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2 Single nucleotide polymorphism in the cytolethal distending toxin B gene confers
3 heterogeneity in the cytotoxicity of *A. actinomycetemcomitans*

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14 Running head: SNP in cytolethal distending toxin gene
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19 Key words: Cdt, SNP, *A. actinomycetemcomitans*, toxin, cytolethal distending toxin,
20 single nucleotide polymorphism

21

1 **ABSTRACT**

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3 Clinical *Actinobacillus actinomycetemcomitans* produces cytolethal

4 distending toxin (CDT) with titers varying from 10^2 to 10^8 U/mg. SNP analysis of the

5 Cdt gene in clinical isolates identified a variation of single amino acid at residue 281 of

6 CdtB, which significantly affected CDT toxicity by modulating chromatin degrading

7 activity of CdtB.

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9

Actinobacillus actinomycetemcomitans may be one of the key pathogens in the etiology of human periodontal disease. It produces a variety of virulence factors including cytotoxic factors. We (18) and others (9) show *A. actinomycetemcomitans* Y4 produces a cytolethal distending toxin (Cdt); and this cytotoxic factor is secreted into the bacterial culture supernatant (18).

Cdt is a toxin produced by a wide variety of pathogenic bacteria (For recent reviews, see (4, 13, 15, 16, 19)). It inhibits the cell proliferation of cultured cells by arresting cell cycle at G2/M (17). The intoxicated cells show distension of the cell and nucleus and eventually die. Cdt is a trimer holotoxin where CdtB is the active subunit and CdtA and CdtC are a heterodimeric subunit apparatus required to deliver CdtB into the cell (6, 7). Nuclear entry of CdtB relies on the atypical nuclear localization signal (10, 12); and is essential for the cytotoxic activity (5, 12). CdtB bears structural similarity to members of the metallo-enzyme super-family including nucleases and various phosphatases (1). Mutations in amino acids in the DNase I active site residues of CdtB abolish the cytotoxic activity (2, 5). Upon entering the nucleus, CdtB appears to induce DNA double strand breaks by phosphorylation of histone H2AX (8) and re-localization of the DNA repair complex, Mre11-Rad50 (3). However, it is still unknown whether CdtB acts as a genuine DNase. The possible mode of DNA damage by CdtB may activate a checkpoint control which results in G2 arrest in the intoxicated cells (13, 15, 19). Cdt may be involved in the pathogenesis of bacterial chronic infections; however, the molecular patho-physiological role of Cdt is unknown.

Identification of SNP in the *cdtB* gene of strains with elevated cytotoxic activity. We previously reported 40 (89 %) of 45 *A. actinomycetemcomitans* isolated from periodontitis patients possess the *cdtABC* genes (21). The Cdt activity was found in the culture supernatant of the 40 strains; however, the titer of the toxin varied from 10^2 U to 10^8 U among these strains. To determine the mechanism of the variation in the Cdt titer from the culture supernatant, we selected three isolates, 330, 1773, and 2102, that show high Cdt titers (> 10^7 U) (21). The concentration of the Cdt produced in the culture supernatant by these strains was compared to strain Y4, a low titer strain (ca. 10^2 U). Immunoblot analysis shows the concentration of Cdt from the three high titer strains were similar to strain Y4 (Data not shown). We then determined if there was a difference in the primary structure in the Cdt proteins that could account for the increased specific activity for the Cdt holotoxin. The *cdtABC* genes were amplified from the genomic DNA from the clinical strains by PCR and were directly sequenced; and compared with sequences of genes derived from strain Y4. Two amino acid substitutions resulting from two single nucleotide alterations were found in the CdtB sequence of strain 330, 1773, and 2102 where the fourth valine residue, valine4 (V4), was substituted with alanine (A, GTA→GCA) and histidine281 (H281) was substituted with arginine (R, CAT→CGT). Since the fourth V or A residue is in the signal peptide of CdtB, this substitution would not affect the Cdt activity because the signal sequence is clipped-off during the maturation and secretion process. Therefore, this indicated the mature CdtB in these strains possessed a single amino acid substitution of H281 to R that possibly accounted for the increased titer.

Site-directed mutagenesis of H281 affects the holotoxin activity. To determine the contribution of a single amino acid substitution on the Cdt activity, we performed a site-directed mutagenesis using the Y4 CdtB gene where the mutation conferred an amino acid substitution of H281 to R281. The Y4 type *cdt* locus and corresponding DNA with the mutation was cloned into the expression vector, pQE60, so that the C-terminal of CdtC was tagged with six histidine residues. The Cdt holotoxin, complex of three subunits was purified using Ni-NTA beads as described previously (14, 20). Immunoblot analysis shows the amino acid substitution did not affect the protein ratio among the subunit components (not shown). The purified holotoxins were titered. Fig. 1 shows $CdtAB^{H281R}C$ had 10^9 times higher activity than the Y4 type, $CdtAB^{H281}C$. To determine the function of the amino acid residue at position 281, we created mutants with different substitutions changing H281 to A281, K281, or D281; and purified the recombinant CdtB holotoxins. When H281 is substituted with the neutral, non-polar amino acid, A281, the holotoxin ($CdtAB^{H281A}C$) lost most Cdt activity. When H281 is replaced with another basic amino acid, K, the holotoxin ($CdtAB^{H281K}C$) showed a 10^6-fold elevated activity though it was less than $CdtAB^{H281R}C$. When H281 is substituted with an acidic residue, D, the holotoxin ($CdtAB^{H281D}C$) showed decreased activity that was less than the Y4 type. The site directed mutagenesis suggests a positively charged amino acid at position 281 in CdtB is critical for the *A. actinomycetemcomitans* Cdt activity where a single amino acid substitution from H to R (K) at position 281 in CdtB elevates the specific activity of the Cdt holotoxin.

