakes, R. W. & Downham, M. (1970). Toxic reaction to *Corynebacterium ulcerans*. *Lancet* **I**, 298.

Galazka, A. M. (1993). *Module 2: Diphtheria, WHO/EPI/GEN/93.12*. Geneva: World Health Organization.

Galbraith, N. S., Forbes, P. & Clifford, C. (1982). Communicable disease associated with milk and dairy products in England and Wales 1951–80. *Br Med J (Clin Res Ed)* **284**, 1761–1765.

Grimont, P. A. D., Grimont, F., Efstratiou, A., De Zoysa, A., Mazurova, I. K., Ruckly, C., Lejay-Collin, M., Martin-Delautre, S., Regnault, B. & other authors (2004). International nomenclature for *Corynebacterium diphtheriae* ribotypes. *Res Microbiol* **155**, 162–166.

Hart, R. J. C. (1984). *Corynebacterium ulcerans* in humans and cattle in North Devon. *J Hyg (Camb)* **92**, 161–164.

Hasselhorn, H.-M., Nübling, M., Tiller, F. W. & Hofmann, F. (1998). Factors influencing immunity against diphtheria in adults. *Vaccine* **16**, 70–75.

Hatanaka, A., Tsunoda, A., Okamoto, M., Ooe, K., Nakamura, A., Miyakoshi, M., Komiya, T. & Takahashi, M. (2003). *Corynebacterium ulcerans* diphtheria in Japan. *Emerg Infect Dis* **9**, 752–753.

Hendrickson, D. A. & Krenz, H. J. (1991). Reagents and stains. In *Manual of Clinical Microbiology*, 5th edn, pp. 1289–1314. Edited by A. Balows, W. J. J. Hausler, K. L. Herrmann, H. D. Isenberg & H. J. Shadomy. Washington, DC: American Society for Microbiology.

Kisely, S. R., Proce, S. & Ward, T. (1994). '*Corynebacterium ulcerans*': a potential cause of diphtheria. *Commun Dis Rep CDR Rev* **4**, R63–R64.

Lartigue, M.-F., Monnet, X., Le Flèche, A., Grimont, P. A. D., Benet, J.-J., Durrbach, A., Fabre, M. & Nordmann, P. (2005). *Corynebacterium ulcerans* in an immunocompromised patient with diphtheria and her dog. *J Clin Microbiol* **43**, 999–1001.

Mikhailovich, V. M., Melnikov, V. G., Mazurova, I. K., Wachsmuth, I. K., Wenger, J. D., Wharton, M., Nakao, H. & Popovic, T. (1995).

Application of PCR for detection of toxigenic *Corynebacterium diphtheriae* strains isolated during the Russian diphtheria epidemic, 1990 through 1994. *J Clin Microbiol* 33, 3061–3063.

Miyamura, K., Tajiri, E., Ito, A., Murata, R. & Kono, R. (1974a). Micro cell culture method for determination of diphtheria toxin and antitoxin titres using VERO cells. II. Comparison with the rabbit skin method and practical application for seroepidemiological studies. *J Biol Stand* 2, 203–209.

Miyamura, K., Nishio, S., Ito, A., Murata, R. & Kono, R. (1974b). Micro cell culture method for determination of diphtheria toxin and antitoxin titres using VERO cells. I. Studies on factors affecting the toxin and antitoxin titration. *J Biol Stand* 2, 189–201.

Murray, B. E., Singh, K. V., Heath, J. D., Sharma, B. R. & Weinstock, G. M. (1990). Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. *J Clin Microbiol* 28, 2059–2063.

Nakao, H., Pruckler, J. M., Mazurova, I. K., Narvskaia, O. V., Glushkevich, T., Marijevski, V. F., Kravetz, A. N., Fields, B. S., Wachsmuth, I. K. & other authors (1996). Heterogeneity of diphtheria toxin gene, *tox*, and its regulatory element, *dtxR*, in *Corynebacterium diphtheriae* strains causing epidemic diphtheria in Russia and Ukraine. *J Clin Microbiol* 34, 1711–1716.

PHLS (2000). Three cases of toxigenic *Corynebacterium ulcerans* infection. *Commun Dis Rep CDR Wkly* 10, 52.

Regnault, B., Grimont, F. & Grimont, P. A. D. (1997). Universal ribotyping method using a chemically labelled oligonucleotide probe mixture. *Res Microbiol* 148, 649–659.

Reinhardt, D. J., Lee, A. & Popovic, T. (1998). Antitoxin-in-membrane and antitoxin-in-well assays for detection of toxigenic *Corynebacterium diphtheriae. J Clin Microbiol* 36, 207–210.

