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Single Nucleotide Polymorphism in the Cytolethal Distending Toxin B Gene Confers Heterogeneity in the Cytotoxicity of *Actinobacillus actinomycetemcomitans*▽†

Shuichi Nishikubo,[1,2] Masaru Ohara,[1] Masae Ikura,[1] Katsuo Katayanagi,[3] Tamaki Fujiwara,[1] Hitoshi Komatsuzawa,[1] Hidemi Kurihara,[2] and Motoyuki Sugai[1]*

Departments of Bacteriology[1] and Periodontal Medicine,[2] Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan, and Department of Mathematical and Life Sciences, Hiroshima University Graduate School of Science, Higashi-Hiroshima 739-8526, Japan[3]

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Clinical *Actinobacillus actinomycetemcomitans* produces cytolethal distending toxin (CDT) with titers ranging from 10^2 to 10^8 U/mg. Single nucleotide polymorphism analysis of the *cdt* gene in clinical isolates identified a variation of a single amino acid at residue 281 of CdtB, which significantly affected CDT toxicity by modulating the chromatin-degrading activity of CdtB.

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) have shown that *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 references 4, 13, 15, 16, and 19). It inhibits the cell proliferation of cultured cells by arresting the cell cycle at G_2/M (17). The intoxicated cells show distensions of the cells and nuclei and eventually die. CDT is a trimer holotoxin: CdtB is the active subunit, and CdtA and CdtC comprise 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 metalloenzyme superfamily, 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 followed by the phosphorylation of histone H2AX (8) and the relocalization of the DNA repair complex Mre11-Rad50 (3). However, whether CdtB acts as a genuine DNase is still unknown. The possible mode of DNA damage by CdtB may activate a checkpoint control which results in G_2 arrest in the intoxicated cells (13, 15, 19). CDT may be involved in the pathogenesis of bacterial chronic infections; however, the molecular pathophysiological role of CDT is unknown.

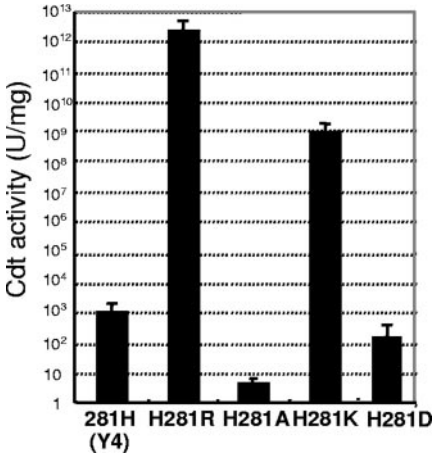

FIG. 1. CDT activity of the purified CDT holotoxin prepared from a recombinant *E. coli* carrying the Y4-type *cdt* gene (H281) and *cdt* genes with the mutated *cdtB*. HeLa cells were placed in a 96-well plate (Falcon; Becton Dickinson) at a concentration of 2×10^3 cells at 100 μl per well 1 day before the experiment. The Y4-type *cdt* locus and corresponding DNA with the mutation were cloned into the expression vector pQE60 so that the C terminus of CdtC was tagged with six-histidine residues. Various CDT holotoxins containing *cdtB* genes with H281 (Y4 type), R281 (H281R), A281 (H281A), K281 (H281K), and D281 (H281D) were purified using Ni-nitrilotriacetic acid beads as described previously (14, 20). The purified holotoxins were dialyzed against phosphate-buffered saline (NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 8.1 mM; KH_2PO_4, 1.5 mM; pH 7.3) overnight, and the protein concentration was adjusted to 0.1 μg/μl by using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). One hundred microliters of filter-sterilized (0.22 μm) sample was inoculated on the HeLa cell monolayer 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 day 1 to day 3. Cell distension was defined as a greater than five times expansion of the cell size compared to that of control cells. The cytodistending activity (total activity) was titrated by using as the end point the highest twofold dilution of culture supernatant showing 50% transformed cells after 72 h incubation. A unit of CDT activity was defined as the reciprocal of the dilution, and the specific activity was defined as units per milligram of protein. Error bars indicate standard deviations.

