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

## 1 ABSTRACT

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.
8	

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

4 Y4 produces a cytolethal distending toxin (Cdt); and this cytotoxic factor is secreted

5 into the bacterial culture supernatant (18).

6 Cdt is a toxin produced by a wide variety of pathogenic bacteria (For recent 7 reviews, see (4, 13, 15, 16, 19)). It inhibits the cell proliferation of cultured cells by 8 arresting cell cycle at G2/M (17). The intoxicated cells show distension of the cell and 9 nucleus and eventually die. Cdt is a trimer holotoxin where CdtB is the active 10 subunit and CdtA and CdtC are a heterodimeric subunit apparatus required to deliver 11 CdtB into the cell (6, 7). Nuclear entry of CdtB relies on the atypical nuclear 12 localization signal (10, 12); and is essential for the cytotoxic activity (5, 12). CdtB 13 bears structural similarity to members of the metallo-enzyme super-family including 14 nucleases and various phosphatases (1). Mutations in amino acids in the DNase I 15 active site residues of CdtB abolish the cytotoxic activity (2, 5). Upon entering the 16 nucleus, CdtB appears to induce DNA double strand breaks by phosphorylation of 17 histone H2AX (8) and re-localization of the DNA repair complex, Mre11-Rad50 (3). 18 However, it is still unknown whether CdtB acts as a genuine DNase. The possible 19 mode of DNA damage by CdtB may activate a checkpoint control which results in G2 20 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 21 22 unknown.

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1	Identification of SNP in the <i>cdtB</i> gene of strains with elevated cytotoxic
2	activity. We previously reported 40 (89 %) of 45 A. actinomycetemcomitans isolated
3	from periodontitis patients possess the <i>cdtABC</i> genes (21). The Cdt activity was found
4	in the culture supernatant of the 40 strains; however, the titer of the toxin varied from
5	$10^2$ U to $10^8$ U among these strains. To determine the mechanism of the variation in
6	the Cdt titer from the culture supernatant, we selected three isolates, 330, 1773, and
7	2102, that show high Cdt titers (> $10^7$ U) (21). The concentration of the Cdt produced
8	in the culture supernatant by these strains was compared to strain Y4, a low titer strain
9	(ca. $10^2$ U). Immunoblot analysis shows the concentration of Cdt from the three high
10	titer strains were similar to strain Y4 (Data not shown). We then determined if there
11	was a difference in the primary structure in the Cdt proteins that could account for the
12	increased specific activity for the Cdt holotoxin. The <i>cdtABC</i> genes were amplified
13	from the genomic DNA from the clinical strains by PCR and were directly sequenced;
14	and compared with sequences of genes derived from strain Y4. Two amino acid
15	substitutions resulting from two single nucleotide alterations were found in the CdtB
16	sequence of strain 330, 1773, and 2102 where the fourth valine residue, valine4 (V4),
17	was substituted with alanine (A, GTA $\rightarrow$ GCA) and histidine281 (H281) was substituted
18	with arginine (R, CAT $\rightarrow$ CGT). Since the fourth V or A residue is in the signal peptide
19	of CdtB, this substitution would not affect the Cdt activity because the signal sequence
20	is clipped-off during the maturation and secretion process. Therefore, this indicated
21	the mature CdtB in these strains possessed a single amino acid substitution of H281 to R
22	that possibly accounted for the increased titer.