We compared the cell-cycle inhibition activity of the variant Cdt holotoxins,

CdtAB^{H281A}C, CdtAB^{H281R}C and CdtAB^{H281K}C to the Y4 type holotoxin. We measured the relative DNA concentration in the HeLa cells intoxicated with the various concentrations of the purified Cdt variant holotoxins. Fig. 2 shows CdtAB^{H281R}C and CdtAB^{H281K}C blocked the cell cycle at a low concentration of 6 ng/ml; whereas Y4 type holotoxin required 6 μg/ml to completely inhibit the cell cycle; and CdtAB^{H281A}C was not able to block the cell cycle at 6 μg/ml. These results roughly agree with the cytodistending titers of the Y4 type and variant holotoxins. However it should be noted that CdtAB$^{H281R/K}$C was 6-9 orders of magnitude more active than the Y4-type holotoxin in cellular distension assay whereas it was approximately only 3 orders of magnitude more active in cell cycle arresting assay. Moreover in cellular distension assay CdtAB^{H281R}C was apparently stronger than CdtAB^{H281K}C but they showed similar activities in cell cycle arresting assay. We cannot explain these discrepancies since molecular mechanism of cellular distension by Cdt treatment and its relation to cell cycle arrest by DNA double strand breaks are virtually unknown.

Amino acid substitution at position 281 affects CdtB-induced chromatin degradation. We show amino acid substitution at position 281 in CdtB affects the specific activity of Cdt holotoxin. The substitution may affect the DNase activity of the CdtB. We determined the DNase activity using the *in vitro* nuclease assay (2, 11). Fig. 3A shows the weak nuclease activity of Y4 type CdtB. CdtB^{H281R} shows approximately 4-fold stronger activity than CdtB^{H281}; whereas CdtB^{H281A} had a weaker activity than the Y4 type CdtB. To determine the direct effect of the amino acid substitution at position 281, we used a CdtB-microinjection assay and compared the

kinetics of the nuclear entry and subsequent chromatin degradation of the variant CdtBs to that of the Y4 type. After microinjection of the purified CdtBs into the cytoplasm of HeLa cells, the injected CdtBs migrated into the nucleus within an hour as detected by immunofluorescence and started to disintegrate the chromatin DNA structure as visualized using the PI stain (Fig. 3B). Fig. 3C showed that $CdtB^{H281R}$ completely disintegrated the chromatin in 3 h after microinjection; whereas the Y4 type CdtB took 8 h; whereas, $CdtB^{H281A}$ showed a much weaker activity in disintegrating the chromatin DNA. Therefore, the results suggest that amino acid substitution of CdtB at position 281 may strongly affect the chromatin disintegrating activity of CdtB *in vivo*.

Single Nucleotide Polymorphism (SNP) analysis of the *cdtB* gene in clinically isolated strains. We investigated the SNP of the *cdtB* gene in clinically isolated strains. The *cdtB* genes amplified from the genomic DNA of clinical strains by PCR were directly sequenced. DNA sequencing identified three SNPs, two correspond to those identified in the three strains showing very high Cdt titers. Accordingly, 41 strains including the standard strain, Y4, were categorized into four types based on the SNP in the *cdtB* gene (Fig. 4). Eleven strains (26.8%) belong to type I where the CdtB sequence is the same as strain Y4 (the 281st amino acid is H). Another eleven strains (26.8%) belong to type II where the 281st amino acid of CdtB sequence is R. Eighteen strains (43.9%) belong to type III containing two amino acid substitutions in the CdtB, V4 to A and H281 to R. And one strain belongs to type IV that contains three amino acid substitutions, V4 to A, D199 to G, and H281 to R in the CdtB amino acid sequence. In summary, 73.2 % of the investigated *A.*

actinomycetemcomitans clinical strains possess a CdtB sequence with R281, and the rest 26.8 % possess a CdtB sequence with H281. Comparing this using the *Hin*dIII RFLP typing we performed previously (21) show the SNP type of the CdtB gene has some relation to the genomic variation in *A. actinomycetemcomitans*. Most strains of RFLP type I and II belong to SNP type I. Similarly most of RFLP type III and IV belong to SNP type II; and for the rest of the strains, the RFLP type V and VI belong to SNP type III. There was no direct relation between the SNP of the CdtB gene and the serotype of the strain (not shown). The Cdt titers of strains belonging to SNP type I are relatively low ($>10^3$ U). In contrast, 19 strains (including 330, 1773 and 2102) out of the 29 in SNP type II or III produced higher titers ($>10^3$ U).