Schuhegger, R., Schoerner, C., Dlugaiczyk, J., Lichtenfeld, I., Trouillier, A., Zeller-Peronnet, V., Busch, U., Berger, A., Kugler, R. & other authors (2009). Pigs as source for toxigenic *Corynebacterium ulcerans. Emerg Infect Dis* 15, 1314–1315.

SCIEH (2002). Toxigenic *Corynebacterium ulcerans* in cats. *SCIEH Weekly Rep* 36, 69–75.

Sing, A., Hogardt, M., Bierschenk, S. & Heesemann, J. (2003). Detection of differences in the nucleotide and amino acid sequences of diphtheria toxin from *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* causing extrapharyngeal infections. *J Clin Microbiol* 41, 4848–4851.

Sing, A., Bierschenk, S. & Heesemann, J. (2005). Classical diphtheria caused by *Corynebacterium ulcerans* in Germany: amino acid sequence differences between diphtheria toxins from *Corynebacterium diphtheriae* and *C. ulcerans. Clin Infect Dis* 40, 325–326.

Tasman, A. & Van Ramhorst, J. D. (1951). On the relation between toxin production and protein synthesis by *Corynebacterium diphtheriae* and the iron content of the culture medium. *Antonie van Leeuwenhoek* 17, 153–158.

Taylor, D. J. & Efstratiou, A. (2002). Diphtheria toxin production by *Corynebacterium ulcerans* from cats. *Vet Rec* 150, 355.

Tchorbanov, A. I., Dimitrov, J. D. & Vassilev, T. L. (2004). Optimization of casein-based semisynthetic medium for growing of toxigenic *Corynebacterium diphtheriae* in a fermenter. *Can J Microbiol* 50, 821–826.

Tiwari, T. S. P., Golaz, A., Yu, D. T., Ehresmann, K. R., Jones, T. F., Hill, H. E., Cassiday, P. K., Pawloski, L. C., Moran, J. S. & other authors (2008). Investigations of 2 cases of diphtheria-like illness due to toxigenic *Corynebacterium ulcerans. Clin Infect Dis* 46, 395–401.

Von Hunolstein, C., Alfarone, G., Scopetti, F., Pataracchia, M., La Valle, R., Franchi, F., Pacchiani, L., Manera, A., Giammanco, A. & other authors (2003). Molecular epidemiology and characteristics of *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* strains isolated in Italy during the 1990s. *J Med Microbiol* 52, 181–188.

Wagner, J., Ignatius, R., Voss, S., Höpfner, V., Ehlers, S., Funke, G., Weber, U. & Hahn, H. (2001). Infection of the skin caused by *Corynebacterium ulcerans* and mimicking classical cutaneous diphtheria. *Clin Infect Dis* 33, 1598–1600.

Novel *Corynebacterium diphtheriae* in Domestic Cats

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Novel nontoxigenic *Corynebacterium diphtheriae* was isolated from a domestic cat with severe otitis. Contact investigation and carrier study of human and animal contacts yielded 3 additional, identical isolates from cats, although no evidence of zoonotic transmission was identified. Molecular methods distinguished the feline isolates from known *C. diphtheriae*.

The clinical relevance of *Corynebacterium diphtheriae* recovered from a cat with otitis is poorly understood. Historically, humans have been thought to be its sole reservoir, and the few human cases reported annually in the United States are generally associated with international travel (*1*). Therefore, when *C. diphtheriae* was isolated from the ears of a cat, an investigation was initiated to evaluate potential sources of the cat's infection and potential public health risks and to preliminarily characterize the *C. diphtheriae* isolate.

The cat, an 8-month-old female domestic shorthair, was examined at a West Virginia veterinary hospital on 5 occasions during January–June 2007. Pertinent findings included severe bilateral otitis, vestibular signs, mild ataxia, anorexia, and failure to gain weight; the cat had a history of ear, eye, and lung infections. Results of diagnostic tests showed no evidence of systemic disease and were negative for feline immunodeficiency and leukemia viruses and feline infectious peritonitis. Culture of an otic swab collected from the cat in May 2007 yielded 4 organisms: *C. diphtheriae*, *Streptococcus equi zooepidemicus*, *Staphylococcus* spp., and *Achromobacter xylosoxidans*. The cat was treated with oral clindamycin, otic enrofloxacin, and an ear-flushing solution.