1	Site-directed mutagenesis of H281 affects the holotoxin activity. To
2	determine the contribution of a single amino acid substitution on the Cdt activity, we
3	performed a site-directed mutagenesis using the Y4 CdtB gene where the mutation
4	conferred an amino acid substitution of H281 to R281. The Y4 type <i>cdt</i> locus and
5	corresponding DNA with the mutation was cloned into the expression vector, pQE60, so
6	that the C-terminal of CdtC was tagged with six histidine residues. The Cdt holotoxin,
7	complex of three subunits was purified using Ni-NTA beads as described previously (14,
8	<u>20).</u> Immunoblot analysis shows the amino acid substitution did not affect the protein
9	ratio among the subunit components (not shown). The purified holotoxins were titered.
10	Fig. 1 shows CdtAB <sup>H281R</sup> C had 10 <sup>9</sup> times higher activity than the Y4 type, CdtAB <sup>H281</sup> C.
11	To determine the function of the amino acid residue at position 281, we created mutants
12	with different substitutions changing H281 to A281, K281, or D281; and purified the
13	recombinant CdtB holotoxins. When H281 is substituted with the neutral, non-polar
14	amino acid, A281, the holotoxin (CdtAB <sup>H281A</sup> C) lost most Cdt activity. When H281 is
15	replaced with another basic amino acid, K, the holotoxin (CdtAB <sup>H281K</sup> C) showed a
16	$10^{6}$ -fold elevated activity though it was less than CdtAB <sup>H281R</sup> C. When H281 is
17	substituted with an acidic residue, D, the holotoxin (CdtAB <sup>H281D</sup> C) showed decreased
18	activity that was less than the Y4 type. The site directed mutagenesis suggests a
19	positively charged amino acid at position 281 in CdtB is critical for the A.
20	actinomycetemcomitans Cdt activity where a single amino acid substitution from H to R
21	(K) at position 281 in CdtB elevates the specific activity of the Cdt holotoxin.
22	We compared the cell-cycle inhibition activity of the variant Cdt holotoxins,

1	$CdtAB^{H281A}C$ , $CdtAB^{H281R}C$ and $CdtAB^{H281K}C$ to the Y4 type holotoxin. We measured
2	the relative DNA concentration in the HeLa cells intoxicated with the various
3	concentrations of the purified Cdt variant holotoxins. Fig. 2 shows CdtAB <sup>H281R</sup> C and
4	CdtAB <sup>H281K</sup> C blocked the cell cycle at a low concentration of 6 ng/ml; whereas Y4 type
5	holotoxin required 6 $\mu$ g/ml to completely inhibit the cell cycle; and CdtAB <sup>H281A</sup> C was
6	not able to block the cell cycle at $6 \mu g/ml$ . These results roughly agree with the
7	cytodistending titers of the Y4 type and variant holotoxins. However it should be
8	noted that CdtAB <sup>H281R/K</sup> C was 6-9 orders of magnitude more active than the Y4-type
9	holotoxin in cellular distension assay whereas it was approximately only 3 orders of
10	magnitude more active in cell cycle arresting assay. Moreover in cellular distension
11	assay CdtAB <sup>H281R</sup> C was apparently stronger than CdtAB <sup>H281K</sup> C but they showed similar
12	activities in cell cycle arresting assay. We cannot explain these discrepancies since
13	molecular mechanism of cellular distension by Cdt treatment and its relation to cell
14	cycle arrest by DNA double strand breaks are virtually unknown.
15	Amino acid substitution at position 281 affects CdtB-induced chromatin
16	degradation. We show amino acid substitution at position 281 in CdtB affects the
17	specific activity of Cdt holotoxin. The substitution may affect the DNase activity of
18	the CdtB. We determined the DNase activity using the <i>in vitro</i> nuclease assay (2, 11).
19	Fig. 3A shows the weak nuclease activity of Y4 type CdtB. CdtB <sup>H281R</sup> shows
20	approximately 4-fold stronger activity than CdtB <sup>H281</sup> ; whereas CdtB <sup>H281A</sup> had a weaker
21	activity than the Y4 type CdtB. To determine the direct effect of the amino acid
22	substitution at position 281, we used a CdtB-microinjection assay and compared the