* Corresponding author. Mailing address: Department of Bacteriology, Hiroshima University Graduate School of Biomedical Sciences, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan. Phone: 81 82 257 5635. Fax: 81 82 257 5639. E-mail: sugai@hiroshima-u.ac.jp.

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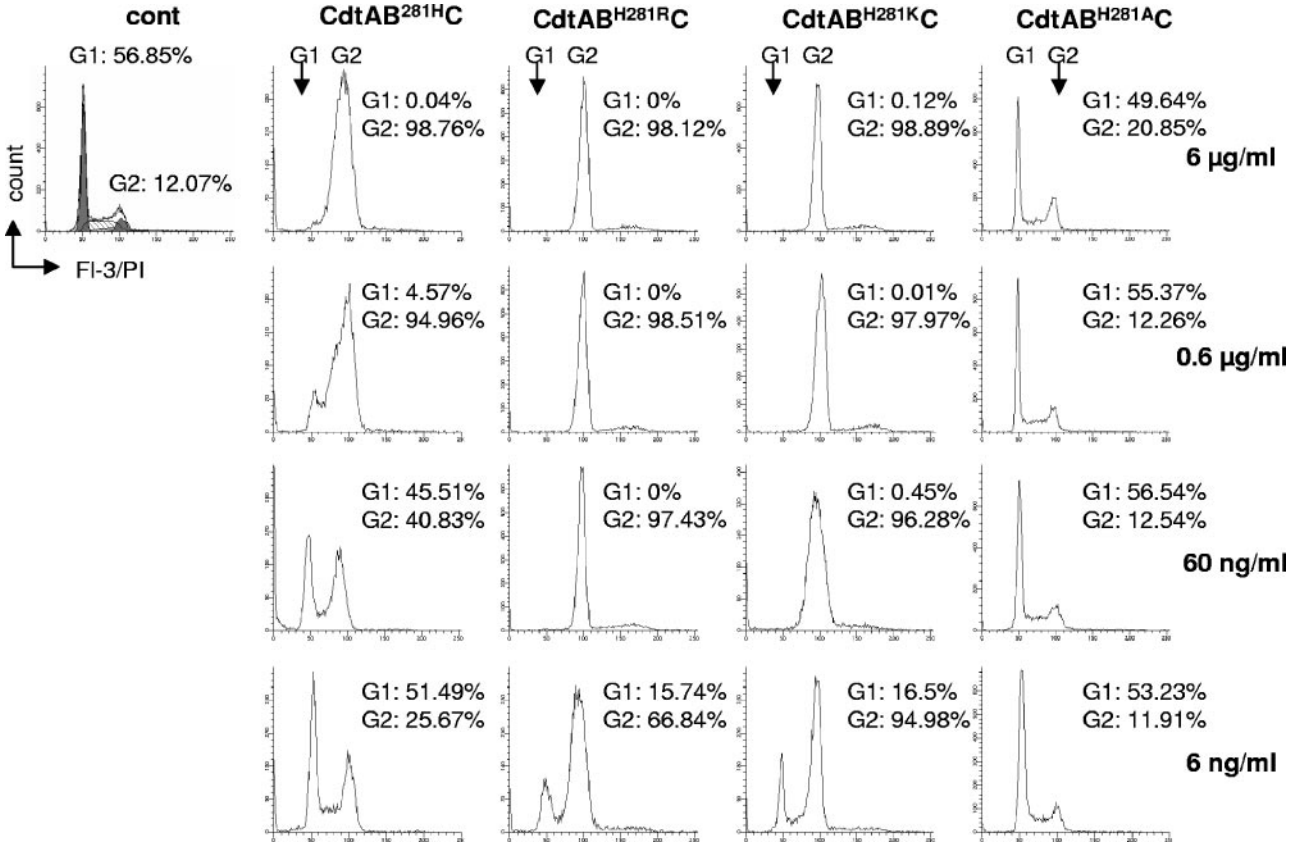

FIG. 2. Flow cytometry of HeLa cell DNA treated with Y4-type CDT holotoxin or variant holotoxins. The DNA of HeLa cells was analyzed by flow cytometric analysis. CDT holotoxin or its variant (CdtAB^{H281R}C, CdtAB^{H281K}C, or CdtAB^{H281A}C) was added to the HeLa cell culture at concentrations of 6 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 propidium iodide (PI). Ten thousand cells were scanned using a FACScan flow cytometer (Becton Dickinson) to detect PI fluorescence to measure the relative DNA concentration. The histogram of G_1 and G_2 populations was analyzed by Modifit software (Verity Software, Inc.). FI-3, fluorescence detector type 3.