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The Study

In June 2007, investigators visited the veterinary clinic and the household of the index cat and conducted a contact investigation and carrier study. Interviews of 2 household members and 8 veterinary staff members indicated no recent respiratory illness, skin infection, or risk factors for diphtheria (e.g., travel to countries to which diphtheria is endemic or contact with known case-patients). Half of these 10 contacts had received diphtheria vaccination within the previous 5 years. Cultures of oropharyngeal swab samples obtained from each person were negative, including cystine tellurite blood agar, which is selective for *C. diphtheriae*. Household members also were interviewed about medical history of a convenience sample of household animals (4 cats, including the index cat; 2 dogs; and 1 horse). Each animal was briefly examined, and oropharyngeal, otic, or ocular swab samples were collected. Otitis was observed in all 4 cats and 1 dog. The horse reportedly had had an eye infection ≈5 years earlier. No other abnormal findings were noted. Animal specimens yielded 3 additional isolates of *C. diphtheriae*: 1 from each ear of the index cat and 1 from the left ear of a 2-year-old domestic medium-hair cat. Both cats had been born on the premises and had remained with the same household since birth.

Feline *C. diphtheriae* and reference isolates used are described in the Table. Tinsdale agar plate growth (Remel, Lenexa, KS, USA) gave rise to black colonies with a brown halo, typical of cysteinase-producing *C. diphtheriae*, *C. ulcerans*, or *C. pseudotuberculosis*. After 24 hours on blood agar, 1–2-mm grey-white or opaque, rounded, convex colonies with no hemolysis were observed. Microscopically, the bacteria were gram-positive, club-shaped rods, 1 µm in diameter, arranged singly or at angles. Biochemical profiles to determine species and biotype were done by using an API Coryne strip (bioMérieux, Durham, NC, USA, and St-Laurent, Quebec, Canada). Query of API Coryne code 0010304 obtained for all isolates by APIWEB (https://apiweb.biomerieux.com) indicated a decreased level of confidence of *C. diphtheriae* biotype *mitis* or *belfanti* (89.5%) because of a maltose-negative result. Isolates were further characterized morphologically and biochemically by using tube substrates (*2*) and were identified by using a standard taxonomic scheme (*3*). Feline isolates were biochemically identical with each other and phenotypically consistent with *C. diphtheriae* biotype *belfanti*, except for the lack

Table. Feline *Corynebacterium diphtheriae* isolates and reference strains used for comparison, West Virginia, 2008*

Strain	Culture collection	Source	Diphtheria toxin	GenBank accession no.		
				16S rRNA	*rpoB*	*tox* gene
CD443	ATCC BAA-1774	Cat 1, right ear	Nontoxigenic	FJ409572	FJ415317	FJ376656
CD448	ND	Cat 1, right ear	Nontoxigenic	FJ409573	ND	FJ422272
CD449	ND	Cat 1, left ear	Nontoxigenic	FJ409574	ND	FJ422273
CD450	ND	Cat 2, left ear	Nontoxigenic	FJ409575	FJ415318	FJ422274
C. diphtheriae biotype *mitis*	NCTC 10356†	Human nose	Nontoxigenic	GQ118340	GQ409648	ND
C. diphtheriae biotype *gravis*	NCTC 10648	Unknown	Toxigenic	ND	ND	ND
C. diphtheriae biotype *gravis*	NCTC 11397^T ATCC 27010^T	Unknown	Nontoxigenic	GQ118341	GQ409649	ND
C. diphtheriae biotype *gravis*	NCTC 13129 ATCC 700971	Human throat	Unknown	GQ118344	GQ409650	ND
C. pseudotuberculosis	NCTC 3450^T	Sheep gland	Unknown	GQ118342	GQ409651	ND
C. ulcerans	NCTC 12077	Human throat	Unknown	GQ118343	ND	ND
C. ulcerans	NCTC 7910	Human throat	Unknown	GQ118345	ND	ND

*CD, Centers for Disease Control and Prevention identifier number; ATCC, American Type Culture Collection; ND, not deposited in this study; NCTC, National Collection of Type Cultures, London, UK. Additional strains used as controls for specific assays: toxigenic *C. diphtheriae* biotype *belfanti* isolates used for real-time PCR of *tox* gene were 718, G4182, C59, C60, C75, C76, C77; toxigenic *C. diphtheriae* ATCC 27012 used as positive control for Elek; *C. diphtheriae* NCTC 10481 and *C. ulcerans* CD199 used as positive and negative controls for Vero cell assay.
†NCTC 10356 is described in the NCTC catalogue as *C. diphtheriae* biotype *mitis*; however, analyses in this study found this strain to be nitrate negative and therefore consistent with *C. diphtheriae* biotype *belfanti*. Thus, it was used in this study as a *belfanti* reference strain.

of maltose fermentation, which was considered an unusual finding (*3*).