1	kinetics of the nuclear entry and subsequent chromatin degradation of the variant CdtBs
2	to that of the Y4 type. After microinjection of the purified CdtBs into the cytoplasm of
3	HeLa cells, the injected CdtBs migrated into the nucleus within an hour as detected by
4	immunofluorescence and started to disintegrate the chromatin DNA structure as
5	visualized using the PI stain (Fig. 3B). Fig. 3C showed that CdtB <sup>H281R</sup> completely
6	disintegrated the chromatin in 3 h after microinjection; whereas the Y4 type CdtB took 8
7	h; whereas, CdtB <sup>H281A</sup> showed a much weaker activity in disintegrating the chromatin
8	DNA. Therefore, the results suggest that amino acid substitution of CdtB at position
9	281 may strongly affect the chromatin disintegrating activity of CdtB in vivo.
10	Single Nucleotide Polymorphism (SNP) analysis of the <i>cdtB</i> gene in
11	clinically isolated strains. We investigated the SNP of the <i>cdtB</i> gene in clinically
12	isolated strains. The <i>cdtB</i> genes amplified from the genomic DNA of clinical strains
13	by PCR were directly sequenced. DNA sequencing identified three SNPs, two
14	correspond to those identified in the three strains showing very high Cdt titers.
15	Accordingly, 41 strains including the standard strain, Y4, were categorized into four
16	types based on the SNP in the $cdtB$ gene (Fig. 4). Eleven strains (26.8%) belong to
17	type I where the CdtB sequence is the same as strain Y4 (the 281 <sup>st</sup> amino acid is H).
18	Another eleven strains (26.8%) belong to type II where the 281 <sup>st</sup> amino acid of CdtB
19	sequence is R. Eighteen strains (43.9%) belong to type III containing two amino acid
20	substitutions in the CdtB, V4 to A and H281 to R. And one strain belongs to type IV
21	that contains three amino acid substitutions, V4 to A, D199 to G, and H281 to R in the
22	CdtB amino acid sequence. In summary, 73.2 % of the investigated A.

1	actinomycetemcomitans clinical strains possess a CdtB sequence with R281, and the
2	rest 26.8 % possess a CdtB sequence with H281. Comparing this using the <i>Hin</i> dIII
3	RFLP typing we performed previously (21) show the SNP type of the CdtB gene has
4	some relation to the genomic variation in A. actinomycetemcomitans. Most strains of
5	RFLP type I and II belong to SNP type I. Similarly most of RFLP type III and IV
6	belong to SNP type II; and for the rest of the strains, the RFLP type V and VI belong to
7	SNP type III. There was no direct relation between the SNP of the CdtB gene and the
8	serotype of the strain (not shown). The Cdt titers of strains belonging to SNP type I
9	are relatively low (> $10^3$ U). In contrast, 19 strains (including 330, 1773 and 2102) out
10	of the 29 in SNP type II or III produced higher titers (> $10^3$ U).
11	Since CdtB induces DNA damage in the target nucleus, most of the previous
12	functional studies have focused on the amino acids corresponding to the DNase
13	catalytic sites, metal binding, and possible DNA binding sites. Elwell and Dreyfus (2)
14	demonstrate mutations at H134, D212, H252, or D251 that correspond to the catalytic
15	sites or metal binding si65jwhere H152 and D185 were important for the C. jejuni Cdt
16	activity (5). Nesic et al. show the mutations at positions R144, N201 and R117 which
17	correspond to the DNA-contact sites of DNase I resulted in complete loss of activity
18	(11). Although data is accumulating where several mutations in the $cdtB$ gene reduce
19	or abolish the Cdt activity, little is known about the amino acid residue(s) whose
20	mutation elevates the Cdt activity. We show in this study that substitution of H281
21	with R in <u>Y4 type</u> -CdtB significantly elevates the specific activity of Cdt holotoxin.

