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-

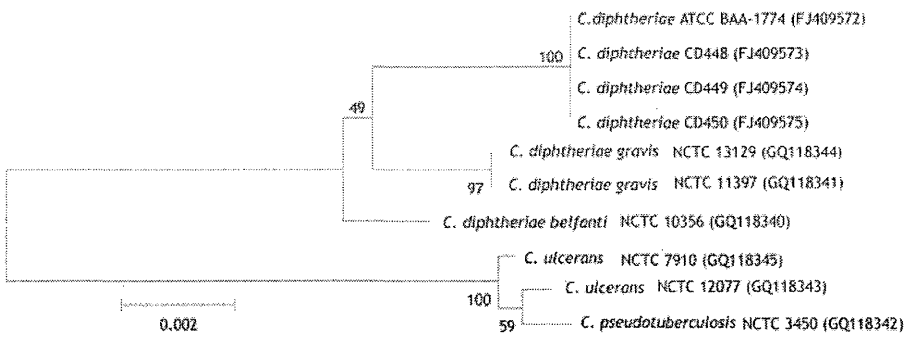

Figure 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence analysis of *Corynebacterium diphtheriae* isolates, including 4 feline isolates from West Virginia, 2008 (ATCC BAA-1774, CD 448, CD 449, CD 450). The tree was constructed from a 1,437-bp alignment of 16S rRNA gene sequences 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.

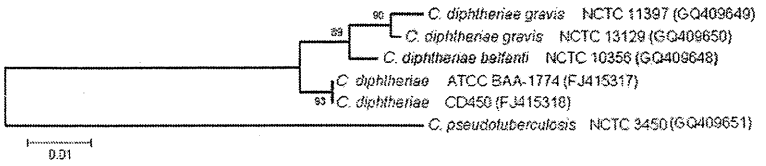

Figure 2. Jukes-Cantor–derived phylogenetic tree based on sequence analysis of a selected region of the *rpoB* gene of *Corynebacterium* isolates, including 2 feline isolates from West Virginia, 2008 (ATCC BAA-1774, CD 450). Feline isolates had 100% identity with each other and 97.7% identity with *C. 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.

Acknowledgments

We gratefully recognize Amy Isaac, Gary Kinder, and Katrina Kretsinger for collaborative assistance in the epidemiologic investigation and Tamara Burdz, Christi Clark, Tiffany Jackson, Brenda Keavey, Betty Ng, Chris Paddock, and Deborah Wiebe for laboratory assistance.

Work performed at University of Warwick was funded in part by the Biotechnology and Biological Sciences Research Council, Micropathology Ltd, and the Medical Research Fund.

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.

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INFECTION AND IMMUNITY, Sept. 2010, p. 3791–3800 Vol. 78, No. 9
0019-9567/10/$12.00 doi:10.1128/IAI.00049-10

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

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Received 15 January 2010/Returned for modification 19 May 2010/Accepted 6 June 2010

***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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† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 14 June 2010.

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 × *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 × *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

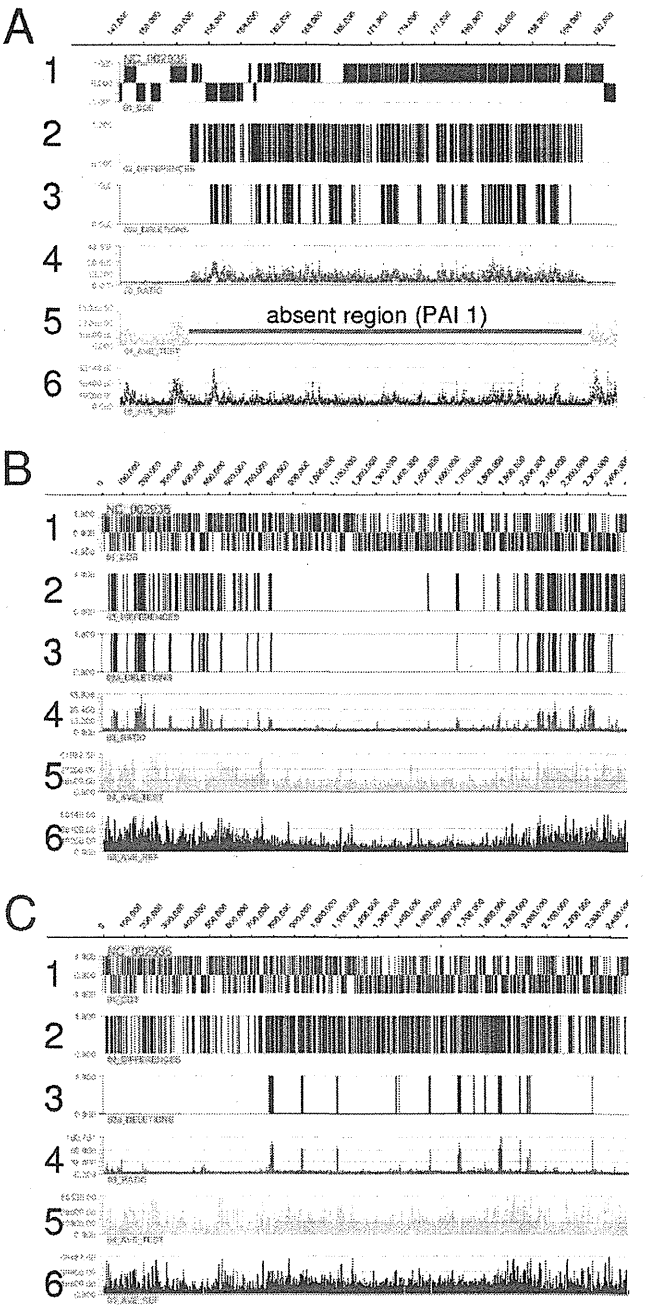

FIG. 1. Comparison of genomes by CGH. Genomic DNAs of *C. diphtheriae* C7(−) or PW8 (test) and the reference strain (reference) were subjected to comparative genomic hybridization with NimbleGen-type tiling arrays covering the entire genome of the *C. diphtheriae* reference strain NCTC13129. By comparing 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.

desired cell density for an inoculum. The exact bacterial titer of the inoculum was determined by appropriate dilution and plating. Prior to challenge, the backs of animals were shaved and then depilated with barium sulfide. The inocula (0.05 to 0.1 ml) were injected intradermally into the backs of the rabbits (1 or 2 rabbits/group) or the mice (3 to 5 mice/group) under anesthesia. Two to 4 days after challenge, the animals were killed by deep anesthesia, the injected sites were surface disinfected with a 1:1 mixture of 70% ethanol and benzalkonium chloride solution or 70% ethanol, and the skin of the back was removed. The abscesses were excised and homogenized in 500 μl of sterile saline in 1.5-ml microtubes using a conical homogenizer (Toyobo Co., Osaka, Japan). The homogenates were assayed for bacterial CFU by 10-fold serial dilution with sterile saline and plating on BHI agar plates. If no detectable colonies appeared at the lowest dilution applied, the data were excluded from consideration. Occasionally, colonies were picked from the plates, and the nucleotide sequence of the 16S RNA gene was determined (52) to confirm the bacterial species. Contamination by surface-resident bacteria was negligible, as confirmed by the absence of colonies detected by plating 100 μl of undiluted skin homogenate from noninoculated regions (data not shown). Statistical analysis (Student's *t* test) was performed using Excel software. The animal experiments were carried out with the approval of the Animal Experiment Committee of the National Institute of Infectious Diseases. The abscesses were also examined by electron microscopy.

Electron microscopy. Ultrathin (80-nm) sections of tissue fixed in phosphate-buffered saline (PBS) containing 2% paraformaldehyde and 2.5% glutaraldehyde were postfixed with 2% osmium tetroxide and embedded in Epon resin. Sections were stained with uranyl acetate and lead acetate and examined using a JEM-1220 electron microscope at 80 kV (Jeol Ltd., Tokyo, Japan) as described previously (57).

Hemagglutination and hemolysis. Fresh human blood (type AB) was suspended in Alsever's solution (20.5 g glucose, 4.2 g NaCl, 8.0 g sodium citrate per liter, pH 6.1), and erythrocytes were prepared by centrifugation (2,300 × *g*) after being washed three times in PBS and subjected to hemagglutination and hemolysis assays directly or after neuraminidase and/or trypsin treatments performed as follows. Neuraminidase (from *Arthrobacter ureafaciens*; Nacalai Tesque, Osaka, Japan) was added to the erythrocyte suspension at 0.1 unit/ml, and the suspension was incubated for 1 to 2 h at 37°C. After the incubation period, the erythrocytes were washed with PBS three times and subjected to assays or subsequently treated with trypsin. Trypsin solution (2.5%; Invitrogen Co., Tokyo, Japan) was added to the cell suspension, followed by incubation at 37°C for 1 h, and the reaction was terminated by adding phenylmethylsulfonyl fluoride to a final concentration of 1 mM. The cells were immediately washed three times with PBS and were finally resuspended in PBS at 0.75 to 1% (vol/vol). Removal of sialic acid from the erythrocyte surface by neuraminidase was confirmed by loss of hemagglutination with sialic acid-specific MAL-II lectin (Vector Laboratories, Burlingame, CA) (data not shown).

Bacteria were cultured in BHI broth overnight at 37°C. Bacterial cells were collected by centrifugation (2,300 × *g*) and resuspended in PBS at an OD_{600} of 3 to 4. The suspension was serially 1.5-fold diluted in PBS in a U-bottom microtiter plate (the volume was adjusted to 100 μl), and then 100 μl of the above-mentioned erythrocyte suspension was added. The plate was kept for 16 h at room temperature, after which hemagglutination was recorded by macroscopic observation and hemolysis was assayed by measuring absorbance at 545 nm.