Since CdtB induces DNA damage in the target nucleus, most of the previous functional studies have focused on the amino acids corresponding to the DNase catalytic sites, metal binding, and possible DNA binding sites. Elwell and Dreyfus (2) demonstrate mutations at H134, D212, H252, or D251 that correspond to the catalytic sites or metal binding si65jwhere H152 and D185 were important for the *C. jejuni* Cdt activity (5). Nesic et al. show the mutations at positions R144, N201 and R117 which correspond to the DNA-contact sites of DNase I resulted in complete loss of activity (11). Although data is accumulating where several mutations in the *cdtB* gene reduce or abolish the Cdt activity, little is known about the amino acid residue(s) whose mutation elevates the Cdt activity. We show in this study that substitution of H281 with R in Y4 type-CdtB significantly elevates the specific activity of Cdt holotoxin. The activity correlates with the degree of protonation of the side chain in the amino acid

281 suggesting a positive charge at position 281 is important for the Cdt activity. Fig. 5 shows the alignment of CdtB protein sequences from various pathogenic bacteria including pathogenic *E. coli* and *Haemophilus ducreyi*. This shows all but *E. coli* CDT I of the aligned amino acid residues corresponding to position 281 in *A. actinomycetemcomitans* CdtB is positively charged. *A. actinomycetemcomitance* CdtB and *H. ducreyi* Cdt share 97% amino acid identity (18). Recently, the crystal structure of *H.ducreyi* Cdt was determined; and they demonstrated the structures of CdtB and DNase I are similar (11). The amino acid position at 281 of CdtB (R in *H. ducreyi*) is remote from those corresponding to essential amino acids for catalysis of DNase I and those for putative DNA binding (Supplement 2). However, H281 is exposed to the surface of the CdtB molecule. Fig. 3A shows the *in vitro* nuclease assay where amino acid substitution at position 281 of CdtB somehow affects endogenous nuclease activity although its effect is very small. The effect of amino acid substitution at 281 of CdtB on nuclease activity was much more apparent when the *in vivo* chromatin-disintegrating assay was used. There is an observed disparity in the *in vivo* and *in vitro* results (13, 16). We do not understand why there is a very low *in vitro* nuclease activity in CdtB and there is a very high specific activity to induce cell cycle arrest, cell distension and chromatin degradation *in vivo*.

Our study suggests that the SNP partly explains the significant heterogeneity of cytotoxic activity present in the culture supernatant of *A. actinomycetemcomitans*. An epidemiological study to determine the relevance of severity of periodontitis with *A. actinomycetemcomitans* as the primary isolate as compared to the SNP of the CdtB gene

is underway.

Bacterial strains, plasmids and primers used in this study are listed in Supplement 1.

This work was supported, in part, by a grant-in aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan. We thank K. Okuda and H. Ohta for providing the clinical isolates of *A. actinomycetemcomitans*. We are grateful to Reiko Yamaguchi and Hiroyuki Shima for their technical assistance, and Jim Nelson for editorial assistance. We thank the Research Facilities, Hiroshima University School of Dentistry and School of Medicine, for the use of their facilities.

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FIGURE LEDGENDS

Fig. 1 Cdt activity of the purified Cdt holotoxin prepared from a recombinant *E. coli* carrying Y4 type *cdt* gene (H281) and *cdt* genes with the mutated *cdtB*.

HeLa cells were placed in a 96-well plate (Falcon; Becton Dickenson) at a concentration of 2 x 10^3 cells at 100 µl per well one day before the experiment. The Y4 type *cdt* locus and corresponding DNA with the mutation was cloned into the expression vector, pQE60, so that the C-terminal of CdtC was tagged with six histidine residues. Various Cdt holotoxins containing *cdtB* genes with H281 (Y4 type), R281 (H281R), A281 (H281A), K281 (H281K), D281 (H281D) were purified using Ni-NTA beads as described previously (14, 20). The purified holotoxins were dialyzed against PBS (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM, pH7.3) over night, and the protein concentration was adjusted to 0.1 µg/µl by BioRad protein assay kit (BioRad, Hercules, CA) respectively. One hundred micro-liter of filter sterilized (0.22 µm) sample was inoculated on the HeLa cell mono-layer of the first well and serially diluted 1:2 in succeeding wells (20). Morphological changes were monitored using phase contrast microscopy (Nikon DIAPHOT 300) from day1 to day3. Cell distension was defined as greater than five times expansion of the cell size compared to control cells. The cytodistendig activity (total activity) was titrated by using as the end point the highest two-fold dilution of culture supernatant showing 50% transformed cells after 72 h incubation. The unit (U) of Cdt activity was defined as the reciprocal of

the dilution and the specific activity was defined as U/mg of protein (U/mg).

Fig. 2 Flow cytometry of HeLa cell DNA treated with Y4 type Cdt holotoxin or variant holotoxins. The DNA of HeLa cells was analyzed by flow cytometric analysis. Cdt holotoxin or its variant ($CdtAB^{H281R}C$, $CdtAB^{H281K}C$, or $CdtAB^{H281A}C$) was added to the HeLa cell culture at concentrations of 6 ng/ml, 60 ng/ml, 0.6 μg/ml, and 6 μg/ml. After 24 h, harvested cells were fixed with ethanol and stained with PI. Ten thousand cells were scanned using a FACScan flow cytometer (Becton Dickinson to detect PI-fluorescence to measure the relative DNA concentration. The histgram of G1 and G2 population was analyzed by Modifit software (Verity Software, Inc.).