22 The activity correlates with the degree of protonation of the side chain in the amino acid

1 281 suggesting a positive charge at position 281 is important for the Cdt activity. Fig. 2 5 shows the alignment of CdtB protein sequences from various pathogenic bacteria 3 including pathogenic E. coli and Haemophilus ducreyi. This shows all but E. coli CDT 4 I of the aligned amino acid residues corresponding to position 281 in A. 5 actinomycetemcomitans CdtB is positively charged. A. actinomycetemcomitance CdtB 6 and *H. ducreyi* Cdt share 97% amino acid identity (18). Recently, the crystal structure 7 of *H.ducreyi* Cdt was determined; and they demonstrated the structures of CdtB and 8 DNase I are similar (11). The amino acid position at 281 of CdtB (R in *H. ducreyi*) is 9 remote from those corresponding to essential amino acids for catalysis of DNase I and 10 those for putative DNA binding (Supplement 2). However, H281 is exposed to the 11 surface of the CdtB molecule. Fig. 3A shows the *in vitro* nuclease assay where amino 12 acid substitution at position 281 of CdtB somehow affects endogenous nuclease activity 13 although its effect is very small. The effect of amino acid substitution at 281 of CdtB 14 on nuclease activity was much more apparent when the *in vivo* chromatin-disintegrating 15 assay was used. There is an observed disparity in the *in vivo* and *in vitro* results (13, 16 16). We do not understand why there is a very low *in vitro* nuclease activity in CdtB 17 and there is a very high specific activity to induce cell cycle arrest, cell distension and 18 chromatin degradation in vivo. 19 Our study suggests that the SNP partly explains the significant heterogeneity 20 of cytotoxic activity present in the culture supernatant of A. actinomycetemcomitans.

- 21 An epidemiological study to determine the relevance of severity of periodontitis with *A*.
- 22 actinomycetemcomitans as the primary isolate as compared to the SNP of the CdtB gene

1 is underway.

2 Bacterial strains, plasmids and primers used in this study are listed in 3 Supplement 1. 4 This work was supported, in part, by a grant-in aid for scientific research from 5 the Ministry of Education, Science, Sports and Culture of Japan. We thank K. Okuda 6 and H. Ohta for providing the clinical isolates of A. actinomycetemcomitans. We are 7 grateful to Reiko Yamaguchi and Hiroyuki Shima for their technical assistance, and Jim 8 Nelson for editorial assistance. We thank the Research Facilities, Hiroshima 9 University School of Dentistry and School of Medicine, for the use of their facilities.

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production in periodontopathogenic bacteria. J. Clin. Microbiol. **41:**1391-1398. FIGURE LEDGENDS

4	Fig. 1 Cdt activity of the purified Cdt holotoxin prepared from a recombinant <i>E</i> .
5	<i>coli</i> carrying Y4 type <i>cdt</i> gene (H281) and <i>cdt</i> genes with the mutated <i>cdtB</i> .
6	HeLa cells were placed in a 96-well plate (Falcon; Becton Dickenson) at a
7	concentration of 2 x $10^3$ cells at 100 µl per well one day before the experiment.
8	The Y4 type <i>cdt</i> locus and corresponding DNA with the mutation was cloned into the
9	expression vector, pQE60, so that the C-terminal of CdtC was tagged with six histidine
10	residues. Various Cdt holotoxins containing <i>cdtB</i> genes with H281 (Y4 type), R281
11	(H281R), A281 (H281A), K281 (H281K), D281 (H281D) were purified using Ni-NTA
12	beads as described previously (14, 20). The purified holotoxins were dialyzed against
13	PBS (NaCl 137 mM, KCl 2.7 mM, Na <sub>2</sub> HPO <sub>4</sub> 8.1 mM, KH <sub>2</sub> PO <sub>4</sub> 1.5 mM, pH7.3) over
14	night, and the protein concentration was adjusted to 0.1 $\mu$ g/ $\mu$ l by BioRad protein assay
15	kit (BioRad, Hercules, CA) respectively. One hundred micro-liter of filter sterilized
16	$(0.22 \ \mu m)$ sample was inoculated on the HeLa cell mono-layer of the first well and
17	serially diluted 1:2 in succeeding wells (20). Morphological changes were monitored
18	using phase contrast microscopy (Nikon DIAPHOT 300) from day1 to day3. Cell
19	distension was defined as greater than five times expansion of the cell size compared to
20	control cells. The cytodistendig activity (total activity) was titrated by using as the end
21	point the highest two-fold dilution of culture supernatant showing 50% transformed
22	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).