RESULTS

Genome organization of *C. diphtheriae* C7(−) and PW8. Genomic DNA from *C. diphtheriae* C7(−), PW8, and the reference strain, differentially labeled with Cy3 and Cy5, were independently hybridized with the CGH tiling array covering the entire genome sequence of the reference strain. The intensities of the hybridization signals were normalized, and the ratio of the test sample [C7(−) or PW8] to the reference sample (reference strain) was calculated and used as an index to estimate the presence or absence of the respective regions in C7(−) or PW8, as described in Materials and Methods. It should be noted that regions present in test strains but absent in the reference strain cannot be detected by the CGH technology. Figure 1A shows a typical pattern of signal distribution, observed for C7(−) in a region surrounding the lysogenized

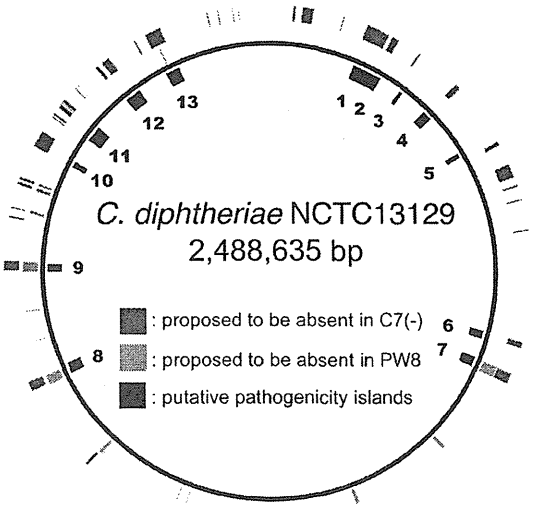

FIG. 2. Circle representation of genome regions proposed to be absent in the C7(−) and PW8 genomes. The solid circle represents the whole genome of *C. diphtheriae* NCTC13129 (reference strain). The putative PAIs are 13 PAIs reported in NCTC13129 by Cerdeño-Tarrága et al. (9). The numbers correspond to PAI numbers in the text and Fig. 3. The artwork was prepared using PlasMapper Web software (14).

corynephage (PAI 1). In the reference row (row 6), signals were seen throughout the region, while in the test row (row 5), signals were lacking in the span corresponding to the lysogenized phage genome (red bar), confirming that the nucleotide sequence for the phage genome was lacking in the C7(−) (test) genome.

Likewise, such deletions were found scattered throughout the genome, as shown in Fig. 1B. The results are also summarized in a circular map, suggesting that they may nonetheless be concentrated in certain areas (Fig. 2). In total, by CGH analysis, at least 37 regions from the published NCTC13129 genome—accounting for 300 CDSs—appeared to be absent from the C7(−) genome (Fig. 2) (for more detail, see Table S2 in the supplemental material).

In contrast, the distribution pattern of absent regions and possibly mutated regions in the PW8 strain was largely different from that observed in the C7(−) strain (Fig. 1C, row 2). The PW8 genome lacked as few as 14 regions, accounting for 98 CDSs. Six of the 14 regions were shared with C7(−), at least partially (Fig. 2) (for more detail, see Tables S2 and S3 in the supplemental material).

In C7(−), 11 of the 13 PAIs previously identified by Cerdeño-Tárraga et al. in 2003 in the reference genome (9) were found to completely or partially overlap the regions found by CGH to be absent. In five cases, the deletion spanned the entire or almost the entire PAI. These cases included PAIs coding for a corynephage genome containing a diphtheria toxin gene (PAI 1), another potential phage genome (PAI 9), structural genes for major and minor pilins, and one putative sortase (PAI 10), as well as two other putative sortases and three putative sortase target pilin genes (PAI 13). In 7 other PAIs (PAIs 2, 4, 6 to 8, 11, and 12), a part of the DNA sequence was still present, but in most cases, it spanned less

than half of each island. In two other small PAIs, PAI 3 and PAI 5, no such large deletions were detected.

In contrast, the PAIs were fairly conserved in the PW8 genome. The two PAIs 7 and 8 were partially absent, and PAI 9 was entirely absent in the genome, but the other PAIs were considered to be present in the genome of the vaccine strain. However, as shown in Fig. 1C, row 2, the putative mutated sites at the single-nucleotide level were suggested to be present in much greater abundance than in the C7(−) genome and were considered to span the whole PW8 genome.

In addition to the PAIs, CGH analysis suggested that at least 26 regions from the reference genome were proposed to be absent in C7(−), as mentioned above. Each region absent in the C7(−) strain contained 1 to 27 CDSs. Twenty-two regions bore 3 or more CDSs. Among them, 19 were flanked by or included inside CDSs annotated as transposases, integrases or their pseudogenes, or tRNA genes. Three exceptions were a 17-CDS region related to nitrogen fixation (CDS identifiers [ID], DIP0492 to -0508) and two 4-CDS regions (DIP0589 to -0592 and DIP1760 to -1763), which were not accompanied by such genes. Another 15 regions, composed of 1 or 2 CDSs per region, did not include and were not associated with any transposases, integrases or their pseudogenes, or tRNA genes, except one (DIP2084) adjacent to a putative transposase gene. In the PW8 strain, the sizes of the 14 absent regions were smaller than those in C7(−). Three major regions corresponded to PAIs 7, 8, and 9 and contained 21, 14, and 22 CDSs, respectively. All of the other regions corresponded to not more than 8 CDSs. Nine of the 14 regions were accompanied or surrounded by transposases and tRNA sequences. The details are summarized in Tables S2 and S3 in the supplemental material for C7(−) and PW8, respectively.

Amplification of DNA fragments corresponding to the selected CDSs located in the 37 regions proposed to be absent in C7(−) was performed with template DNA from C7(−), PW8, the reference strain, ATCC 11951 (C4B), and 10 Japanese clinical isolates using primers listed in Table S1 in the supplemental material. The results are summarized in Fig. 3. The pattern of amplification was diverse, with some CDSs (DIP1820 and DIP1836) detectable only in the reference strain and others, such as DIP2012 (*srtA*) and DIP2300, detectable in most strains, except for a few, including C7(−). The results may suggest greater genetic diversity among *C. diphtheriae* strains than might have been anticipated. It should be noted that putative sortases (DIP2012 [*srtA*], DIP0233 [*srtB*], DIP0236 [*srtC*], DIP2225 [*srtD*], and DIP2224 [*srtE*]) and pilin genes (DIP2013 [*spaA*], DIP2011 [*spaB*], and DIP2010 [*spaC*]), previously shown to be crucial for the adhesion of *C. diphtheriae* to Detroit 562 cells (34), were detected in the PW8 genome, but not in the C7(−) genome, by PCR with primers designed for detection of these CDSs.

Cell adhesion activity of *C. diphtheriae* C7(−) and PW8. We then attempted to assess the pathogenicity of C7(−) and PW8 strains in comparison to that of the reference strain by *in vitro* and *in vivo* experiments. First, we examined adhesion of the *C. diphtheriae* strains to Detroit 562 cells (Fig. 4). Bacterial cells were coincubated with Detroit 562 cells at an approximate multiplicity of infection (MOI) of 5 at 37°C for 1 h. We found that 10 to 20% of the added cells of the reference strain were associated with the human cell surface. The strain C7(−), suggested to lack *srtA* sortase and *spaB* and *spaC* pilin genes, showed reduced adhesion to Detroit 562 cells compared to the reference strain. However, the PW8 strain, in which CGH and PCR analyses suggested the presence of pilin genes, also showed a reduced level of adherence.

Persistence of bacterial cells after intradermal injection. Barksdale et al. (3) reported that toxigenic and nontoxigenic strains of *C. diphtheriae* formed abscesses when injected intradermally into rabbits. We first confirmed their results (see Fig. S1 and S2 in the supplemental material). Toxigenic strains (the reference strain and PW8) formed abscesses surrounded by edema 1 day after injection. A large contribution by diphtheria toxin to the pathogenicity of *C. diphtheriae* was confirmed. Neutralization of diphtheria toxin by 1 IU/site of antitoxin, coinjected with the bacteria, was effective in eliminating the edema, but abscess formation was not affected. The nontoxigenic C7(−) strain formed abscesses to a degree comparable to that of the toxigenic strains, regardless of antitoxin treatment. These results were consistent with those reported by Barksdale et al. (3). On day 2, necrotic plaques developed on sites where toxigenic strains had been injected without neutralization. On the neutralized sites, the sizes of the abscesses varied depending on the strains and numbers of inoculated bacterial cells. The abscess formed by approximately 10^6 CFU of PW8 was smaller than that formed by an equivalent number of the reference strain or C7(−). Not more than 2% of inoculated bacterial cells were recovered from homogenates of any of these abscesses (see Fig. S1 in the supplemental material). Another rabbit was inoculated with the bacteria, and observation was done on day 4. Extension of necrotic plaques was observed, and abscesses were still observable in nonneutralized and neutralized sites. The abscesses caused by PW8 (neutralized) were smaller than those caused by the other strains. In most sites, recovery of viable bacterial cells (see Fig. S2 in the supplemental material) was less than on day 2 in the separate experiment described above.