Identification of SNP in the *cdtB* gene of strains with elevated cytotoxic activity. We previously reported that 40 (89%) of 45 *A. actinomycetemcomitans* isolates from periodontitis patients possessed the *cdtABC* genes (21). The CDT activity was found in the culture supernatant of the 40 strains; however, the titer of the toxin ranged 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 ($>10^7$ U) CDT titers (21). The concentration of the CDT produced in the culture supernatant by these strains was compared to that of strain Y4, a low-titer (ca. 10^2 U) strain. Immunoblot analysis shows that the concentrations of CDT from the three high-titer strains were similar to that of strain Y4 (data not shown). We then determined whether 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 of the clinical strains by PCR and were directly sequenced, and the results were compared with sequences of genes derived from strain Y4. Two amino acid substitutions, resulting from two single-nucleotide alterations, were found in the CdtB sequences of strains 330, 1773, and 2102: the fourth valine residue, valine 4 (V4), was substituted with alanine (A, GTA 224 GCA), and histidine 281 (H281) was substituted with arginine (R, CAT to 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 that 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 to the CDT activity, we performed site-directed mutagenesis by 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 terminus of CdtC was tagged with six-histidine residues. The CDT holotoxin complex of three subunits was purified using Ni-nitrilotriacetic acid beads as described previously (14, 20). Immunoblot analysis showed that the amino acid substitution did not affect the protein ratio among the subunit components (not shown). The titers of the purified holotoxins were determined. Figure 1 shows that CdtAB^{H281R}C had 10^9 times higher activity than that of the Y4-type, CdtAB^{H281}C. To determine the function of the amino acid residue at position 281, we