2

3 Fig. 2 Flow cytometry of HeLa cell DNA treated with Y4 type Cdt holotoxin or 4 variant holotoxins. The DNA of HeLa cells was analyzed by flow cytometric analysis. Cdt holotoxin or its variant (CdtAB<sup>H281R</sup>C, CdtAB<sup>H281K</sup>C, or CdtAB<sup>H281A</sup>C) 5 6 was added to the HeLa cell culture at concentrations of 6 ng/ml, 60 ng/ml, 0.6 µg/ml, 7 and 6 µg/ml. After 24 h, harvested cells were fixed with ethanol and stained with PI. 8 Ten thousand cells were scanned using a FACScan flow cytometer (Becton Dickinson to 9 detect PI-fluorescence to measure the relative DNA concentration. The histgram of G1 10 and G2 population was analyzed by Modifit software (Verity Software, Inc.). 11 12 Fig. 3 In vitro nuclease and microinjection assays of Y4 type CdtB and variants. A, *In vitro* DNase activity of Y4 type CdtB (CdtB<sup>281H</sup>), CdtB<sup>H281R</sup> and CdtB<sup>H281A</sup>. The 13 Y4 type cdtB or  $cdtB^{mut281}$  were sub-cloned into the expression vector pET28. The 14 15 His-tag recombinant proteins were purified as described (14). The plasmid pGEM 16 DNA (1 µg BioRad) was incubated in buffer (20mM Hepes pH7.5, 150mM NaCl, 5mM 17  $CaCl_2$ , 5mM MgCl<sub>2</sub>) with various concentrations of purified CdtB (0 µg to 10 µg) for 5 18 h at 37 °C. The linear and super-coiled plasmid DNAs were separated using 1 % 19 agarose-gel electrophoresis and visualized with ethidium bromide. B, Y4 type (CdtB<sup>281H</sup>) or variant CdtB (CdtB<sup>H281R</sup>, CdtB<sup>H281A</sup>) with the His-tag at the C-terminus 20 21 was expressed and purified by Ni-agarose chromatography. Microinjection was 22 performed using an Eppendorf Injectman NI2 (12). Purified proteins were adjusted to

1	a concentration of 0.5 $\mu$ g/ $\mu$ l and injected into the cytosol at a pressure of 50-120 hpa for
2	0.2 s. The cells were incubated at $37^{\circ}$ C in 5 % CO <sub>2</sub> 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 <sup>281H</sup> ), Square: CdtB <sup>H281R</sup> ,
9	Triangle: CdtB <sup>H281A</sup> .
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	<u>Sparks, MD) with 0.6% yeast extract (TSBYE, Becton Dickinson) for 3 days.</u> After the total protein concentration <u>of culture supernatant</u> was adjusted <u>at 0.1 <math>\mu</math>g/<math>\mu</math>l by</u> .
18 19	<u>Sparks, MD) with 0.6% yeast extract (TSBYE, Becton Dickinson) for 3 days.</u> After the total protein concentration <u>of culture supernatant</u> was adjusted <u>at 0.1 μg/μl by</u> <u>diluting the sample with PBS, 100μl of</u> the supernatant was added to the culture of
18 19 20	Sparks, MD) with 0.6% yeast extract (TSBYE, Becton Dickinson) for 3 days.Afterthe total protein concentration of culture supernatant was adjusted at 0.1 µg/µl bydiluting the sample with PBS, 100µl of the supernatant was added to the culture ofHeLa cells using a 1:2 serial dilution in a 96 multi-well plate.
18 19 20 21	Sparks, MD) with 0.6% yeast extract (TSBYE, Becton Dickinson) for 3 days. Afterthe total protein concentration of culture supernatant was adjusted at 0.1 µg/µl bydiluting the sample with PBS, 100µl of the supernatant was added to the culture ofHeLa cells using a 1:2 serial dilution in a 96 multi-well plate. After 3 days,morphological cyto-distention was determined after fixing with 100 % methanol and

1 Cdt titer (>10<sup>7</sup> 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* 2 3 genes. Single nucleotide polymorphism (SNP) typing and *Hin*dIII restriction enzyme 4 fragment length polymorphism (RFLP) (21) are shown. B. Polymorphism of CdtB 5 amino acid sequences. Four types of amino acid sequences of CdtB identified in 40 6 clinical strains were aligned. Conserved amino acid residues are shown in gray. An 7 arrowhead shows the signal peptide cleavage site. The predicted catalytic sites 8 conserved in DNase I is shown by stars. The predicted metal binding site conserved in 9 DNase I are shown by a cross.