Fig. 3 *In vitro* nuclease and microinjection assays of Y4 type CdtB and variants. A, *In vitro* DNase activity of Y4 type CdtB ($CdtB^{281H}$), $CdtB^{H281R}$ and $CdtB^{H281A}$. The Y4 type *cdtB* or $cdtB^{mut281}$ were sub-cloned into the expression vector pET28. The His-tag recombinant proteins were purified as described (14). The plasmid pGEM DNA (1 μg BioRad) was incubated in buffer (20mM Hepes pH7.5, 150mM NaCl, 5mM $CaCl_2$, 5mM $MgCl_2$) with various concentrations of purified CdtB (0 μg to 10 μg) for 5 h at 37 ˚C. The linear and super-coiled plasmid DNAs were separated using 1 % agarose-gel electrophoresis and visualized with ethidium bromide. B, Y4 type ($CdtB^{281H}$) or variant CdtB ($CdtB^{H281R}$, $CdtB^{H281A}$) with the His-tag at the C-terminus was expressed and purified by Ni-agarose chromatography. Microinjection was performed using an Eppendorf Injectman NI2 (12). Purified proteins were adjusted to

1 a concentration of 0.5 μg/μl and injected into the cytosol at a pressure of 50-120 hpa for
2 0.2 s. The cells were incubated at 37˚C in 5 % CO_2 for 1-8 h; and then the injected
3 cells were stained by immunohistochemistry for CdtB (12) and by PI, followed by
4 observation using confocal microscopy (Carl Zeiss LSM 401). Arrows indicate the
5 cells microinjected with CdtB. C, The relative chromosomal DNA was measured by a
6 Mac Scope image analysis system where the reduction of intensity of propidium iodide
7 stain was plotted against the relative decrease of the signal showing the relative
8 concentration of intact DNA. Circle: Y4 type CdtB ($CdtB^{281H}$), Square: $CdtB^{H281R}$,
9 Triangle: $CdtB^{H281A}$.
10
11 **Fig. 4 Polymorphism of the CdtB amino acid sequence in clinically isolated *A.***
12 ***actinomycetemcomitans*.** A. Cytodistending activity, RFLP typing and CdtB typing of
13 *A. actinomycetemcomitans* clinical strains. The culture supernatant was prepared from
14 exponentially growing *A. actinomycetemcomitans*. The protein concentration of the
15 culture supernatant was measured by BioRad protein assay kit (BioRad) at the O.D.=
16 0.3 after culturing *A.actinomycetemcomitans* in trypticase soy broth (Becton Dickinson,
17 Sparks, MD) with 0.6% yeast extract (TSBYE, Becton Dickinson) for 3 days. After
18 the total protein concentration of culture supernatant was adjusted at 0.1 μg/μl by
19 diluting the sample with PBS, 100μl of the supernatant was added to the culture of
20 HeLa cells using a 1:2 serial dilution in a 96 multi-well plate. After 3 days,
21 morphological cyto-distention was determined after fixing with 100 % methanol and
22 staining with 2% Giemsa solution. The arrowheads show the strains with very high

Cdt titer (>10^7 U). Presence or absence of the *cdt* genes was shown by + and -. Dotted Y-axis line divided the strains with (left side) or without (right side) the *cdtABC* genes. Single nucleotide polymorphism (SNP) typing and *Hin*dIII restriction enzyme fragment length polymorphism (RFLP) (21) are shown. B. Polymorphism of CdtB amino acid sequences. Four types of amino acid sequences of CdtB identified in 40 clinical strains were aligned. Conserved amino acid residues are shown in gray. An arrowhead shows the signal peptide cleavage site. The predicted catalytic sites conserved in DNase I is shown by stars. The predicted metal binding site conserved in DNase I are shown by a cross.

Fig. 5 Alignment of CdtBs from various microorganisms and DNaseI.

Predicted C-terminal sequences of CdtBs from various pathogenic bacteria were aligned. The conserved amino acid residues are in gray. The predicted catalytic sites conserved in DNase I is shown by stars. The predicted metal binding site conserved in DNase I is shown by a cross. GenBank accession numbers were: *A. actinomycetemcomitans* Y4 AB011405, *H. ducreyi* 35000, *C. jejuni* 81-176: U51121, *Helicobacter hepaticus*: AAF19158, *E. coli* CDT I / *E. coli* E6468/62: U03293, *E. coli* CDT II / *E. coli* 9142-88: U04208, *E. coli* CDT III / *E. coli* 1404: U89305, DNase I / *Mus musculus*: AAH30394 .