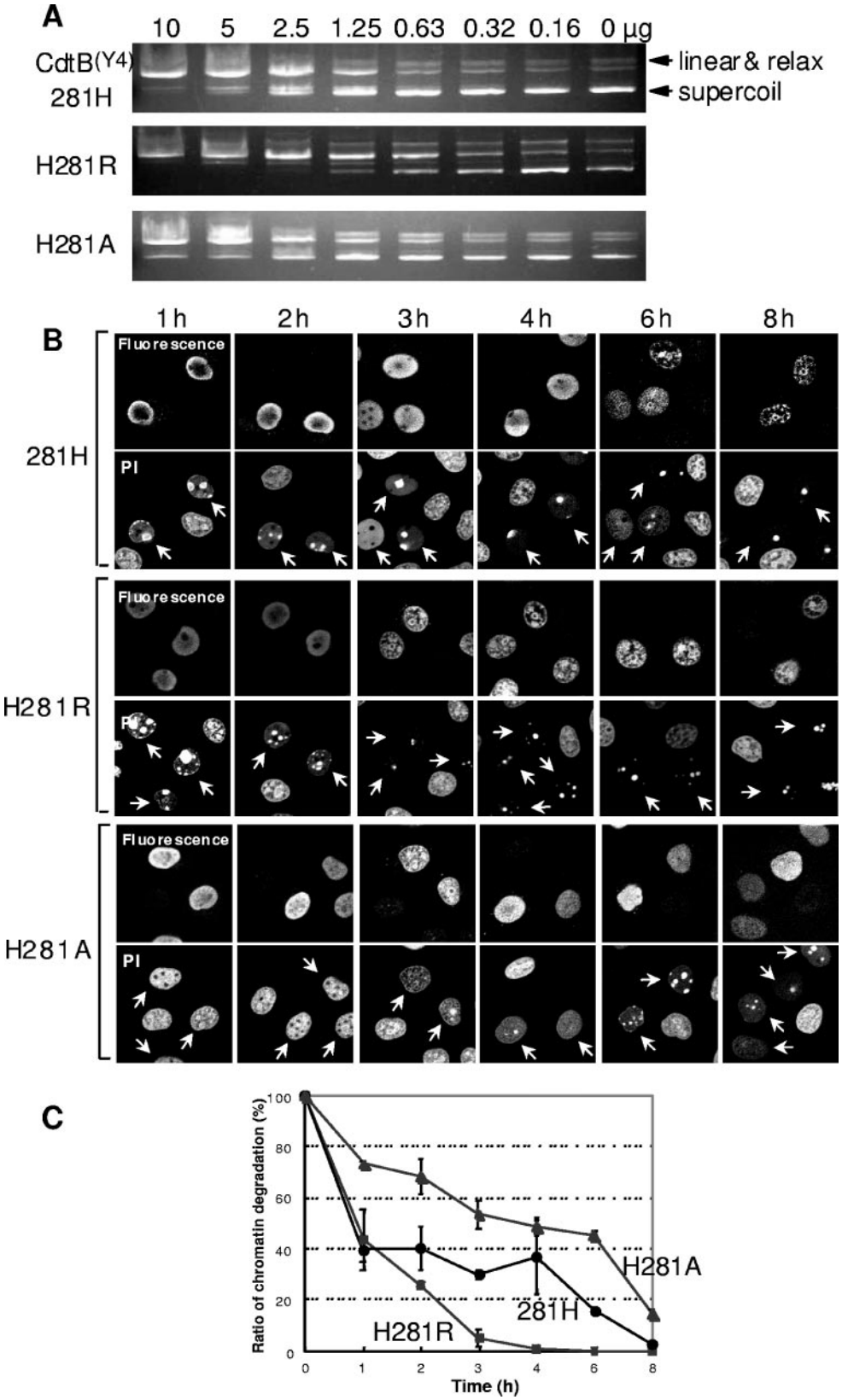

FIG. 3. In vitro nuclease and microinjection assays of Y4-type CdtB and variants. (A) In vitro DNase activity of Y4-type CdtB ($CdtB^{281H}$), $CdtB^{H281R}$, and $CdtB^{H281A}$. The Y4-type *cdtB* or $cdtB^{mut281}$ was subcloned into the expression vector pET28. The His tag recombinant proteins were purified as described previously (14). The plasmid pGEM DNA (1 μg; Bio-Rad) was incubated in buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$) with various concentrations of purified CdtB (0 μg to 10 μg) for 5 h at 37°C. The linear and supercoiled plasmid

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, nonpolar amino acid A281, the holotoxin (CdtAB^{H281A}C) lost most of its CDT activity. When H281 is replaced with another basic amino acid, K, the holotoxin (CdtAB^{H281K}C) showed elevated activity of 10^6-fold, though it was less than that of CdtAB^{H281R}C. When H281 is substituted with an acidic residue, D, the holotoxin (CdtAB^{H281D}C) showed decreased activity that was less than that of the Y4 type. The site-directed mutagenesis suggested that a positively charged amino acid at position 281 in CdtB is critical for CDT activity of *A. actinomycetemcomitans*, while 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 that of 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. Figure 2 shows that CdtAB^{H281R}C and CdtAB^{H281K}C blocked the cell cycle at a low concentration of 6 ng/ml, whereas the 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 to 9 orders of magnitude more active than was the Y4-type holotoxin in the cellular distension assay, whereas it was approximately only 3 orders of magnitude more active in the cell cycle-arresting assay. Moreover, in the cellular distension assay, CdtAB^{H281R}C was apparently stronger than CdtAB^{H281K}C but the two showed similar activities in the cell cycle-arresting assay. We cannot explain these discrepancies since the 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 that 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 by using the in vitro nuclease assay (2, 11). Figure 3A shows the weak nuclease activity of Y4-type CdtB. CdtB^{H281R} shows activity approximately fourfold stronger than that of CdtB^{H281}, whereas CdtB^{H281A} had a weaker activity than that of 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 the 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 by using propidium iodide (PI) staining (Fig. 3B). Figure 3C showed that CdtB^{H281R} completely disintegrated the chromatin in 3 h after microinjection, whereas the Y4-type CdtB took 8 h and whereas CdtB^{H281A} showed a much weaker activity in disintegrating the chromatin DNA. Therefore, the results suggest that the amino acid substitution of CdtB at position 281 may strongly affect the chromatin-disintegrating activity of CdtB in vivo.