Although a large part of *C. diphtheriae* pathogenicity can be attributed to the main virulence factor, diphtheria toxin, the results with the nontoxigenic C7(−) strain indicated that the toxin is not the only virulence factor. Using an animal species insensitive to the toxin, concentrated analysis of factors other than the toxin might be possible. Mice are insensitive to diphtheria toxin, except when challenged intracerebrally with a large amount (45) and thus are considered to be suitable for such a purpose. In fact, mice developed abscesses after intradermal injection of *C. diphtheriae* cells. Figure 5A shows the inside of the skin of a mouse 3 days after inoculation of ca. 10^6 (Fig. 5A, a) and ca. 10^7 (Fig. 5A, b) CFU of C7(−), PW8, or the reference strain. Saline was inoculated as a negative control. The abscesses formed by C7(−) or the reference strain were easily separable from the muscle and tightly attached to the skin. The yellowish contents of the abscesses were encapsulated, as revealed by light microscopy (data not shown). In contrast, no abscesses were observed, at least by macroscopic observation, in the mice injected with PW8 or saline alone.

The numbers of viable bacteria recovered from the homogenates of the abscesses were assessed. Figure 5B and C show the ratio of recovered viable cells relative to the inoculum 3 days after inoculation. For an inoculum of approximately 10^7 CFU (Fig. 5C), the fractions of recovered viable bacteria from

Region No.	PAI	CDS ID	Putative function of product	TM1	TM2	TM3	TM4	TM5	TM6	TM7	TM8	TM9	TM10	C4B	C7(-)	PW8	reference	H2O
1		DIP0047	transposase (pseudogene)															
2		DIP0063	two-component sensor															
2		DIP0064	two-comp. response regulator															
2		DIP0075	hypothetical protein															
3		DIP0139	membrane protein															
4	1	DIP0222 (*tox*)	diphtheria toxin															
5	2	DIP0233 (*srtB*)	sortase															
5	2	DIP0235 (*spaD*)	pili component															
5	2	DIP0236 (*srtC*)	sortase															
5	2	DIP0237 (*spaE*)	pili component															
5	2	DIP0238 (*spaF*)	pili component															
6		DIP0278	surface-anchored memb. prot															
7	4	DIP0357	starch degradation															
8		DIP0385	hypothetical protein															
9		DIP0450	secreted protein															
10		DIP0501	nitrate/nitrite transport															
11		DIP0523	membrane protein															
12		DIP0543	sialidase precursor															
13		DIP0589	membrane protein															
13		DIP0592	hypothetical protein															
14		DIP0711	hypothetical protein															
15	6	DIP0756	lantibiotic ABC-transport															
16	7	DIP0816	exported protein															
17		DIP1520	membrane protein															
18	8	DIP1647	membrane protein															
19		DIP1761	exported protein															
20	9	DIP1820	membrane protein															
20	9	DIP1836	collagen-like repeat protein															
21		DIP1895	DNA methylase															
22		DIP1908	phosphate permease															
23		DIP1950	hypothetical protein															
24		DIP1960	exported protein															
25	10	DIP2010 (*spaC*)	pili component															
25	10	DIP2011 (*spaB*)	pili component															
25	10	DIP2012 (*srtA*)	sortase															
25	10	DIP2013 (*spaA*)	pili component															
26		DIP2062	surface anchored protein															
27		DIP2066	fimbrial associated															
28	11	DIP2078	membrane transport															
28	11	DIP2084	hypothetical (pseudogene)															
29		DIP2116	membrane anchored protein															
30		DIP2146	integral membrane protein															
30	12	DIP2153	bacteriophage holin															
31		DIP2161	peptide synthase															
32		DIP2195	integral membrane protein															
33	13	DIP2223 (*spaI*)	pili component															
33	13	DIP2224 (*srtE*)	sortase															
33	13	DIP2225 (*srtD*)	sortase															
33	13	DIP2226 (*spaH*)	pili component															
33	13	DIP2227 (*spaG*)	pili component															
34		DIP2300	DNA-binding protein															
35		DIP2313	restriction/modification															
36		DIP2330	membrane protein															
37		DIP2338	hypothetical protein															

FIG. 3. PCR analysis of selected CDSs in *C. diphtheriae* clinical isolates and laboratory strains. Amplification of DNA fragments corresponding to selected CDSs located in the regions proposed to be absent in the C7(−) genome was performed with template DNA from C7(−), the reference strain, ATCC 11951 (C4B), the vaccine strain PW8, and 10 Japanese clinical isolates, with primers listed in Table S1 in the supplemental material. Pink shading indicates that amplification was successful. Yellow shading indicates that weak bands were observed at positions identical to those of the band observed for the reference strain. Green shading indicates one or more bands observed at a different position(s) from that observed for the reference strain. These bands probably represent nonspecific amplification, because raising the annealing temperature from 54°C to 56°C eliminated such bands as far as we tested. No amplification was observed for the white squares. *, not determined.

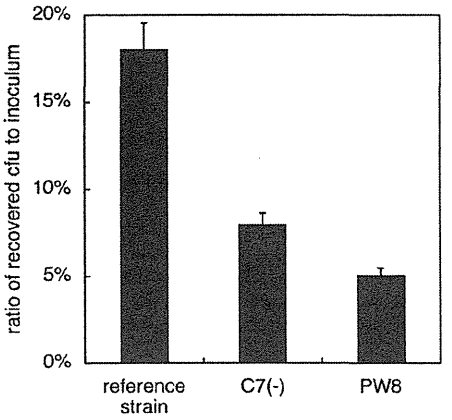

FIG. 4. Adhesion of *C. diphtheriae* to Detroit 562 cells. Adhesion of the *C. diphtheriae* reference strain, C7(−), and PW8 was assayed as described in Materials and Methods. The inoculum sizes were 1.2×10^7 (reference strain), 3.2×10^7 [C7(−)], and 7.1×10^6 (PW8). The bars indicate standard errors from quadruplicate assays. Assays were repeated 6 times, and representative results from one of the assays are shown. $P = 0.004$ [reference versus C7(−)], 0.004 (reference versus PW8), and 0.010 [C7(−) versus PW8] by Student's t test.

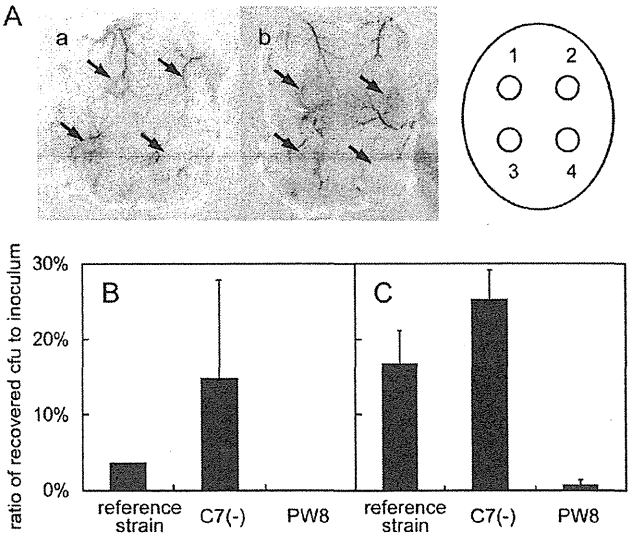

FIG. 5. Mouse intradermal challenge. The backs of female ICR mice (6 weeks of age) were depilated and then intradermally inoculated with *C. diphtheriae* C7(−), the reference strain, and PW8. The mice were sacrificed 3 days after inoculation, and the skins were removed. (A) Internal views of abscesses formed by 7.0×10^6 CFU (a, site 1) and 7.0×10^7 CFU (b, site 1) *C. diphtheriae* C7(−); 2.0×10^6 CFU (a, site 2) and 2.0×10^7 CFU (b, site 2) *C. diphtheriae* PW8; 6.4×10^6 CFU (a, site 3) and 6.4×10^7 (b, site 3) CFU *C. diphtheriae* reference strain; and 0.05 ml of saline (a and b, sites 4). Three mice were used for each of the experiments (a and b), and representative results from each are shown. (B) Recovery of viable cells from abscesses formed by ca. 10^6 CFU of bacterial cells. Abscesses from tissues (shown in panel A, a) were homogenized, and the numbers of viable bacteria in homogenates were measured as described in Materials and Methods. Assays were done in triplicate. $P = 0.56$ [reference strain versus C7(−)]. The error bar [C7(−)] indicates the standard error. The standard error could not be calculated for the reference strain because a set of three data could not be obtained (from one of three mice, viable bacteria were not recovered). The actual numbers of CFU recovered were 1.2×10^5 (reference strain) and 5.2×10^5 [C7(−)]. PW8 was not recovered from any of three mice. (C) Recovery of viable cells from abscesses formed by ca. 10^7 CFU of bacterial cells. Recovery from abscesses from tissues (shown in panel A, b) are shown. $P = 0.046$ [reference strain versus C7(−)], 0.059 (reference strain versus PW8), and 0.018 [C7(−) versus PW8]. The actual numbers of CFU recovered were 5.4×10^6 (reference strain), 8.9×10^6 [C7(−)]), and 7.9×10^4 (PW8). The mouse assays were repeated twice with consistent results.

abscesses for both *C. diphtheriae* C7(−) and the reference strain were around 20%. At an inoculum of approximately 10^6 CFU (Fig. 5B), slightly lower recovery was found, but it was proportionally similar. Recovery of PW8 was much lower than that of the other strains at an inoculum of 10^7 CFU and was undetectable at an inoculum of 10^6 CFU.