Antimicrobial drug susceptibility testing was performed according to the Clinical and Laboratory Standards Institute's recommended methods and interpretative criteria (*4*). All 4 feline isolates were sensitive to ampicillin, cefepime, cefotaxime, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, clindamycin, daptomycin, erythromycin, ertapenam, gatifloxacin, gentamicin, levofloxacin, linezolid, meropenem, moxifloxacin, penicillin, quinupristin/dalfopristin, rifampin, telithromycin, tetracycline, tigecycline, trimethoprim/sulfamethoxazole, and vancomycin. Cellular fatty acid composition analysis was performed as described (*5*) by using the Sherlock system (MIDI, Inc., Newark, DE, USA), except that version 4.5 of the operating software was used. The cellular fatty acid composition profiles were consistent for *C. diphtheriae, C. ulcerans*, or *C. pseudotuberculosis*, including a substantial proportion (28%–30% of total) of C16:1ω7c (*5*). All feline isolates produced 7–15 meq/L of propionic acid among fermentation products, a feature associated with C. *diphtheriae* (*2*).

Results from use of the modified Elek test (*6*) indicated that all feline isolates were negative for production of diphtheria toxin; however, an atypical precipitation was observed after 36 h of incubation. Lack of toxin expression was corroborated by negative Vero cell assay results (*7*) and confirmed by using Western blot. Real-time PCR selective for the *C. diphtheriae* and *C. ulcerans* toxin gene (*tox*) (*8*) was positive for all feline isolates. However, real-time PCR for A and B subunits of *tox* (*9*) amplified subunit A but not subunit B. Sequence analysis of the *tox* gene was performed as previously outlined (*10*) and compared with a reference *tox* gene, GenBank accession no. K01722. The 4 feline *tox* sequences were identical to each other but con-

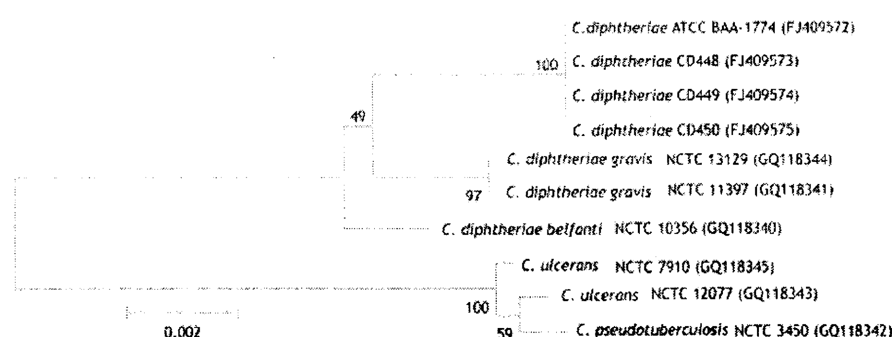

Figure 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence analysis of *Corynebacterium diphtheriae* isolates, including 4 feline isolates from West Virginia, 2008 (ATCC BAA-1774, CD 448, CD 449, CD 450). The tree was constructed from a 1,437-bp alignment of 16S rRNA gene sequences by using the neighbor-joining method and Kimura 2-parameter substitution model. Bootstrap values (expressed as percentages of 1,000 replicates) >40% are illustrated at branch points. Feline isolates had 100% identity with each other and ≥99.1% identity with *C. diphtheriae* biotypes *gravis* and *belfanti*. GenBank accession nos. given in parentheses. ATCC, American Type Culture Collection; CD, Centers for Disease Control and Prevention identifier number; NCTC, National Collection of Type Cultures. Scale bar indicates number of substitutions per site.

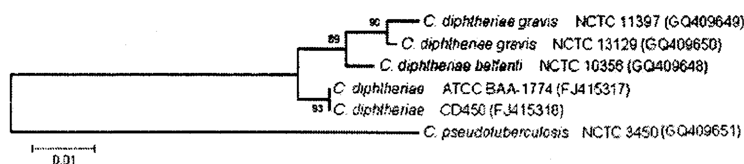

Figure 2. Jukes-Cantor–derived phylogenetic tree based on sequence analysis of a selected region of the *rpoB* gene of *Corynebacterium* isolates, including 2 feline isolates from West Virginia, 2008 (ATCC BAA-1774, CD450). Feline isolates had 100% identity with each other and 97.7% identity with *C. diphtheriae* biotypes *gravis* and *belfanti*. GenBank accession nos. given in parentheses. ATCC, American Type Culture Collection; CD, Centers for Disease Control and Prevention identifier number; NCTC, National Collection of Type Cultures. Scale bar indicates number of substitutions per site.

tained multiple nucleotide substitutions and deletions compared with the reference gene. By NCBI BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the feline *tox* had higher sequence identity (97%–98%) to the *tox* sequences of *C. ulcerans*, compared with those from C. diphtheriae (94%–95%). A deletion at nt 55, coupled with a cytosine-to-thymine substitution at nt 74, prematurely terminated the peptide at aa 25.