1 Supplement 2 **Van der Waals surface representation of *H. ducreyi* CdtB.**

2 Conserved residues for DNase catalytic (H274), metal binding (N273) and

3 DNA binding (R117, R144, and N201) in CdtB were shown in cyan, pink, and yellow,

4 respectively. R281 is shown in red. This figure was drawn with graphic program

5 RasMol and atomic coordinates of CdtB (accession code 1SR4) (11) are from the

6 Protein Data Bank.

7

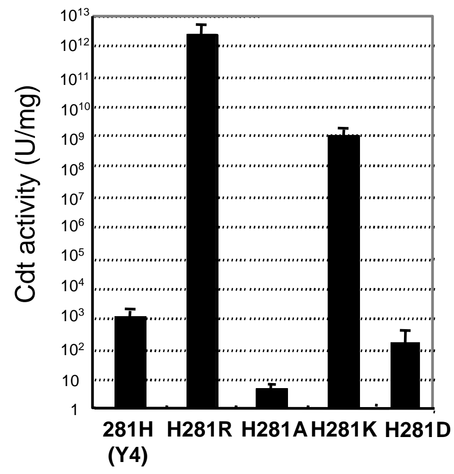

Fig.1 Nishikubo, et al

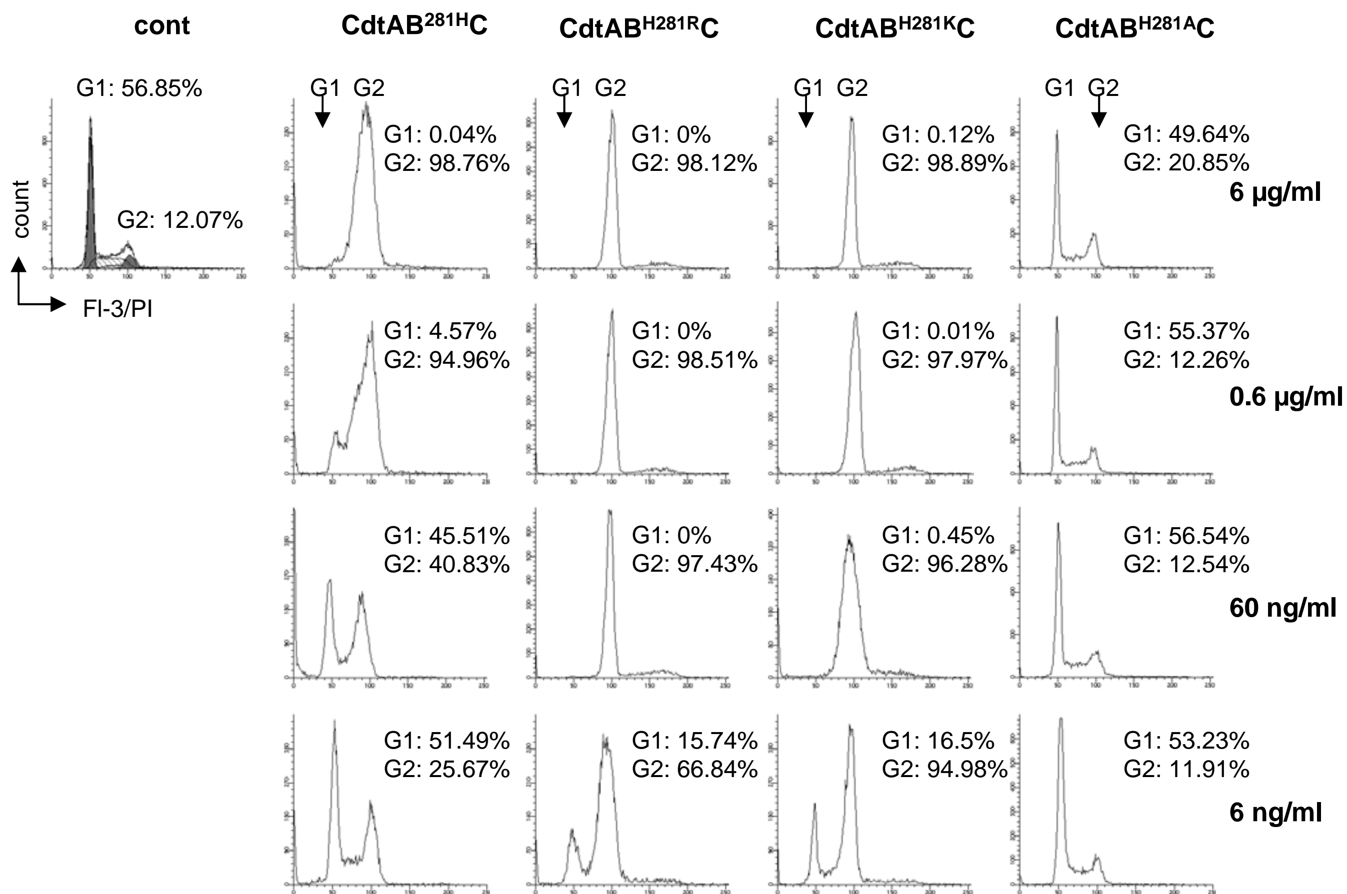

Fig.2 Nishikubo, et al

A

10 5 2.5 1.25 0.63 0.32 0.16 0 μg

CdtB(Y4)

281H

linear& relax

supercoil

H281R

H281A

B

1h 2h 3h 4h 6h 8h

281H

Fluorescence

PI

H281R

Fluorescence

PI

H281A

Fluorescence

PI

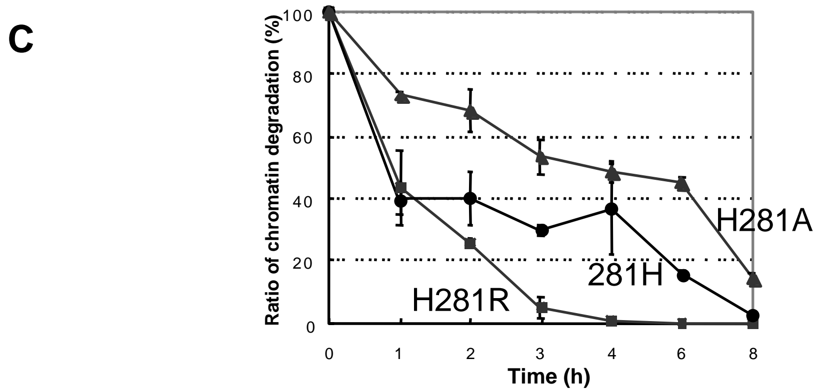

Fig.3 Nishikubo, et al

A

Cdt activity (U/mg)