Sections of abscesses formed by *C. diphtheriae* C7(−) were investigated by electron microscopy 3 days after inoculation. In abscesses formed by inoculation of C7(−), bacteria were found in vacuoles isolated from the cytosol of phagocytic cells (Fig. 6A, arrows). Bacteria were also found in the cytosol outside vacuoles (Fig. 6A, arrowheads). Lysosomes (Fig. 6A, asterisks) that were not fused with vacuoles were also found within the phagocytic cells. Strain C7(−) was capable of disrupting phagocytic cells (Fig. 6B, arrow), resulting in release of bacteria into the milieu of the abscess contents. Propagating bacterial cells were found in the cytosol (Fig. 6B, arrowhead), showing that the cytosol of mouse phagocytic cells was capable of providing an environment suitable for the growth of this bacterial strain.

C. diphtheriae C7(−) propagation was also observed inside vacuoles. Figure 6C shows growing cells with septa typical of dividing *C. diphtheriae* (arrowheads) (63). The growing bacteria may be released from the vacuole into the abscess milieu outside the phagocytic cells (Fig. 6B, arrow).

Hemagglutination and hemolysis. Log-phase cultures of the *C. diphtheriae* reference strain, C7(−), and PW8 were serially diluted in PBS as described in Materials and Methods, and a human erythrocyte suspension was added and then incubated at room temperature for 16 h. Hemagglutination was induced by the reference strain and was enhanced by pretreatment of erythrocytes with neuraminidase and/or trypsin (Fig. 7A). The removal of sialic acid residues from the cell surface saccharides by neuraminidase and the removal of cell surface protein by trypsin, probably resulting in the unmasking of glycolipids (58), might have contributed to the enhancement. In contrast, for C7(−), hemolysis was prominent (Fig. 7A). Figure 7B illustrates the absorbance of the erythrocyte supernatant at 545 nm after 16 h of incubation with serial dilutions of a C7(−) suspension. Interestingly, neuraminidase or trypsin pretreatment of erythrocytes did not affect the hemolysis (Fig. 7B). The reference strain exhibited much lower hemolytic activity than C7(−), with an A_{545} of <0.05 with a bacterial suspension at an OD of 2.0. The PW8 strain did not show either hemagglutination or hemolysis.

The primary structure of the *srtA-spaABC* region and the DIP1281 locus. The region (PAI 10) previously shown to be crucial for adhesion of the reference strain NCTC13129 to Detroit 562 cells (*srtA-spaABC*) by Mandlik et al. (34) was shown to be present in the PW8 strain. However, the vaccine strain showed greatly reduced adhesion activity to the human pharyngeal cells. We therefore determined the nucleotide se-

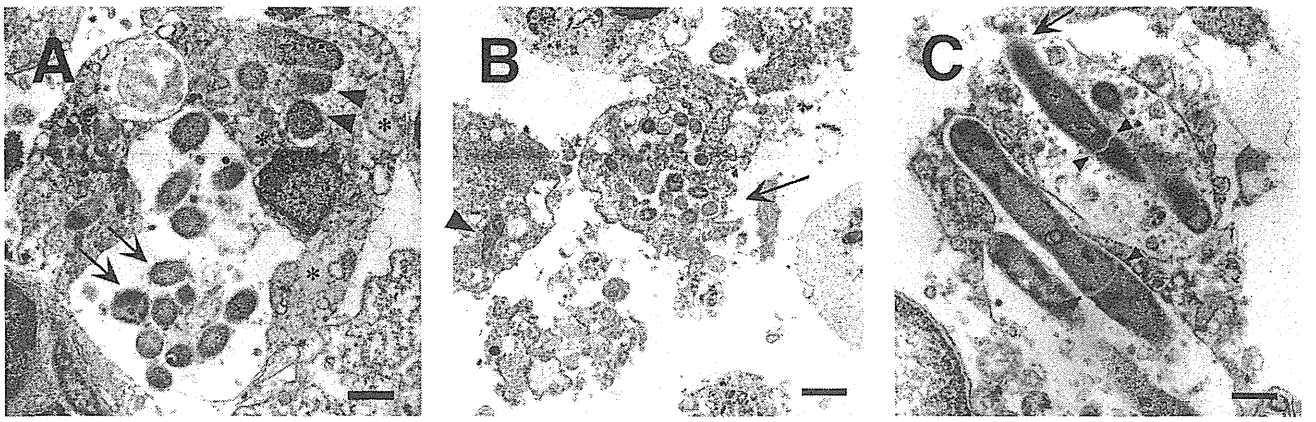

FIG. 6. Electron microscopic appearance of abscess contents. Mice were inoculated with *C. diphtheriae* C7(−) (9.5×10^7 CFU), and the abscesses were removed and prepared for electron microscopy as described in Materials and Methods 3 days after inoculation. (A) Mouse phagocytic cells containing *C. diphtheriae* C7(−). The arrows indicate bacteria enclosed in a vacuole-like structure. The arrowheads indicate bacteria in the cytosol. The asterisks indicate lysosomes. Original magnification, ×5,000; bar, 1 μm. (B) Disintegrating mouse phagocytic cells containing *C. diphtheriae* C7(−). The arrow indicates a disrupted cytoplasmic membrane and vacuole-like structure from which bacteria could escape into the abscess milieu. The arrowhead shows a dividing bacterial cell in the cytosol. Original magnification, ×3,000; bar, 2 μm. (C) Dividing *C. diphtheriae* C7(−) cells in vacuole-like structures in a mouse phagocytic cell. The arrow indicates a disrupted membrane. The arrowheads show septa, typical of dividing *C. diphtheriae* cells. Original magnification, ×10,000; bar, 500 nm.

quence of the region. The *srtA-spaABC* region was not in an intact form. The sequence of the sortase gene *srtA* (DIP2012; accession number AB562324) was 95.4% identical to that in the reference strain. At the amino acid level, 273 out of 289 residues (94.5%), including His160 and Cys222 at the active center (23, 61), were identical. A lower identity, i.e., 90.2% at the nucleotide level and 89.1% at the amino acid level, was observed for the gene for the pilus backbone subunit (*spaA* [DIP2013; accession number AB562325]), with a conserved LPLTG motif at the C terminus. The genes for the other two important minor pilins (*spaB* and *spaC*) contained large and small internal deletions. The *spaB* gene (DIP2011; accession number AB562326) contained two in-frame deletions, 9 bp and 3 bp, exactly accounting for 3 and 1 amino acid residues, respectively. That is, the PW8 gene was 92.7% identical to that of the reference strain at the nucleotide level. The *spaC* gene (DIP2010; accession number AB562327) was largely impaired by a 470-bp deletion, which resulted in a frameshift mutation. Two other deletions (3 and 9 bp) were found near the 5′ and 3′ ends of the *spaC* region, respectively. The overall sequence identity with the *spaC* gene sequence from the reference strain, except the deletions, was 94.7% at the nucleotide level.

Recently, another surface-anchored protein (DIP1281), initially annotated as a putative invasion protein, was shown to function as an adhesion factor of *C. diphtheriae* to Detroit 562 cells (43). We thus compared the nucleotide sequences of the gene among the three strains {accession numbers AB562328 [C7(−)] and AB562329 [PW8]}. All three of the strains possessed the DIP1281 gene, and no internal deletion or insertion was found in any of them. The identities of the nucleotide sequences were 98.3% [reference strain versus C7(−)], 98.5% (reference strain versus PW8), and 99.3% [C7(−) versus PW8], respectively. The differences in the nucleotide sequences were reflected in 8 [reference strain versus C7(−)], 10 (reference strain versus PW8), and 4 [C7(−) versus PW8] different amino acid residues, respectively.

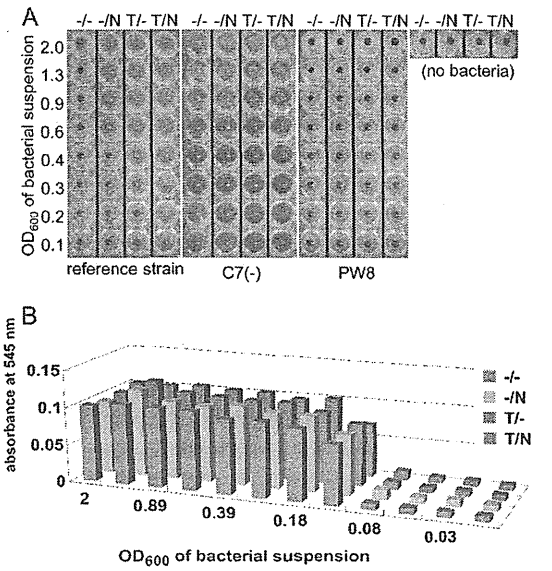

FIG. 7. Hemagglutinating and hemolytic activities of *C. diphtheriae*. Dilutions of the *C. diphtheriae* reference strain, C7(−), and a human erythrocyte suspension were prepared as described in Materials and Methods. The trypsin and neuraminidase treatments are also described in the text. The bacterial suspensions were mixed with erythrocyte suspensions in a 96-well microtiter plate; after incubation at room temperature for 16 h, hemagglutination was scored macroscopically and hemolysis was measured by absorbance at 545 nm. (A) Hemagglutination by the reference strain. −/−, untreated erythrocytes; T/−, treated with trypsin; −/N, treated with neuraminidase; T/N, treated with both trypsin and neuraminidase; no bacteria, control without bacterial suspension. (B) Measurement of hemolytic activity by C7(−). OD_{600} of bacterial suspension is the dilution of C7(−) suspension expressed in OD units; absorbance at 545 nm is the absorbance of the supernatant of the mixture after incubation for 16 h. −/−, untreated erythrocytes; −/N, treated with neuraminidase; T/−, treated with trypsin; T/N, treated with both trypsin and neuraminidase.