Species characterization was corroborated by using 16S rRNA (*11*) and partial *rpoB* (*12*) gene sequencing. By 16S rRNA gene sequence analysis, the feline strains had 100% identity with each other and ≥99.1% identity with various reference sequences for *C. diphtheriae* biotype *gravis* and *belfanti* sequences, including NCTC 11397^T. Partial *rpoB* sequence analyses indicated 100% identity among the feline isolates and 97.7% identity with *C. diphtheriae* NCTC 11397^T. Neighbor-joining phylogenetic trees based on both 16S rRNA (Figure 1) and partial *rpoB* gene sequencing (Figure 2) positioned the feline isolate sequences within the *C. diphtheriae* clade but clearly distinguished them from the other *C. diphtheriae* isolates. Comprehensive molecular analyses to characterize differences between biotype *belfanti* strains, including these feline isolates, with other *C. diphtheriae* biotypes, are the subject of a separate publication (C.G. Dowson, pers. comm.).

Conclusions

We identified a potentially novel biotype of *C. diphtheriae* recovered from domestic cats in West Virginia but found no evidence of zoonotic transmission. Although rare, isolation of *C. diphtheriae* from animals has been reported, including *C. diphtheriae* biotype *belfanti* from a skin lesion of a cow (*13*) and toxigenic *C. diphtheriae* biotype *gravis* from a wound of a horse (*14*). *C. ulcerans* is a known animal pathogen, and zoonotic transmission of toxigenic *C. ulcerans* from companion animals has been reported, often associated with predisposing concurrent illnesses (*15*).

The feline strains isolated during this investigation differed phenotypically from previously described biotypes but were otherwise regarded as typical of *C. diphtheriae*. However, isolates were nontoxigenic and harbored a modified *tox* gene with sequence differences from *Corynebacterium* spp. capable of expressing diphtheria toxin. On the basis of published criteria (*11*), the feline strain might represent a novel subspecies of *C. diphtheriae* because it shares <98% sequence homology to the type strain within the *rpoB* gene. Potential for zoonotic transmission of this novel, cat-associated *C. diphtheriae* and associated public health implications are unknown. Additional studies are needed to further characterize these isolates and determine their appropriate taxonomy. Large-scale screening of domestic cat populations is recommended to determine the prevalence of *C. diphtheriae* and its pathogenic potential and to identify additional isolates for more formal description and classification.

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Dr Hall is a public health veterinarian who completed this study while serving as an Epidemic Intelligence Service Officer of the Centers for Disease Control and Prevention (CDC), assigned to the state of West Virginia. Currently he is an epidemiologist on the CDC Viral Gastroenteritis Team. His research interests focus on public health issues involving interactions between humans, domestic animals, wildlife, and the environment.

References

1. Vitek CR, Wharton M. Diphtheria toxoid. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines. 5th ed. Philadelphia: Elsiever; 2008. p. 139–56.
2. Bernard KA, Munro C, Wiebe D, Ongsansoy E. Characteristics of rare or recently described *Corynebacterium* species recovered from human clinical material in Canada. J Clin Microbiol. 2002;40:4375–81. DOI: 10.1128/JCM.40.11.4375-4381.2002

3. Funke G, Bernard KA. Coryneform gram-positive rods. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, editors. Manual of clinical microbiology. 9th ed. Washington: ASM Press; 2007. p. 485–514.
4. Clinical Laboratory Standards Institute. M45-A. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. Wayne (PA): The Institute; 2006.
5. Bernard KA, Bellefeuille M, Ewan EP. Cellular fatty acid composition as an adjunct to the identification of asporogenous, aerobic gram-positive rods. J Clin Microbiol. 1991;29:83–9.
6. Engler KH, Glushkevich T, Mazurova IK, George RC, Efstratiou A. A modified Elek test for detection of toxigenic corynebacteria in the diagnostic laboratory. J Clin Microbiol. 1997;35:495–8.
7. Miyamura K, Nishio S, Ito A, Murata R, Kono R. Micro cell culture method for determination of diphtheria toxin and antitoxin titres using VERO cells. I. Studies on factors affecting the toxin and antitoxin titration. J Biol Stand. 1974;2:189–201. DOI: 10.1016/0092-1157(74)90015-8
8. Schuhegger R, Lindermayer M, Kugler R, Heesemann J, Busch U, Sing A. Detection of toxigenic *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* strains by a novel real-time PCR. J Clin Microbiol. 2008;46:2822–3. DOI: 10.1128/JCM.01010-08
9. Mothershed EA, Cassiday PK, Pierson K, Mayer LW, Popovic T. Development of a real-time fluorescence PCR assay for rapid detection of the diphtheria toxin gene. J Clin Microbiol. 2002;40:4713–9. DOI: 10.1128/JCM.40.12.4713-4719.2002
10. Cassiday PK, Pawloski LC, Tiwari T, Sanden GN, Wilkins PP. Analysis of toxigenic *Corynebacterium ulcerans* strains revealing potential for false-negative real-time PCR results. J Clin Microbiol. 2008;46:331–3. DOI: 10.1128/JCM.01251-07
11. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991;173:697–703.
12. Khamis A, Raoult D, La Scola B. *rpoB* gene sequencing for identification of *Corynebacterium* species. J Clin Microbiol. 2004;42:3925–31. DOI: 10.1128/JCM.42.9.3925-3931.2004
13. Corboz L, Thoma R, Braun U, Zbinden R. Isolation of *Corynebacterium diphtheriae* subsp. *belfanti* from a cow with chronic active dermatitis [in German]. Schweiz Arch Tierheilkd. 1996;138:596–9.
14. Henricson B, Segarra M, Garvin J, Burns J, Jenkins S, Kim C, et al. Toxigenic *Corynebacterium diphtheriae* associated with an equine wound infection. J Vet Diagn Invest. 2000;12:253–7.
15. Bonmarin I, Guiso N, Le Fleche-Mateos A, Patey O, Patrick AD, Levy-Bruhl D. Diphtheria: a zoonotic disease in France? Vaccine. 2009;27:4196–200. DOI: 10.1016/j.vaccine.2009.04.048