10^0 10^1 10^2 10^3 10^4 10^5 10^6 10^7 10^8 10^9

Y4 129 126 29524 29523 29522 ATY4 2510 108 99 57 9710 2267 2303 2232 211 2242 2241 2213 2212 301-b 2211 10219 10224 2339 2101 2403 350 347 330 328 322 342 341 315 312 2102 1773 2404 314 IDH 67 9 8 6 2

cdtABC gene

Typing

I II III IV

Hind III RFLP Typing

I II III IV V VI VII

B

	10	20	30	40	50
Type I	MQWVKQLNVV	FCTMLFSFSS	YANLSDFKVA	TWNLQGSSAV	NESKWNINVR
Type II	MQWVKQLNVV	FCTMLFSFSS	YANLSDFKVA	TWNLQGSSAV	NESKWNINVR
Type III	MQWAKQLNVV	FCTMLFSFSS	YANLSDFKVA	TWNLQGSSAV	NESKWNINVR
Type IV	MQWAKQLNVV	FCTMLFSFSS	YANLSDFKVA	TWNLQGSSAV	NESKWNINVR

	60	70	80	90	100
Type I	QLLSGEQGAD	ILMVQEAGSL	PSSAVRTSRV	IQHGGTPIEE	YTWNLGTRSR
Type II	QLLSGEQGAD	ILMVQEAGSL	PSSAVRTSRV	IQHGGTPIEE	YTWNLGTRSR
Type III	QLLSGEQGAD	ILMVQEAGSL	PSSAVRTSRV	IQHGGTPIEE	YTWNLGTRSR
Type IV	QLLSGEQGAD	ILMVQEAGSL	PSSAVRTSRV	IQHGGTPIEE	YTWNLGTRSR

	110	120	130	140	150
Type I	PNMVYIYYSR	LDVGANRVNL	AIVSRRQADE	AFIVHSDSSV	LQSRPAVGIR
Type II	PNMVYIYYSR	LDVGANRVNL	AIVSRRQADE	AFIVHSDSSV	LQSRPAVGIR
Type III	PNMVYIYYSR	LDVGANRVNL	AIVSRRQADE	AFIVHSDSSV	LQSRPAVGIR
Type IV	PNMVYIYYSR	LDVGANRVNL	AIVSRRQADE	AFIVHSDSSV	LQSRPAVGIR

	160	170	180	190	200
Type I	IGTDVFFTVH	ALATGGSDAV	SLIRNIFTTF	TSSPSSPERR	GYSWMVVGDF
Type II	IGTDVFFTVH	ALATGGSDAV	SLIRNIFTTF	TSSPSSPERR	GYSWMVVGDF
Type III	IGTDVFFTVH	ALATGGSDAV	SLIRNIFTTF	TSSPSSPERR	GYSWMVVGDF
Type IV	IGTDVFFTVH	ALATGGSDAV	SLIRNIFTTF	TSSPSSPERR	GYSWMVVGGF

	210	220	230	240	250
Type I	NRAPVNLEAA	LRQEPAVSEN	TIIIAPTEPT	HRSGNILDYA	ILHDAHLPRR
Type II	NRAPVNLEAA	LRQEPAVSEN	TIIIAPTEPT	HRSGNILDYA	ILHDAHLPRR
Type III	NRAPVNLEAA	LRQEPAVSEN	TIIIAPTEPT	HRSGNILDYA	ILHDAHLPRR
Type IV	NRAPVNLEAA	LRQEPAVSEN	TIIIAPTEPT	HRSGNILDYA	ILHDAHLPRR

	260	270	280	
Type I	EQARERIGAS	LMLNQLRSQI	TSDHFPVSFV	HDR
Type II	EQARERIGAS	LMLNQLRSQI	TSDHFPVSFV	RDR
Type III	EQARERIGAS	LMLNQLRSQI	TSDHFPVSFV	RDR
Type IV	EQARERIGAS	LMLNQLRSQI	TSDHFPVSFV	RDR