SNP analysis of the *cdtB* gene in clinically isolated strains. We investigated the single nucleotide polymorphism (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 of which corresponded 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, for which the CdtB sequence is the same as that of strain Y4 (the 281st amino acid is H). Another 11 strains (26.8%) belong to type II, for which the 281st amino acid of CdtB sequence is R. Eighteen strains (43.9%) belong to type III and contain two amino acid substitutions in the CdtB, V4 to A and H281 to R. And one strain belongs to type IV and contains three amino acid substitutions in the CdtB amino acid sequence, V4 to A, D199 to G, and H281 to R. 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 HindIII restriction fragment length polymorphism (RFLP) typing we performed previously (21) shows that 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 were 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

DNAs were separated by using 1% agarose gel electrophoresis and visualized with ethidium bromide. (B) Y4-type (CdtB^{281H}) or variant CdtB (CdtB^{H281R} or 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 NI 2 (12). Purified proteins were adjusted to a concentration of 0.5 μg/μl and injected into the cytosol at a pressure of 50 to 120 hPa for 0.2 s. The cells were incubated at 37°C in 5% CO_2 for 1 to 8 h, and then the injected cells were stained by immunohistochemistry for CdtB (12) and with propidium iodide, followed by observation using confocal microscopy (Carl Zeiss LSM 401). Arrows indicate the cells microinjected with CdtB. (C) The relative chromosomal DNA was measured by a Mac Scope image analysis system, for which the reduction of intensity of propidium iodide stain was plotted against the relative decrease of the signal showing the relative concentration of intact DNA. Circle, Y4-type CdtB (CdtB^{281H}); square, CdtB^{H281R}; triangle, CdtB^{H281A}. Error bars indicate standard deviations.