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Genome Organization and Pathogenicity of *Corynebacterium diphtheriae* C7(−) and PW8 Strains▽†

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***Corynebacterium diphtheriae* is the causative agent of diphtheria. In 2003, the complete genomic nucleotide sequence of an isolate (NCTC13129) from a large outbreak in the former Soviet Union was published, in which the presence of 13 putative pathogenicity islands (PAIs) was demonstrated. In contrast, earlier work on diphtheria mainly employed the C7(−) strain for genetic analysis; therefore, current knowledge of the molecular genetics of the bacterium is limited to that strain. However, genomic information on the NCTC13129 strain has scarcely been compared to strain C7(−). Another important *C. diphtheriae* strain is Park-Williams no. 8 (PW8), which has been the only major strain used in toxoid vaccine production and for which genomic information also is not available. Here, we show by comparative genomic hybridization that at least 37 regions from the reference genome, including 11 of the 13 PAIs, are considered to be absent in the C7(−) genome. Despite this, the C7(−) strain still retained signs of pathogenicity, showing a degree of adhesion to Detroit 562 cells, as well as the formation of and persistence in abscesses in animal skin comparable to that of the NCTC13129 strain. In contrast, the PW8 strain, suggested to lack 14 genomic regions, including 3 PAIs, exhibited more reduced signs of pathogenicity. These results, together with great diversity in the presence of the 37 genomic regions among various *C. diphtheriae* strains shown by PCR analyses, suggest great heterogeneity of this pathogen, not only in genome organization, but also in pathogenicity.**

Corynebacterium diphtheriae is the causative agent of diphtheria. In 2003, the genomic nucleotide sequence of NCTC13129 (equivalent to ATCC 700971, here referred as the reference strain)—isolated in 1997 during a large outbreak in the former Soviet Union—was published, and 13 putative pathogenicity islands (PAIs) were shown to be present in its genome (9). The 13 PAIs have been annotated based on their unusual GC contents (9). The PAIs include *tox* (the genetic determinant for diphtheria toxin)-bearing corynebacteriophages, sortase genes (*srtA* to *-E* [34]), pilin genes (*spaA* to *-G* [34]), lantibiotic synthesis-related genes, and iron uptake-related genes. However, the contributions of these genes to *C. diphtheriae* pathogenesis have not yet been experimentally determined, except for the toxin, the minor pilins, and some of the sortases (34).

In earlier research on diphtheria, the nontoxigenic strain C7(−) (equivalent to ATCC 27010)—isolated in 1949 from a diphtheria contact in California as "culture 770" and later renamed (5, 17)—has been one of the "standard" strains used for analyses of *C. diphtheriae* in bacteriology and pathogenicity studies (4, 5, 35, 55, 56, 60), including the molecular biology of bacteriophages and diphtheria toxin genes (22, 38, 49–51, 62). More importantly, C7(−) is, in fact, pathogenic to humans. Barksdale et al. documented two cases of laboratory personnel infected with the C7(−) strain and suffering typical clinical manifestations of diphtheria, such as sore throat and pseudomembrane formation (3). The strain is still important in molecular analysis of the bacterium (7, 24, 31, 42). However, information from the reference genome sequence has not yet been fully integrated with other research results, except the proteomics approach of Hansmeier and colleagues (24).