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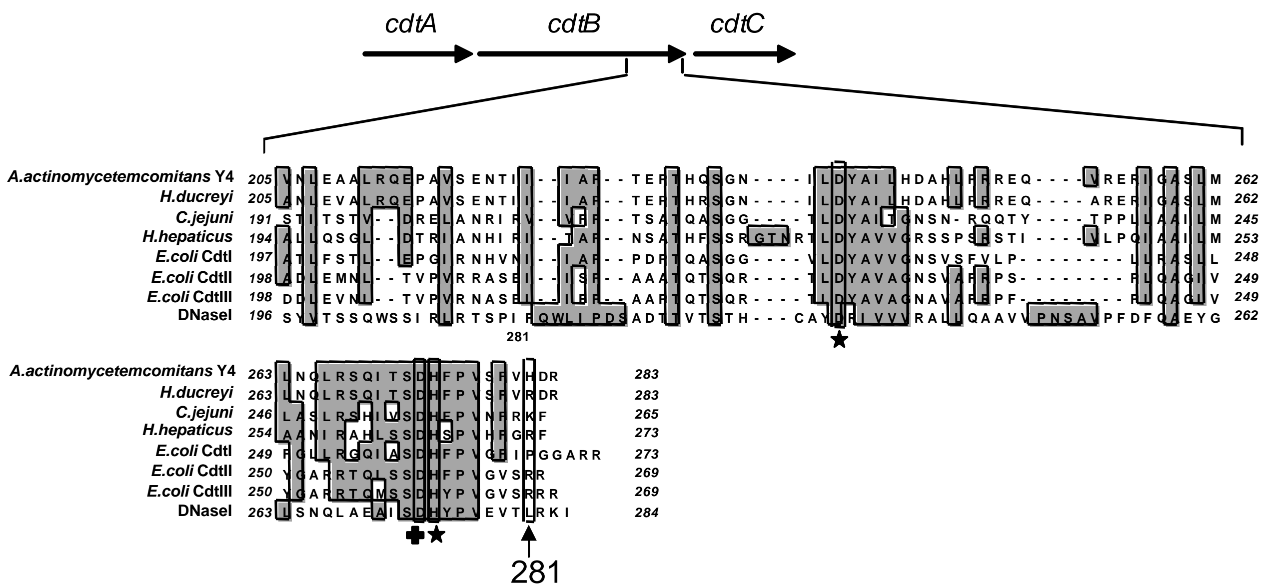
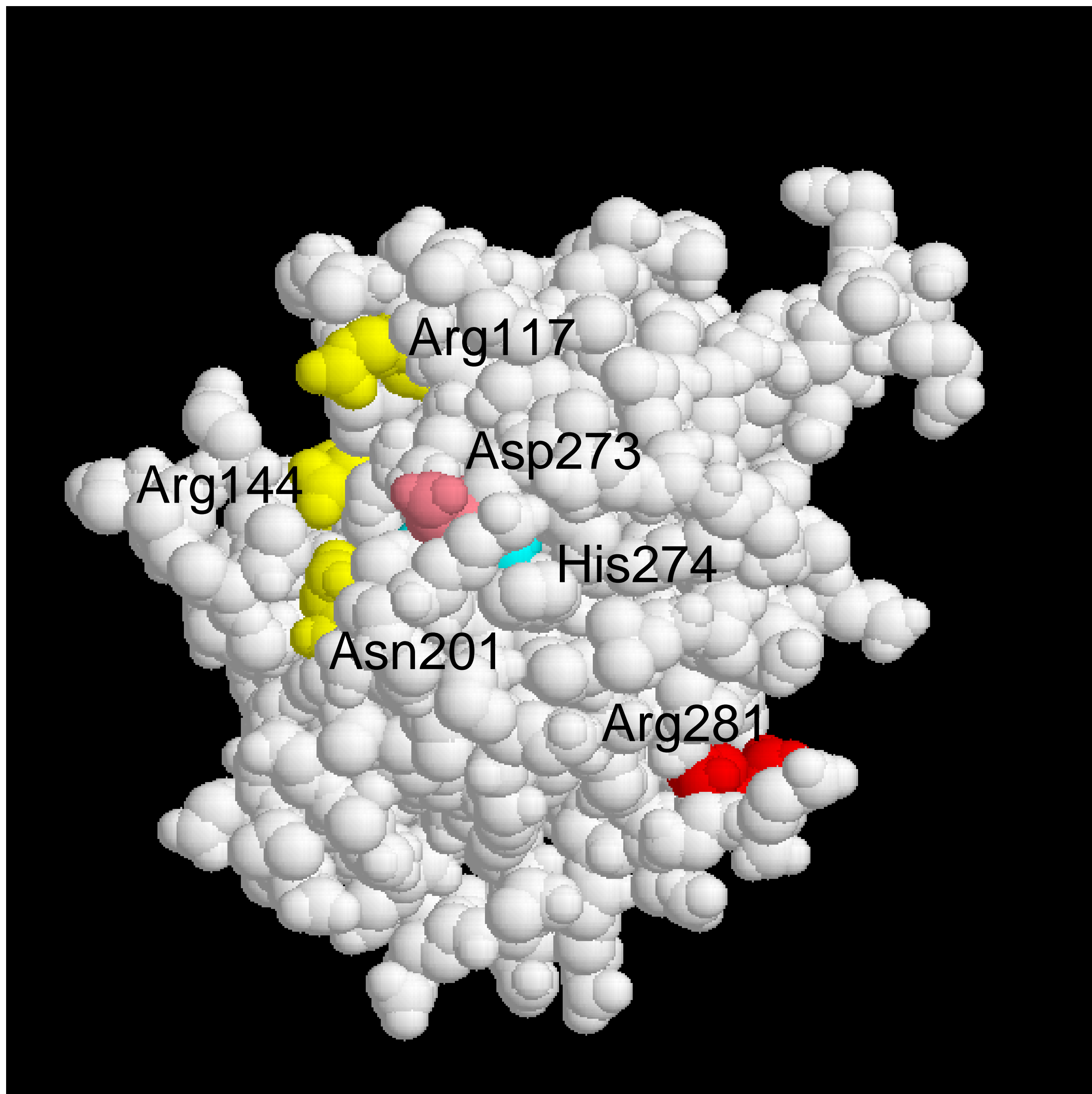