DISCUSSION

The C7(−) and PW8 strains of *C. diphtheriae* are two of the oldest strains available to researchers and are the main standard strains utilized in analysis of *C. diphtheriae* pathogenesis and vaccine production (10, 20, 32, 46, 48–51). The C7(−) strain, which can infect humans (3), was considered to lack most PAIs but still showed signs of pathogenicity, whereas the nonpathogenic (32) PW8 strain retained more PAIs but showed much reduced signs.

By comparative genomic hybridization, the C7(−) strain appeared to lack 11 of 13 PAIs. The total number of NCTC13129 (reference strain) genomic regions that we found to most likely be missing from the C7(−) genome, including PAIs, was at least 37. This corresponds to 300 CDSs, approximately 12.5% of the total number of CDSs in the reference strain.

In contrast, many fewer, i.e., 14, regions were considered to be absent in the vaccine strain PW8, isolated earlier than the C7(−) strain (46). Among the absent regions, only three were related to PAIs. However, the difference at the single-nucleotide level between the PW8 genome and the reference genome was suggested to be greater than that between the C7(−) and reference genomes (Fig. 1C, row 2). In addition, large genomic diversity was observed among various *C. diphtheriae* strains and clinical isolates (Fig. 3).

The regions of the reference genome that appeared to be absent in C7(−) or PW8 were in most cases flanked by, or associated with, insertion sequence (IS)-related transposases, phage-related transposases, or tRNA sequences. Short (less than 100-bp) direct repeats were also seen. In C7(−) and PW8, some of these regions were shown to be replaced by DNA fragments of various sizes, and some were shown simply to be absent, leaving one of the direct repeats (data not shown). Taken together with our preliminary PFGE results showing that the size of the C7(−) genome is larger than that of the reference genome (see Fig. S3 in the supplemental material), it may be possible that at least some of the replacing fragments have a high degree of mobility by horizontal gene transfer. Detailed analysis of such fragments would be possible by genome sequencing of the C7(−) strain, as CGH technology is not capable of detecting and analyzing such regions.

Adhesion of *C. diphtheriae* to human epithelial cells, followed by internalization, was demonstrated and analyzed in detail by Hirata et al. in 2002 (26) using HEp-2 cells and later by Bertuccini et al. in 2004 (6) using HEp-2 and Detroit 562 cells. One of the signs of pathogenicity for C7(−) and PW8 strains was such adherence. In our study, C7(−) showed adherence to Detroit 562 cells at a level comparable to that of the reference strain, and PW8 showed a much reduced level of adherence compared to these strains. Mandlik et al. (34) reported that disruption of the sortase (*srtA*) and/or pilin (*spaB* or *spaC*) gene resulted in almost complete loss of the adhesive properties of the *C. diphtheriae* reference strain (34). Our results (Fig. 4) indicate that the C7(−) strain, lacking all of these genes, showed reduced adhesive properties compared to the reference strain. However, the activity was still higher than that of the Δ*spaBC* mutant investigated by Mandlik et al. (34), in which, based on our recalculation of their data, less than 2% of the inoculum adhered to the Detroit 562 cells. This suggests that the C7(−) strain possesses additional mechanisms that compensate for the lack of minor pilins. As for the larger genome size of C7(−) than of the reference strain, C7(−) could possess genetic information undetectable by CGH analysis, which might include genes sufficient for mediating adhesion to mammalian cells. This is not the case in the PW8 strain, where the *srtA-spaABC* region was present, but not in an intact form. The *srtA* sortase gene could be functional, because the two important residues His160 and Cys222 at the active center (23, 61) were conserved. However, *spaC*, one of the minor pilin genes crucial for attachment to the target cell surface (34), was largely affected by a 470-bp internal deletion and a concomitant frameshift mutation. The reading frame of another minor pilin gene, *spaB*, was not impaired, while two small in-frame deletions were found inside the gene. The observed differences in the pilus structure, especially the impairment in the *spaC* gene, might account for the reduced pathogenicity of the vaccine strain. According to Mandlik et al. (34), single-deletion mutants of *spaB* or *spaC* exhibited reduced adhesive activity, but it was not as reduced as that in the double mutant Δ*spaBC*. This is consistent with our results obtained with PW8, in which the *spaB* structural gene could be functional. Further, concerning the extent of the suggested single-nucleotide level differences from the reference genome scattered throughout the PW8 genome (Fig. 1C, row 2), other possible mechanisms contributing to pathogenicity might also be impaired in PW8. Mandlik et al. (34) also showed that a Δ*srtA* variant of the reference strain NCTC13129 still exhibited approximately 30% adhesion to Detroit 562 cells, indicating that *spaABC*-type pili are not solely responsible for adhesion to Detroit 562 cells. In addition, a Δ(*srtA-srtF*) variant showed further reduced adhesion, suggesting the contribution to adhesion of some unknown factor mediated by at least one of the sortases encoded by *srtB-srtF*. On the other hand, in the present study, C7(−) and PW8, both of which are considered to lack *spaD*- and *spaH*-type pili mediated by sortases encoded by *srtB* and *srtC* (19) and *srtD* and *srtE* (59), respectively (Fig. 3), still exhibited reduced but significant binding to Detroit 562 cells. The possibility remains that factors other than pili contribute to the cell adhesion in the cases of C7(−) and PW8.

On the other hand, a recently identified virulence factor, DIP1281, initially annotated as a putative invasion protein and later revealed to be an adhesion factor of nontoxigenic *C. diphtheriae* to Detroit 562 cells (43), was present in all three of the strains with much less diversity than the *srtA-spaABC* region. The well-conserved DIP1281 gene may account, at least in part, for the cell adhesion still observed in strains lacking functional pili.

Another sign of pathogenicity was persistence of inoculated bacteria in mouse skin abscesses. Comparable levels of persistence of inoculated bacteria in abscesses were also shown for the reference and C7(−) strains when injected into mouse skin. To develop the mouse model, we first reproduced the results of the original experiments of Barksdale et al. (3) using rabbits (see Fig. S1 and S2 in the supplemental material). Toxigenic strains were shown to form abscesses, and the lesions were diminished by neutralizing antibody (antitoxin), indicating the large contribution of the toxin to the pathogenesis of *C. diphtheriae*. However, the nontoxigenic strain C7(−) was also able to form abscesses that were not neutralized by the antitoxin, suggesting the presence of pathogenicity factors

other than the toxin. In the intradermal-injection system of Barksdale et al., physical invasion was used to aid the entry of the bacteria into host tissue. The model reflects one of the major forms of human diphtheria, cutaneous diphtheria, as sometimes associated with physical invasion, such as that due to insect bites (12). Experimental infection models for several *Corynebacterium* species of veterinary importance (*C. kutscheri* [8], *C. bovis* [28], and *C. pseudotuberculosis* [47]) also employ physical invasion mechanisms, including intradermal injection. In our rabbit experiments, we confirmed the necrotizing effect of diphtheria toxin by inoculating toxigenic strains (the reference strain and PW8). In addition, abscess formation by the nontoxigenic C7(−) strain was observed as another sign of pathogenicity, which has been reported by Barksdale et al. (3). Diphtheria antitoxin neutralized the necrotizing effects of toxigenic strains but did not inhibit abscess formation (compare Fig. 5, sites 1 and 2), indicating that the pathogenicity of *C. diphtheriae* could not be fully attributable to the toxin.

We then transferred the rabbit system to mice, an animal species insensitive to diphtheria toxin. The abscesses formed by *C. diphtheriae* were shown to contain viable bacterial cells 3 days after injection, which survived for at least 13 days (data not shown). Due to the greater persistence of viable bacterial cells in mice than in rabbits, mice could be good candidates for a *C. diphtheriae* skin infection model with the additional benefit of easier handling than rabbits, although the system is not suitable for assessing the overall pathogenicity, including the effect of the toxin. The maximum persistence of C7(−) and the reference strain was not very pronounced compared to the results of experimental infection models reported for other bacterial species (2, 11, 30, 53, 64) but was greater than that of PW8. Multiple bacterial cells were found in the vacuoles, some of which exhibited growth septa (Fig. 6B and C) for the C7(−) strain. These results are consistent with previous observations by other groups. Thus, vacuoles containing bacteria have also been observed in *in vitro* studies using HEp2 and Detroit D562 cells infected with nontoxigenic (6) and toxigenic (26) *C. diphtheriae* strains. Recently, dos Santos et al. demonstrated the survival of the C7(−) strain in human U-937 macrophages (16). Bacteria were also found in the cytosol outside vacuoles (Fig. 6A and B, arrowheads). The escape of bacteria from vacuoles into the cytoplasm has been reported for *Listeria*, *Shigella*, and *Rickettsia* (15, 21).

The third sign of pathogenicity was associated with human erythrocytes. The reference strain showed hemagglutination, whereas C7(−) exhibited hemolysis. PW8 showed none of these activities (Fig. 7). Mattos-Guaraldi et al. have also reported great diversity among *C. diphtheriae* strains in hemagglutinating activity (36). The results show that *C. diphtheriae* has diversity in pathogenicity, as well as in genome organization. In addition, the hemagglutination by the reference strain could be dependent on a sugar moiety on the erythrocyte surface, as shown by enhancement by trypsin and/or sialidase pretreatment of erythrocytes. The results suggest that adhesion of the bacterium to target cells upon respiratory infection might also be dependent on cell surface saccharides.