The strain Park-Williams no. 8 (PW8), originally isolated from a very mild diphtheria case during the 1890s (46), has been widely used for toxoid vaccine production because of its great ability to secrete diphtheria toxin into the culture supernatant (46). As PW8 is effectively the only strain employed for vaccine production, its importance in public health and the vaccine industry is incomparable. Despite its high toxin-producing activity, the PW8 strain has been regarded as avirulent, as shown by Lampidis and Barksdale by the fact that their experience with this strain for more than 20 years did not show any detectable rise in the serum antibody titer (32).

Toxigenic strains of *C. diphtheriae* produce a potent extracellular protein toxin, i.e., diphtheria toxin (44). The toxin is recognized as the main virulence factor of the bacterium and has been employed for toxoid vaccine with remarkable success (44). The mode of action of this toxin has been extensively studied (37, 40, 41). In contrast to research and application involving diphtheria toxin, our understanding of other factors and mechanisms underlying *C. diphtheriae* infections remains largely deficient. Nevertheless, several experimental systems have been constructed to clarify the mechanisms, *in vitro* employing HEp-2 and Detroit 562

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cells (6, 26, 27, 34, 43) and *in vivo* using rabbits and guinea pigs (3, 18, 29, 33, 39).

In the present paper, we aimed to relate the genome information of the reference strain to that of the C7(−) and PW8 strains using comparative genomic hybridization (CGH), and we demonstrate that most of the PAIs found in NCTC13129 are considered to be absent in C7(−) but present in PW8. The implications of these findings are discussed in relation to the results of *in vivo* and *in vitro* assays of pathogenicity.

MATERIALS AND METHODS

Bacterial strains and preparation of genomic DNA. *C. diphtheriae* ATCC 27010 [referred as C7(−) in the present paper], ATCC 700971 (equivalent to NCTC13129; referred as the reference strain in the present paper), and ATCC 11951 (equivalent to C4B) were obtained from the American Type Culture Collection (Manassas, VA). The vaccine strain PW8 was from our laboratory stock (39), which originated from Harvard University. Japanese clinical isolates TM1 to -10 were from our laboratory stock. Bacterial genomic DNA was prepared by CsCl density gradient centrifugation or by using a Qiagen genomic buffer set and tips (Qiagen Co., Tokyo, Japan).

Comparative genomic hybridization. The comparative genomic hybridization (1, 25) service was provided by GeneFrontier Co. (now Roche Diagnostics Ltd., Tokyo, Japan) with NimbleGen microarrays. The tiling DNA array (1), composed of 29- to 39-mer oligonucleotide probes covering the entire reference genome at 7-nucleotide intervals, was subjected to hybridization with DNA from CsCl-purified, mechanically disrupted, and then differentially Cy3- and Cy5-labeled C7(−) or PW8 (test) and reference strain (reference) DNAs. The intensity of hybridization signals was extracted and normalized by using NimbleScan software (Roche Diagnostics). Data were analyzed as described previously (1). Briefly, ratios of intensity were calculated for each probe and compared to the global median of intensity ratio. Outliers showing high reference/test intensity ratios were identified and excluded, and identification of outliers was repeated using the remaining data as described previously (1). Data were converted to GFF format, and distribution of such outliers were visualized by SignalMap software (Fig. 1A to C, rows 3). Coding sequences (CDSs) associated with such outlying probes were considered to be absent from the test genome. PCR detection of genes was performed using primers described in Table S1 in the supplemental material. Pulsed-field gel electrophoresis (PFGE) analysis of SfiI-digested genomic DNA was performed as described previously (13) with a Chef DRII apparatus (Bio-Rad Japan, Tokyo, Japan).

Adhesion of bacterial cells to the human pharyngeal cell line Detroit 562. Adhesion of *C. diphtheriae* cells to human pharyngeal Detroit 562 cells was assayed according to the method of Mandlik et al. (34) with slight modifications. Cells were purchased from ATCC (Manassas, VA) and cultured in minimum essential medium (MEM) supplemented with 1 mM pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum (Invitrogen Japan K.K., Tokyo, Japan). Semiconfluent cultures (approximately 1×10^6/well) in 12-well culture plates were washed once with Hanks' balanced salt solution (Sigma-Aldrich Japan K.K., Tokyo, Japan) prior to the addition of bacterial suspensions. Bacteria were cultured overnight in 2 ml of brain heart infusion (BHI) broth at 37°C with vigorous shaking. After sedimentation of the bacterial cells by centrifugation at $2,000 \times g$, they were resuspended in an equal volume of Hanks' balanced salt solution and centrifuged again. The pellet was finally resuspended in Hanks' balanced salt solution at an optical density at 600 nm (OD_{600}) of around 0.1, and 1 ml was added to Detroit 562 cell cultures. The exact viable-cell number in the bacterial-suspension inoculum was determined by appropriate dilution in saline and plating on BHI agar. The culture plates were centrifuged at $600 \times g$ for 5 min at room temperature and then incubated for 1 h at 37°C for adhesion. The wells were then washed gently 3 times with Hanks' balanced salt solution and detached/lysed with 0.5 ml of solution containing 0.25% trypsin and 0.025% Triton X-100, which had been confirmed not to be harmful to bacterial colony formation (data not shown). The lysates containing viable bacterial cells were appropriately diluted in saline and plated onto BHI agar plates for colony counting. Control wells without bacterial cells were similarly treated and assayed, and the possibility of cross-contamination among the wells was excluded. Statistical analysis (Student's t test) was performed using Excel software (Microsoft Co., Tokyo, Japan).