Fig.5 Nishikubo, et al

Supplementary 1. Strains and primers used in this study

Strain		Plasmid	Character	Reference
A.actinomycetemcomitans Y4			standard strain	
A.actinomycetemcomitans			45clinical isolates	21
E.coli	M15	pQE*cdtABC*	Y4 *cdtAB*^{H281}*C* on pQE60 (*Nco* I-*Bgl* II)	14
	M15	pQE*cdtAB*^{H281R}*C*	*cdtAB*^{H281R}*C* on pQE60	This study
	M15	pQE*cdtAB*^{H281A}*C*	*cdtAB*^{H281A}*C* on pQE60	This study
	M15	pQE*cdtAB*^{H281K}*C*	*cdtAB*^{H281K}*C* on pQE60	This study
	M15	pQE*cdtAB*^{H281D}*C*	*cdtAB*^{H281D}*C* on pQE60	This study
	M15	pQE*cdtAB*^{H282H}*C*	*cdtAB*^{D282H}*C* on pQE60	This study
	M15	pQE*cdtAB*^{H282A}*C*	*cdtAB*^{D282A}*C* on pQE60	This study
	M15	pQE*cdtAB*^{H283K}*C*	*cdtAB*^{R283K}*C* on pQE60	This study
	M15	pQE*cdtAB*^{H283A}*C*	*cdtAB*^{R283A}*C* on pQE60	This study
	M15	pQE60	His-tag protein expression vector	Qiagen
	HMS174(DE3)	pET*cdtB*	Y4 *cdtB*^{H281} on pET28a (*Bgl* II-*Eco* RI)	This study
	HMS174(DE3)	pET*cdtB*^{H281R}	Y4 *cdtB*^{H281R} on pET28a	This study
	HMS174(DE3)	pET*cdtB*^{H281A}	Y4 *cdtB*^{H281A} on pET28a	This study
	HMS174(DE3)	pET28a	His-tag protein expression vector	Novagen
	XL-2 blue	pGEM-T easy	PCR cloning vector	BioRad

Primers	sequence (5'-3')		
QIA-U	AGGTACCATGGAAAAGTTT	*cdtA* 5' region with *Nco* I site	21
QIA-L	AAAGATCTGCTACCCTGA	*cdtC* 3' region with *Bgl* II site instead of stop codon	21
mcdtBfbgl	CAAGATCTGCTAACTTGAGT	*cdtB* with B*gl* II site instead of signal sequences	12
L2188	AGTATTCTCCTTAGCGATCATGAA	*cdtB* 3' region	12
H281R-U	AGTTTTGTTcgcGATCGCTAAGG	site-directed mutagenesis	This study
H281R-R	TCCTTAGCGATCgcgAACAAAACT	site-directed mutagenesis	This study
H281A-U	AGTTTTGTTgctGATCGCTAAGGA	site-directed mutagenesis	This study
H281A-R	TCCTTAGCGATCagcAACAAAACT	site-directed mutagenesis	This study
H281D-U	AGTTTTGTTgatGATCGCTAAGGA	site-directed mutagenesis	This study
H281D-R	TCCTTAGCGATCatcAACAAAACT	site-directed mutagenesis	This study
H281K-U	AGTTTTGTTaaaGATCGCTAAGGA	site-directed mutagenesis	This study
H281K-R	TCCTTAGCGATCtttAACAAAACT	site-directed mutagenesis	This study
D282A-U	AGTTTTGTTCATgctCGCTAAGGA	site-directed mutagenesis	This study
D282A-R	TCCTTAGCGagcATGAACAAAACT	site-directed mutagenesis	This study
D282H-U	AGTTTTGTTCATcatCGCTAAGGA	site-directed mutagenesis	This study
D282H-R	TCCTTAGCGatgATGAACAAAACT	site-directed mutagenesis	This study
D283A-U	TTTGTTCATGATgccTAAGGAGAA	site-directed mutagenesis	This study
D283A-R	TTCTTCTTAggcATCATGAACAAA	site-directed mutagenesis	This study
D283K-U	TTTGTTCATGATaaaTAAGGAGAA	site-directed mutagenesis	This study
D283K-R	TTCTCCTTAtttATCATGAACAAA	site-directed mutagenesis	This study
AASPC3'	AAATCACCAACAACCATCCAGCTA	*cdtB* sequencing primer	18
L1812	ATCAGTACCAATGCGGATACCTACT	*cdtB* sequencing primer	This study
L1629	CCAGGTATATTCCTCAATTGGCGTT	*cdtB* sequencing primer	This study
L1527	TGCACCTTGTTCTCCCGATAATAAT	*cdtB* sequencing primer	This study
AASPC5'	GAAAGTAAATGGAATATTAATGTGCG	*cdtB* sequencing primer	18
U1851	TGATGCGGTAAGTTTAATTCGTAATA	*cdtB* sequencing primer	18



Supplement 2. Nishikubo, et al