10

## 11 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:

18 U04208, E. coli CDT III / E. coli 1404: U89305, DNase I / Mus musculus: AAH30394.

1	Supplement 2Van 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.



Fig.1 Nishikubo, et al



Fig.2 Nishikubo, et al









Fig.3 Nishikubo, et al



В

	10	20 20	a 40	50							
Туре Іможуко				P							
Type I M Q W V K Q		S S Y A N L S D F K V A	TWNLQGSSAVNESKWNINV	R							
Type III M Q W A K C	LNVVFCTMLFSFS	SYANLSDFKVA	TWNLQGSSAVNESKWNINV	R							
Туре IV м Q W A К C	LNVVFCTMLFSFS	SYANL SD F K V A	TWNLQGSSAVNESKWNINV	R							
	60	70 80	90 1	00							
Type I QLLSGE	QGADILMVQEAGS	S L P S S A V R T S R V	IQHGGTPIEEYTWNLGTRS	R							
Type II Q L L S G E	QGADILMVQEAGS	S L P S S A V R T S R V	I Q H G G T P I E E Y T W N L G T R S	R							
		I P S S A V R T S R V	I O H G G T P I F F Y T W N I G T R S	R							
lype IV Q L L S G E	QGADILMVQEAGS	SLPSSAVRISRV	' I Q H G G T P I E E Y I W N L G I R S	R							
	110	120 12	0 140 1	50							
Type I PNMVYI	YYSRLDVGANRVN	ILAIVS RR QADE	AFIVHSDSSVLQSRPAVGI	R							
Type    PNMVYI	YYSRLDVGANRVN		AFIVHSDSSVLQSRPAVGI	R							
Type III P N M V Y I	Y Y S R L D V G A N R V N	LAIVSRRQADE	AFIVHSDSSVLQSRPAVGI	R							
Type IV PNMVYI	YYSRLDVGANRVN	I L A I V S R R Q A D E	AFIVHSDSSVLQSRPAVGI	R							
	160	170 18	<sup>0</sup> 190 🗗	00							
Type I IGTDVF	FT V H A L A T G G S D A	A VSLIRNIFTTF	T S S P S S P E R R G Y S W M V V G D	F							
IVPE I GTDVF	FTVHALATGGSDA	VSLIRNI FTTF	T S S P S S P E R R G Y S W M V V G D	E							
	FIVHALATGGSDA	VSLIRNIFTIF	TS SPS SPER RGYSWMVV GD	E.							
Type IV IGIDVF	FIVHALAIGGSDA	VSLIKNIFIIF									
	210	220 230	0 240 2	50							
Type I NRAPVN	ILEAALRQEPAVSE		HR SGNILDYAILHDAHLPR	R							
Type II NRAPVN	ILEAALRQEPAVSE	N T I I I A P T E P T	HR SGNIL DYAILH DAHL PR	R							
Type III NR AP VN	II F A A I R O F P A V S F	. N T I I I A P T F P T	HRSGNII DYAIIHDAHIPR	R							
Type IV NRAPVN	ILEAALRQEPAVSE	. N T I I I A P T E P T	HR SGNILDYAILHDAHLPR	R							
	260	270 280	°								
Type I E Q A R E R	I G A S L M L N Q L R S G	1 T S D H F P V S F V									
	I G A S L M L N Q L R S Q										
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		<b>*</b> *	<b></b>								
			201								



Aactinomycetemicomitans     450       Ecoli     M15     pQEcduBC     Y4 cduB/ <sup>M316</sup> C     or pQE60 (Nco 1-Bg/II)     14       M15     pQEcduB     CduB     add M316     C     add M316     Add M3	Strain		Plasmid	Character	Reference
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	111851	TGATGCGGT	ΔGTTTΔΔTTCGTΔΔTΔ	cdtB sequencing primer	18



Supplement 2. Nishikubo, et al