Intradermal challenge. Intradermal challenge was performed as follows. *C. diphtheriae* C7(−), PW8, and the reference strain were cultured as described above and then resuspended in a volume of saline to give approximately the

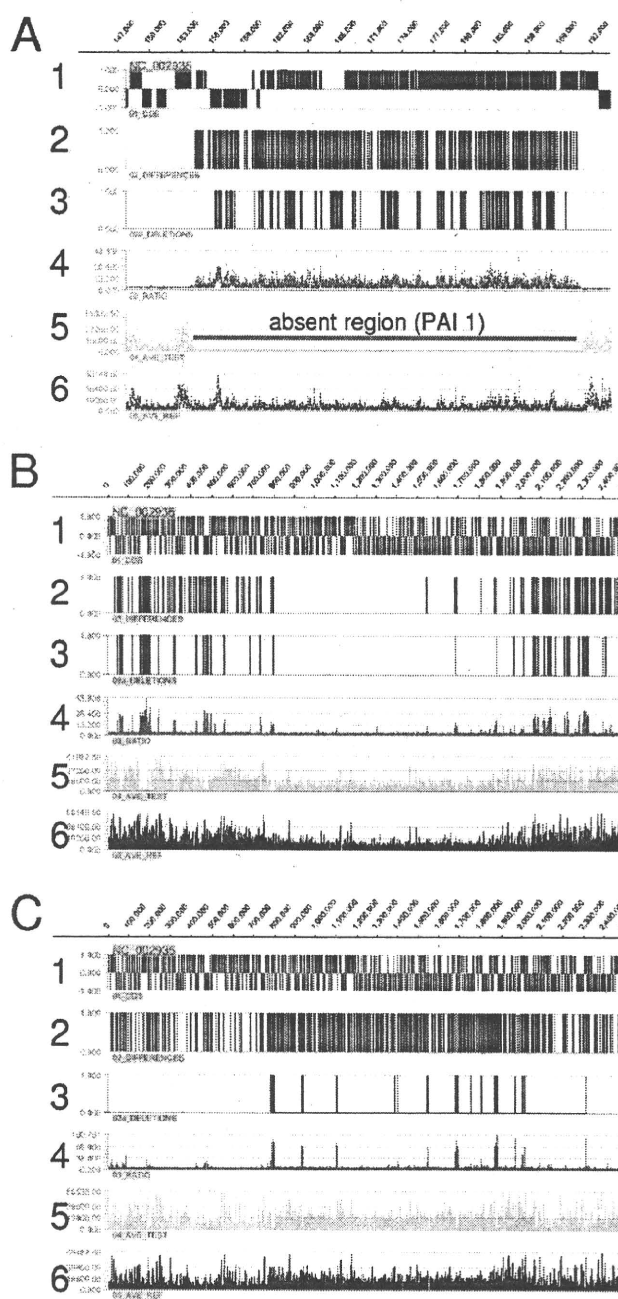

FIG. 1. Comparison of genomes by CGH. Genomic DNAs of *C. diphtheriae* C7(−) or PW8 (test) and the reference strain (reference) were subjected to comparative genomic hybridization with NimbleGen-type tiling arrays covering the entire genome of the *C. diphtheriae* reference strain NCTC13129. By comparing the hybridization signals with reference DNA and with test DNA, regions present in the reference genome but lacking in the test genome were identified. (A) Summary of results for the corynephage region (PAI 1). (B and C) Summary of results from the whole C7(−) and PW8 genomes, respectively. Rows 1, CDSs in the NCTC13129 genome; rows 2, regions in which a difference between signals from the two strains was suggested; rows 3, regions suggested to be absent in the test genome; rows 4, ratio of signal intensity (reference/test, expressed as log_2); rows 5, signal intensity from the test genome; rows 6, signal intensity from the reference genome. The red bar in panel A indicates the span of the corynephage region.