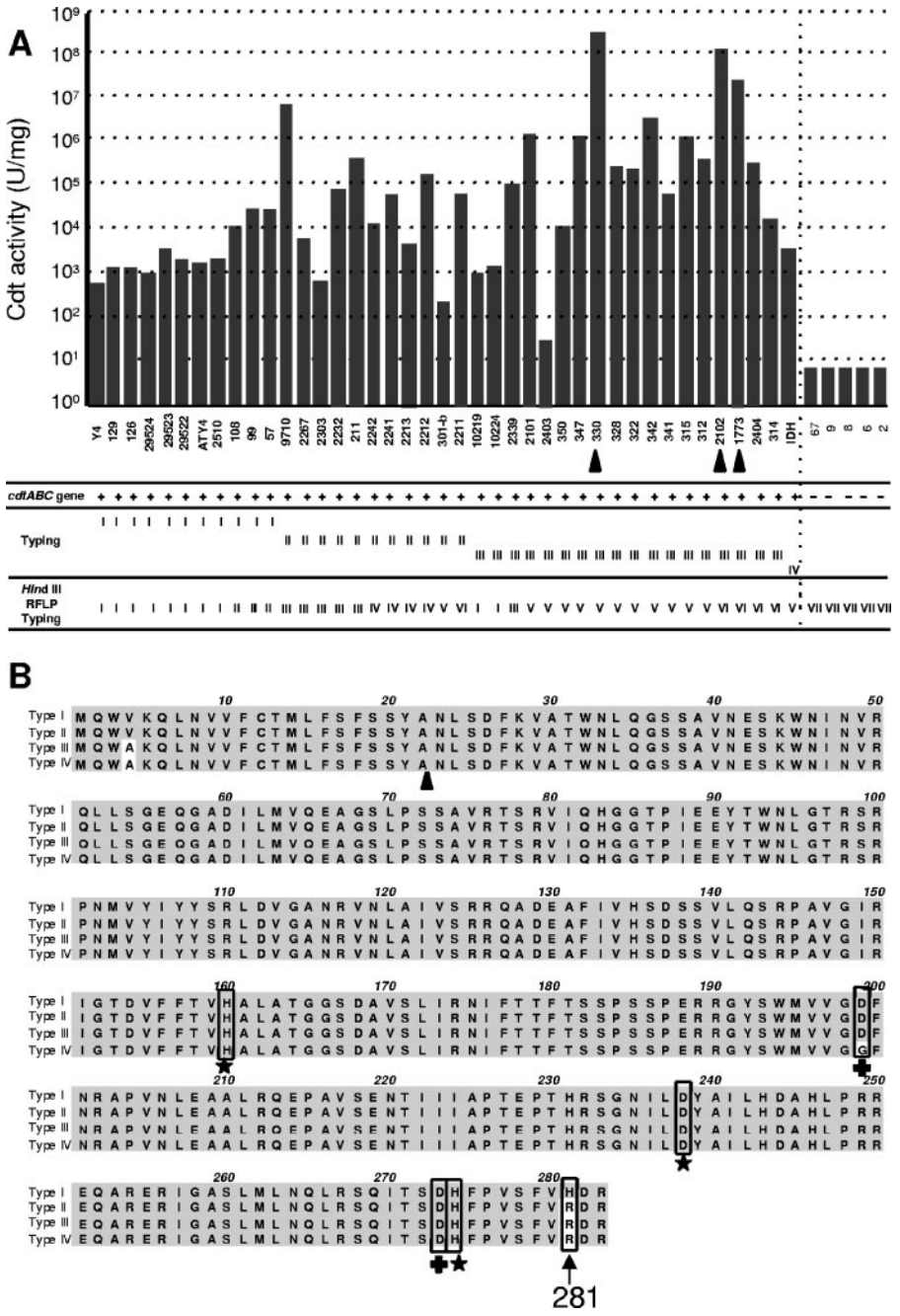

FIG. 4. Polymorphism of the CdtB amino acid sequence in clinically isolated *A. actinomycetemcomitans*. (A) Cytodistending activity, RFLP typing, and CdtB typing of *A. actinomycetemcomitans* clinical strains. The culture supernatant was prepared from exponentially growing *A. actinomycetemcomitans*. The protein concentration of the culture supernatant was measured by using a Bio-Rad protein assay kit (Bio-Rad) at an optical density of 0.3 after culturing *A. actinomycetemcomitans* in Trypticase soy broth (Becton Dickinson, Sparks, MD) with 0.6% yeast extract (TSBYE; Becton Dickinson) for 3 days. After the total protein concentration of culture supernatant was adjusted at 0.1 μg/μl by diluting the sample with phosphate-buffered saline, 100 μl of the supernatant was added to the culture of HeLa cells by using a 1:2 serial dilution in a 96 multiwell plate. After 3 days, morphological cytodistention was determined after fixing with 100% methanol and staining with 2% Giemsa solution. The arrowheads show the strains with very high CDT titers ($>10^7$ U). The presence (+) or absence (−) of the *cdt* genes is shown. The dotted *y* axis line divides the strains with (left side) or without (right side) the *cdtABC* genes. Results from SNP typing and HindIII RFLP are shown (modified from reference 21 with permission). (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 are shown by stars. The predicted metal binding sites conserved in DNase I are shown by crosses.

(2) reported mutations at H134, D212, H252, or D251 that correspond to the catalytic sites or metal binding site where H152 and D185 were important for the *C. jejuni* CDT activity (5). Nesic et al. showed that the mutations at positions R144, N201, and R117, which correspond to the DNA contact sites of DNase I, resulted in a complete loss of activity (11). Although data are accumulating regarding several mutations in the *cdtB* gene that reduce or abolish the CDT activity, little is known