The high degree of genome plasticity in *C. diphtheriae* showed that the species could be more diverse than had been anticipated. Intraspecies genome diversity largely differs from species to species. In species such as certain mycobacteria, chlamydiae, and streptococci, genome diversity has been shown to be small, and the presence of very few or no PAIs has been demonstrated (54). Possibly this is not the case for *C. diphtheriae*. Further studies on various *C. diphtheriae* strains may reveal novel virulence factors of the pathogen.

ACKNOWLEDGMENTS

We thank Yoshiaki Nagaoka, Ayako Harashima, and Ikuyoshi Hatano for excellent experimental support in animal manipulation, light microscopy, and electron microscopy, respectively. We are also grateful to Takuya Iwasaki for helpful and stimulating discussions.

This work was supported in part by Health and Labor Sciences Research Grants (Research on Emerging and Reemerging Infectious Diseases and Research on Pharmaceutical and Medical Safety, H21-Iyaku-Ippan-012) from the Ministry of Health, Labor and Welfare of Japan and in part by a grant from the New Energy and Industrial Technology Development Organization (NEDO) Hatanaka Project (to A.I.).

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Editor: J. B. Bliska

総　説

ジフテリア毒素原性 *Corynebacterium ulcerans* の感染症
Infectious diseases caused by Toxigenic *Corynebacterium ulcerans*

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1　疾患の概要

Coryne 属菌は自然界では常在細菌として知られ，人をはじめ種々な動物の臨床材料から多くの菌種が分離・報告されている．人のジフテリアの起因菌である *Corynebacterium diphtheriae* は，ジフテリア毒素を産生する代表的な *Coryne* 属菌である．ジフテリアの病因分析は古来より多くの報告があり，その感染経路，伝播性，病態等が明らかとなっている．一方，*Corynebacterium ulcerans* (*C. ulcerans*) は，牛の乳房炎 [8] や複数の動物における一般的な化膿を引き起こす菌として知られ [1-3]，海外では人の咽頭炎や扁桃炎の起因菌としての報告もある [4]．さらに，人のジフテリアにおける咽頭痛，発咳，発熱および偽膜・白苔が観察される患者から *C. ulcerans* が分離され，ジフテリア毒素遺伝子を有し，ジフテリア毒素を産生する菌 (*C. ulcerans*$^{tox+}$) であることが明らかとなっている [5-7]．患者の環境調査で，感染経路として牛，馬などの畜産動物や犬，猫などの愛玩動物の関与も強く疑われている [8, 9])．人と動物の共通感染症の分類・定義に従えば，動物から人へ伝播することは明らかであるが (Zooanthroponoses)，人から動物 (Anthropozoonoses) または両方から伝播する (Amphixenose) かは，現在のところ不明である．国内外での *C. ulcerans* 及び *C. ulcerans*$^{tox+}$ の詳細な分布調査報告はなく，本菌による人及び動物の疫学的，細菌学的調査は重要である．

なお，本稿は国立感染症研究所ホームページで公開している情報をもとに再編集および追加記載しており，以下のURLを有効に参照されたい．

http://www.nih.go.jp/niid/bac2/Coryne_ulcerans/

2　病因・症状・病態

C. diphtheriae によるジフテリアは上気道粘膜に主に感染し，鼻腔・咽頭及び喉頭が感染部位の場合は気管や気管支よりも深部気道までに及ぶことも報告されている．一般的に上気道では咽頭ジフテリアが多く，喉頭，鼻腔は比較的少ない．症状としては，発熱 (38～39℃)，狭窄性の夏声，犬吠様の咳が観察される．局所の腫脹 (ブルネック)，偽膜形成，狭窄による呼吸障害，声門等の白苔および呼息性の呼吸困難も報告されている．*C. ulcerans*$^{tox+}$ によるジフテリアも重篤な場合は死亡報告があり，典型的な症状が現れる場合は上記と同様の臨床経過が観察されている．

3　人の感染症

国内のジフテリア患者の年間報告数 (厚生省統計) は昭和20年 (1945年) には8万6千人であったが，昭和60年 (1985年) には100人程度，2000年以降にあってはゼロとなっている．患者の急激な減少には，1950年代から導入されたジフテリアトキソイド製剤 (液状ジフテリアトキソイド，沈降ジフテリア破傷風混合トキソイド，沈降精製百日せきジフテリア破傷風混合ワクチン等) の効果および医療の発展に負うものである．一方，ジフテリア様症状を呈する患者から *C. ulcerans*$^{tox+}$ が報告された事例を表1に示した．国内では2001年に初めて千葉県旭中央病院で確認された．翌年，同じ医師により2例目が，2005，2006年にさらに3例が確認された．最近では，平成21年1月に国内6例目の患者が確認された [10-14]．1例目の患者は，2001年2月に52歳の女性が，呼吸困難，嗄声，咽頭痛，咳，発熱，上咽頭と喉頭前庭に白色偽膜を呈して入院し，一時呼吸困難を呈してICU管理となった．病院検査課で *C. ulcerans* を分離し，後日 *C. ulcerans*$^{tox+}$ であることが確認された．2例目の患者は1例目と同一病院で2002年10月に咽頭痛，発熱，上咽頭と右咽頭側索に偽膜が観察され，比較的軽症であり，1例目の患者と住居は同地区であった．3例目の患者は2005年9月に岡山県 (58歳，男性) で，左耳下腺部腫脹，軽度の咳が観察され，感染前に慢性皮

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日獣会誌　63　813～818 (2010)

表1　国内患者からジフテリア毒素産生性 *C. ulcerans* 分離例

患　者	場　所	発症年月	年齢・性	主　な　症　状	感染と動物の関係
症例1	千　葉　県	2001年2月	52歳　女	呼吸困難，嗄声，咽頭痛，咳，発熱	屋外の猫約20匹に給餌
症例2	千　葉　県	2002年10月	54歳　男	咽頭痛，発熱，水様性鼻漏，偽膜	症例1と同一病院，同一地域
症例3	岡　山　県	2005年9月	57歳　男	左耳下腺部腫脹，軽度の咳	慢性皮膚炎の犬が死亡
症例4	大　分　県	2005年10月	51歳　男	肺に多発性空洞病変，咳，痰，発熱	屋外の猫12匹に給餌
症例5	神奈川県	2006年7月	58歳　女	上咽頭，鼻腔に白苔，若干の喉頭腫脹	
症例6	東　京　都	2009年1月	57歳　女	咽頭痛，発熱，水様性鼻漏，偽膜	屋外の猫5匹に給餌

表2　動物の *C. ulcerans* の分離・調査結果（平成21年度）

都道府県	対象施設	対象動物	材　料	調査数	菌陽性数	抗体陽性数
A	動物管理センター	犬	咽頭スワブ	76	0	ND
B	動物病院	猫	咽頭スワブ	32	2	ND
	畜産課	アライグマ	咽頭スワブ	55	0	ND
		ハ　ト		26	0	ND
C	動物管理センター	猫	咽頭スワブ	78		ND
D	動物管理センター	犬	咽頭スワブ	63	5	ND
	動物管理センター	猫	咽頭スワブ	29		ND
E	動物管理センター	犬	咽頭スワブ	50	1	ND
	動物管理センター	猫	咽頭スワブ	51	4	ND
F	動物病院	犬	咽頭スワブ	36	0	ND
	動物病院	猫	咽頭スワブ	27	0	ND
G	動物管理センター	犬	咽頭スワブ	11	0	ND
	動物管理センター	猫	耳　垢	2	0	ND
H	動物病院	猫	咽頭スワブ	36	0	ND
	動物管理センター	犬	咽頭スワブ	27	0	ND
	動物管理センター	猫	咽頭スワブ	85	5	ND
	と畜場	牛	鼻腔スワブ等	65	0	ND
静岡県	動物病院	猫	鼻水等	1	1	1
香川県	動物病院	猫	鼻水等	85	7	
	動物病院	犬	鼻水等	10	0	11
岐阜大（西日本）	猟　師	猟　犬	鼻腔スワブ等	154	2	13
大阪府立大（大阪府，奈良県）	保健所	乳用牛	乳	75	0	ND
	獣医臨床センター	猫	咽頭スワブ	3	0	ND
	食肉流通センター	肉用牛	咽頭スワブ	124	0	ND
	動物園	サ　ル	咽頭スワブ	22	0	ND
		ペンギン	咽頭スワブ	5	0	ND

膚疾患の飼育犬が死亡していた．4例目の患者は，2005年10月に大分県で咳，痰，発熱症状を呈し，肺の多発性空洞病変部から *C. ulcerans*$^{tox+}$ が分離され，野良猫を含め，猫を自宅で12頭飼育していた．5例目の患者は，2006年7月に神奈川県の慢性関節リウマチ患者（結腸癌患者）が，咽頭痛，鼻閉感，口蓋垂・上咽頭・鼻腔に白苔，喉頭腫脹，咳，嗄声，発熱を訴え，検査の結果，当該菌を検出した．6例目の患者は，2009年1月に1例目と2例目の診断治療を担当した医師が東京都に転勤となり，一般診療中に，くしゃみと水様性鼻漏，鼻かみにて左鼻出血，咽頭痛，嗄声，左鼻腔粘膜，上咽頭，中咽頭後壁に偽膜を伴う炎症性病変，左上不深頸リンパ節の腫脹と圧痛が観察された加療中の関節リウマチ患者の咽頭スワブの検査で *C. ulcerans*$^{tox+}$ を分離した．

4　動物の感染症

平成22年3月までに国内の調査で *C. ulcerans* または *C. ulcerans*$^{tox+}$ が分離された結果を表2に示した．日本国内で発生した6例の患者のうち，3例は風邪様症状を呈した野良猫との接触，及び1例は長期間皮膚炎の犬の飼育歴が確認されている．国内6例目のジフテリア様疾患患者の環境調査で，自宅に集まる野良猫の1匹は風邪様症状を呈してクシャミ，鼻水を飛散していたために自分が感染しないように注意していたが，その後咽頭炎等が発現したことが判明した．野良猫から患者と遺伝子型が一致する *C. ulcerans*$^{tox+}$ を分離し，さらに子猫からも

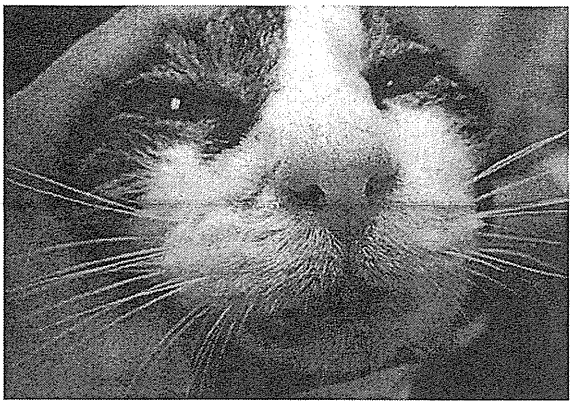

図1　*C. ulcerans*$^{tox+}$が分離された家庭猫に観察された鼻汁　（提供：小川　高獣医師　静岡県）

同菌を分離した［14］.

平成19年11月27日から20年12月26日にかけて大阪府が収容した犬管理センターの犬583頭を調査対象とした調査では，44頭から*C. ulcerans*$^{tox+}$が検出され，41頭からは毒素産生株，2頭からは毒素非産生株，1頭からは毒素産生株及び毒素非産生株が同時に検出された［15］.

21年度の厚生労働科学研究班の調査では，数カ所の自治体における動物愛護センターに搬入された犬や猫の咽頭スワブ，及びと畜場に搬入された牛や豚の咽頭スワブ等から菌分離調査を実施した結果，6カ所の愛護センターで6頭の犬，9頭の猫より*C. ulcerans*$^{tox+}$が分離された.

国内6例目の患者発生事例に基づき，本菌による患者発生状況と情報提供の通知が結核感染症課長から各衛生担当者に行われた．これにより，日本小動物獣医師会の感染症部会等の協力を得て共同調査を実施した結果，一般家庭で飼育している合計10頭の猫から*C. ulcerans*$^{tox+}$が分離され，多頭飼いの同居猫にはジフテリア抗毒素を保有し過去の感染既往も確認した［16］.

犬，猫及び畜産動物に感染させるホスト動物として野生動物が考えられたが，野生動物と接触機会の多い猟犬の血清中のジフテリア抗毒素保有状況を調査した．その結果，複数の地域の猟犬において複数犬で陽性を確認した．これら猟犬で菌分離の追跡調査を実施した結果，同居犬の1頭から*C. ulcerans*$^{tox+}$を分離した．なお，過去の調査で，大阪府及び千葉県内の一般家庭で飼育されている犬の約300頭の血清中の抗毒素抗体及び咽頭スワブからの*C. ulcerans*$^{tox+}$分離調査の結果では，両者とも陰性であった.

畜産動物の調査として，東北，近畿地区のと畜場に搬入された健康牛，病感牛及び一般農家の乳房炎罹患牛の生乳を検査した結果，*C. ulcerans*及び*C. ulcerans*$^{tox+}$いずれも陰性であった.

海外における*C. ulcerans*$^{tox+}$の分離報告は，英国で1986年から2002年の間に47例あり，西欧，米国でも散発的に報告がある［17-20］．フランスの犬は，慢性の口唇部潰瘍やくしゃみが観察されており，飼い主が重篤なジフテリアを呈したために，人への感染が強く疑われている［21］.

5　感染症法の届出義務

ジフテリアは急性灰白髄炎，コレラ，細菌性赤痢，腸チフス及びパラチフスとともに「二類感染症」として扱われる．主な措置の特徴は，擬似症患者も対象となり，入院の勧告・措置及び輸送が適用される．届け出は，厚生労働省が定めた「医師から都道府県知事等への届出のための基準」には，下記のように記されている.

《報告のための基準》

○診断した医師の判断により，症状や所見から当該疾患が疑われ，かつ，以下の方法によって病原体診断がなされたもの.

（材料）病変（感染）部位からの採取材料

・病原体の検出

　ジフテリア菌の分離と同定，ならびに分離菌におけるジフテリア毒素の検出

・病原体の遺伝子の検出

例，PCR法など

http://www.mhlw.go.jp/bunya/kenkou/kekkaku-kansenshou11/01-02-03.html

C. ulcerans^{tox+}については，現状の法解釈ではジフテリアとしての届け出条件をすべて満たしていないために，*C. ulcerans*$^{tox+}$感染症が*C. diphtheriae*による伝播性，患者の重篤性に違いがないかの調査が必要である.

なお，厚生労働省では*C. ulcerans*$^{tox+}$感染による2例の患者報告を受けて，平成14年11月20日付けで健康局結核感染症課長通知（健感発1120001号）として，本菌による患者発生および現場からの情報提供を各地方衛生主管部長宛に以下の通知をしている．さらに，6例目の患者発生に際しては，猫から感染した可能性が高いことについて，以下のように関係者へ再通知した（平成21年7月22日　健感発0722第3号）.

（1）ジフテリア様症状を呈した患者については，ジフテリア菌のみならず*C. ulcerans*による感染の可能性もあること，

（2）*C. ulcerans*による感染が疑われた場合は，都道府県を通じて国立感染症研究所で検査が可能であること，

（3）*C. ulcerans*による患者と診断した場合は，患者の同意を得て保健所を通じて情報提供をするように求めた.

海外の本菌による感染症の取り扱いは，英国では，毒

素原性*C. diphtheriae*, *C. ulcerans*$^{tox+}$による感染症をジフテリアとする．フランスでは，2002年から*C. ulcerans*$^{tox+}$がジフテリアの定義に含まれることとなった．米国では，ジフテリアの実験室診断は*C. diphtheriae*を分離し組織病理的に診断することを求め，*C. ulcerans*, *C. pseudotuberculosis*が分離された場合にも菌株をCDCに送ることとなっている．

6 病原体・発生・流行状況

C. diphtheriae^{tox+}のジフテリア毒素は，易熱性のタンパク毒素であり，ウサギの皮内やモルモットの皮下に注射すると局所の浮腫，充出血・壊死等が観察される．また，発症した患者は毒素により，心不全による循環器系障害，四肢の筋肉及び呼吸筋などの麻痺（ジフテリア後神経麻痺）をおこすと考えられる．毒素の直接作用は，動物細胞の膜レセプターに結合し，細胞膜から細胞内に侵入しペプチド伸長因子（EF-2）を不活化することによりタン白合成を阻害し，細胞を死に至らしめる．

一方，*C. ulcerans*$^{tox+}$の産生する毒素は，*C. diphtheria*の産生毒素やトキソイドで免疫した血清で中和される．*C. ulcerans*ジフテリア毒素ばかりでなく，*Corynebacterium ovis*の産生する毒素と同一の毒素活性（出血活性と壊死活性）を認め，この両毒素は分子量，生化学的及び免疫学的性状に違いあることが証明されている［23］．

国内で分離した*C. ulcerans*$^{tox+}$と*C. diphtheriae*$^{tox+}$の生化学的な相違点は，すべての*C. ulcerans*$^{tox+}$はPhospho Lipase Dを産生することを確認した．また，アミノ酸配列の解析から両菌の毒素遺伝子については異なることが明らかとなった．Phospho Lipase Dは，*C. diphtheriae*の産生するジフテリア毒素の作用は，心筋炎や後麻痺と関連が明らかであるが，*C. ulcerans*のPhospho Lipase Dとの病原性は不明である．

C. ulcerans^{tox+}の海外での分離報告としては，英国では1986年から2002年の間に47例あり，西欧，米国でも散発的に報告がある．フランスの犬は，慢性の口唇部潰瘍やくしゃみが観察されており，飼い主が重篤なジフテリアを呈したために，人への感染が強く疑われている．なお，海外報告については国立感染症研究所のhttp://www.nih.go.jp/niid/bac2/Coryne_ulcerans/world.htmlを参照されたい．

7 検査・診断

犬や猫においては臨床観察だけで本症の確認は困難である．従って，病原体診断には，鼻水，皮膚炎，咽頭スワブ等を採取し，検査を実施する．スワブをチンスダール培地，亜テルル酸塩加血液寒天培地などの選択培地に塗布し培養する．培地上に出現した黒色のコロニーをDSS培地に移植し，上層部が青色，中層部が透明な菌を指標として分離・同定する．この際にPCRによりジフテリア毒素遺伝子の検出試験を組み合わせて分離すると効率的である．分離菌はAPIコリネキット等の簡易生化学試験キットで簡易同定を行なうとともに，毒素産生性の試験を行う．毒素産生性の試験には，培養上清をモルモットやウサギなどの感受性動物に接種して出血や壊死活性を指標とする動物試験，レフレル培地で培養後の凝固水中にVero細胞に対する細胞毒性を指標とする*in vitro*試験，培地中に産生された毒素を免疫学的に検出するエレク試験，菌の毒素遺伝子を検出するPCR試験がある．採取材料から，菌分離を経由せずに直接PCRによる毒素遺伝子の検出が可能な場合があるが，採取材料からジフテリア菌を分離し，分離菌の毒素産生性を確認することが大切である．