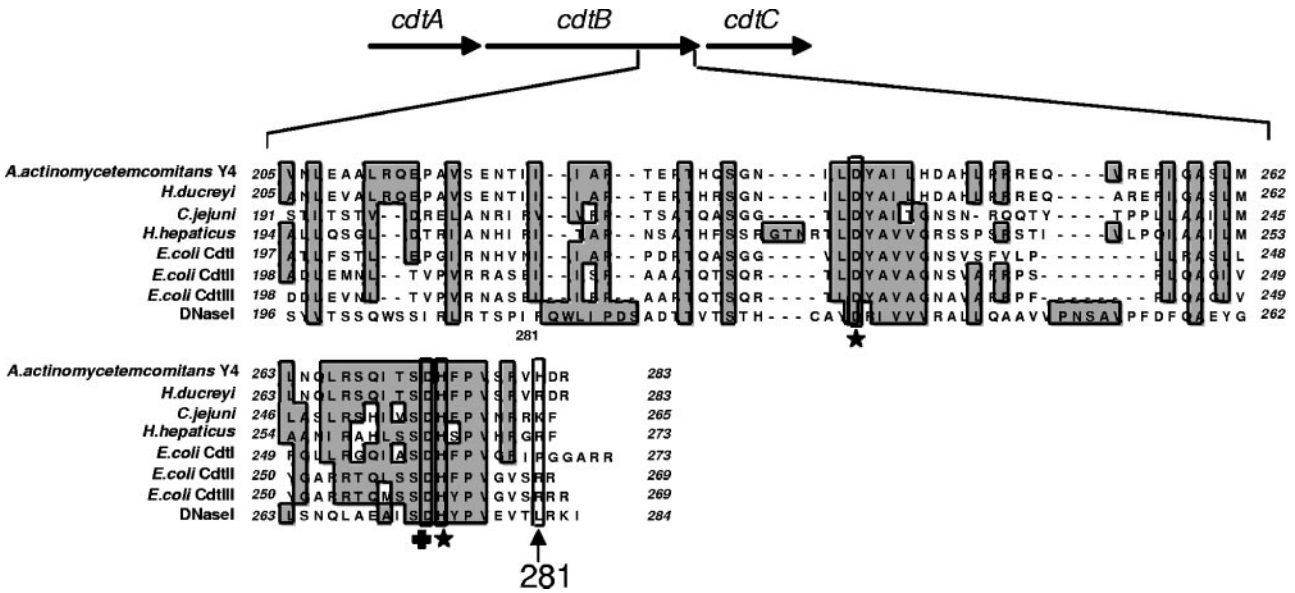

FIG. 5. Alignment of CdtBs from various microorganisms and DNase I. Predicted C-terminal sequences of CdtBs from various pathogenic bacteria were aligned. The conserved amino acid residues are in gray. The predicted catalytic sites conserved in DNase I are shown by stars. The predicted metal binding site conserved in DNase I is shown by a cross. GenBank accession numbers are as follows: for *A. actinomycetemcomitans* Y4, AB011405; for *H. ducreyi*, 35000; for *C. jejuni* 81-176, U51121; for *Helicobacter hepaticus*, AAF19158; for *E. coli* CDT I/*E. coli* E6468/62, U03293; for *E. coli* CDT II/*E. coli* 9142-88, U04208; for *E. coli* CDT III/*E. coli* 1404, U89305; and for DNase I/*Mus musculus*, AAH30394.

about the amino acid residue(s) whose mutation elevates the CDT activity. In this study, we show that the 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 that a positive charge at position 281 is important for CDT activity. Figure 5 shows the alignment of CdtB protein sequences from various pathogenic bacteria, including pathogenic *Escherichia coli* and *Haemophilus ducreyi*. This shows that all but *E. coli* CDT I of the aligned amino acid residues corresponding to position 281 in *A. actinomycetemcomitans* CdtB is positively charged. *A. actinomycetemcomitans* CdtB and *H. ducreyi* CDT share 97% amino acid identity (18). Recently the crystal structure of *H. ducreyi* CDT was determined, and it demonstrated that 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 the catalysis of DNase I and those for putative DNA binding (see Fig. S2 in the supplemental material). However, H281 is exposed to the surface of the CdtB molecule. Figure 3A shows the results from an in vitro nuclease assay where the amino acid substitution at position 281 of CdtB somehow affects endogenous nuclease activity, although its effect is very small. The effect of the 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 level of 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 relative to the SNP of the CdtB gene is underway.

The bacterial strains, plasmids and primers used in this study are listed in Table S1 in the supplemental material.

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