8 予防・治療

C. ulcerans^{tox+}による動物からの感染予防としては，風邪様症状（鼻水，クシャミ等），皮膚炎の動物と接した後には手と衣類の消毒を徹底する．また，多頭飼育で感染動物が発生した場合には，予防目的で人用のジフテリアトキソイドの代用接種も考慮する．このジフテリアトキソイドの開発の歴史は古く，1921年Glennyらにより始まり，現在は乳幼児に接種される有効性と安全性の高いワクチンのひとつである．国内では，1948年に予防接種法が制定されるとともに，液状ジフテリアトキソイドが導入され，その後，数種の混合型のワクチンが接種対象者の目的ごとに開発された．現在では沈降精製百日咳ジフテリア破傷風混合ワクチン（DTaP）が幼児期の免疫用に広く用いられ，患者制圧に効果を挙げている．ジフテリアは，ワクチン接種により防圧可能な疾病であり，また，“予防は治療に勝る”．

治療は抗生剤投与と抗毒素療法が効果的である．ジフテリア菌に対しては抗菌薬としてエリスロマイシンやペニシリンGを2週間投与する．*C. ulcerans*感染症の治療に関しても，英国のガイドラインでは抗毒素療法と抗菌薬の併用が推奨されている．ジフテリアの発症において毒素が極めて重要な役割を演じているので，抗毒素をもちいた毒素の中和が必要である．その他，気道閉塞に対する気道確保や徐脈性不整脈に対するペースメーカーの使用など呼吸循環管理について充分注意し治療を行う．

C. ulcerans^{tox+}を保菌する恐れのある病的な愛玩動物や畜産動物と濃厚または常時接触する場合は，不適当な処理の乳製品［26, 27］については，感染源となる可能性が有るので適切な消毒や滅菌が必要である．

9 おわりに

人と動物の共通感染症をはじめとする新興感染症の制圧は，医者，獣医師と他の科学的な医療専門職が綿密な関係を持ち，人間と動物と，その環境を網羅的に管理することが望ましいことが，近年「One health」「One world, One health」[One medicine] とする標語で提唱されている．*C. ulcerans*$^{tox+}$による感染症についても，国内外の疫学調査や病因・毒素の基礎研究で人と動物の共通感染症として警戒が必要な科学的情報が収集されている．

一般的にコリネ属菌は皮膚等の一般細菌叢として分離され，*C. ulceras*もこれら細菌叢の一部として存在している可能性もある．現在のところ，一般家庭では野外活動時間が長い犬と猫から*C. ulcerans*$^{tox+}$分離が確認されており，それらの動物は皮膚炎，風邪様症状が観察されており，飼い主の適正な管理が求められる．一般家庭で飼育する猫は，特に冬場では鼻水，クシャミは日常的に観察される．これらの猫は猫白血病ウイルスが陽性であることも多く，免疫力が低下していることも指摘されている．さらに，犬，猫の中に*C. ulcerans*$^{tox+}$が潜在している可能性が示されたことは，特にジフテリア抗毒素抗体（免疫）のない，または基礎疾患により治療・投薬により免疫力が低下している人への感染には注意を要する．

稿を終えるにあたり本研究に協力いただいた，国立感染症研究所細菌第二部，同動物管理室，日本小動物獣医師会理事会，栃木県保健環境センター微生物部，同動物愛護センター，群馬県衛生環境研究所，同中央食肉衛生検査所，宮城県保健環境センター，同食肉衛生検査所，東京都健康安全研究センター，千葉県衛生研究所，神奈川県衛生研究所，同動物保護センター，静岡県環境衛生科学研究所，同県小川動物病院，大阪府立公衆衛生研究所，同動物愛護畜産課，同イヌ管理指導所，同府下獣医科医院，富山県衛生研究所，同動物管理センター，岡山県環境保健センター，同動物愛護センター，同食肉衛生検査所，同県下小動物獣医師会，山口県環境保健センター，香川県内小動物獣医師会，愛媛県衛生環境研究所，大分県衛生環境研究センター，大分大学医学部附属病院，東京医科歯科大学耳鼻咽喉科，大阪府立大学大学院獣医感染症学教室，岐阜大学獣医病理学教室の関係者に深謝する．

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らEV71が，1名からCA16が分離された（前ページ表）。EV71が分離された患者のうち1名は，無菌性髄膜炎を併発し入院していたが，重症化せず，早期に退院した。また，EV71が分離された手足口病患者は，すべて4歳以下であった（前ページ表）。

ウイルス分離には，18検体すべてにFL細胞，RD-18S細胞，Vero細胞を用いて，33℃で2週間，回転培養を行った。その結果，細胞変性効果は，Vero細胞では，EV71の11株中10株，RD-18S細胞では，EV71の8株に認められたが，FL細胞では認められず，Vero細胞がEV71分離株に対して最も高い感受性を示した。

EV71の同定には1978年の分離株を，また，CA16の同定には1995年の分離株を用いて作製した自家製抗血清を使用した。今回分離された株の中には，同定が困難な株はなく，判定は比較的容易であった。

流行初期に，CA16が手足口病患者1名から分離されたが，その他はすべてEV71が分離されていることから，今回の愛媛県における大規模な手足口病の流行は，EV71によるものと考えられた。

手足口病の患者報告数は，第22週現在，定点当たり8.3人と依然として多く，5月中旬以降も無菌性髄膜炎を併発している手足口病患者検体が搬入されている。また，本疾患は通常，夏季を中心に流行することから，今後の動向に注意が必要と考える。

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中国からのH1型麻疹ウイルス輸入症例――札幌市

2010年5月，札幌市内の医療機関で麻疹と診断された患者からH1型麻疹ウイルスを検出したので報告する。

患者は中国籍の女性（20代，北京在住）で，5月1日に観光目的で来札し，知人宅に滞在していた。5月6日朝から頭痛，夕方に発熱を呈し，7日に咳，8日に発疹が出現した。さらに10日には，コプリック斑，結膜充血および鼻汁が認められ，市内の医療機関において臨床症状より麻疹と診断された。なお，患者のワクチン接種歴は不明であった。

5月13日に採取された患者の咽頭ぬぐい液，末梢血単核球および尿を用いてRT-nested PCR法による麻疹ウイルス遺伝子の検出を試みた。その結果，すべての検体で麻疹ウイルスのHおよびN遺伝子が増幅された。増幅されたN遺伝子の部分塩基配列はすべて一致し，系統樹解析によりH1型麻疹ウイルスと同定された（図1）。GenBankに登録されている株との相同性検索では，N遺伝子472塩基について，上海で分離されたMVi/Shanghai.PRC/22.06/11（DQ902857）と100%の相同性を示した。また，レファレンスセンター

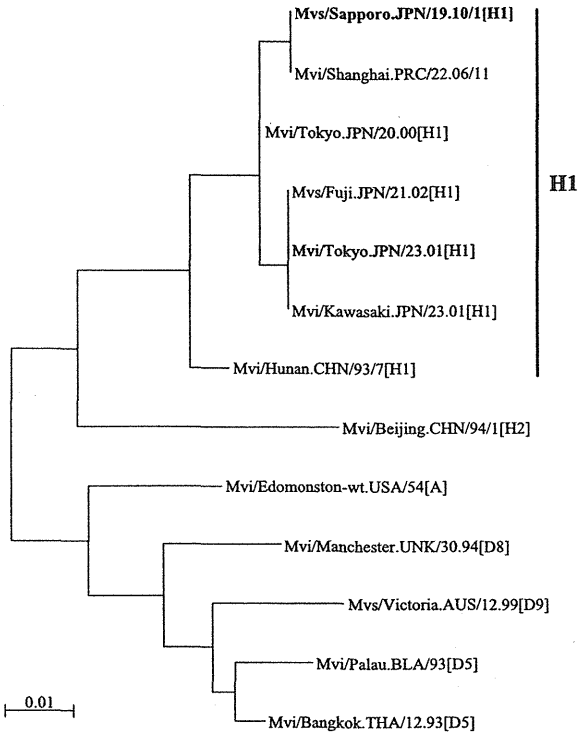

図1. 麻疹ウイルスN遺伝子(456bp)に基づく分子系統樹

である北海道立衛生研究所にて実施しているウイルス分離では末梢血単核球と尿から麻疹ウイルスが分離されており，抗体検査では麻疹IgM＞14.03と強陽性を示した。

今回の患者は海外からの輸入症例と考えられた。届出後，患者の行動調査を実施したうえで，感染機会があったと推定される対象者への注意喚起・健康状況確認を行った結果，6月22日現在，本症例からの二次感染例は確認されていない。今後，本邦における麻疹発生数の低下にともない，輸入症例への注意が必要になると同時に，麻疹ウイルスの分子疫学がさらに重要になると思われる。

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＜国内情報＞

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

2009年1月にジフテリア様症状を呈する患者からジフテリア毒素産生性*Corynebacterium ulcerans*が分離された。ヒトからの検出は本邦第6例目である（次ページ表1）。患者が発症する以前に接触した野良猫は，鼻水，発咳が観察されており，患者と野良猫から分離された菌は同じ遺伝子型であることが確認され