Polymorphisms Influencing Expression of Dermonecrotic Toxin in *Bordetella bronchiseptica*

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Abstract

*Bordetella bronchiseptica* is a pathogenic bacterium causing respiratory infections in a broad range of mammals. Recently, we determined the whole genome sequence of *B. bronchiseptica* S798 strain isolated from a pig infected with atrophic rhinitis and found four single-nucleotide polymorphisms (SNPs) at positions -129, -72, +22, and +38 in the region upstream of *dnt* encoding dermonecrotic toxin (DNT), when compared with a rabbit isolate, RB50. DNT is known to be involved in turbinate atrophy observed in atrophic rhinitis. Immuno blotting, quantitative real-time PCR, and β-galactosidase reporter assay revealed that these SNPs resulted in the increased promoter activity of *dnt* and conferred the increased ability to produce DNT on the bacteria. Similar or identical SNPs were also found in other pig isolates kept in our laboratory, all of which produce a larger amount of DNT than RB50. Our analysis revealed that substitution of at least two of the four bases, at positions -72 and +22, influenced the promoter activity for *dnt*. These results imply that these SNPs are involved in the pathogenicity of bordetellae specific to pig diseases.

Introduction

Pathogenic bordetellae including *Bordetella bronchiseptica*, *B. pertussis*, and *B. parapertussis* are known to cause respiratory infections in mammals. These bordetellae share many virulence factors, such as filamentous hemagglutinin, adenylate cyclase toxin, dermonecrotic toxin (DNT), and type III secretion apparatus and effectors. Comparative analysis of their genome sequences revealed that *B. pertussis* and *B. parapertussis* independently evolved from a *B. bronchiseptica*-like ancestor [1] through genome decay including extensive loss and translocation of genes, which resulted in different host tropism: *B. bronchiseptica* infects various mammals, causing atrophic rhinitis in pigs, kennel cough in dogs, bronchopneumonia in guinea pigs and rabbits, and sporadic infections in humans. *B. pertussis* and *B. parapertussis* of one
lineage infect humans exclusively, while *B. parapertussis* of another distinct lineage is found only in sheep [2]. These bordetellae, which are phylogenetically closely related to each other but show different host tropism, are considered to serve as an appropriate subject of research for understanding how host tropism of pathogenic bacteria could be determined [1,3–5].

Recently, we determined the whole genome sequence of *B. bronchiseptica* S798 strain isolated from a pig infected with atrophic rhinitis [6], the strain that has often been used for the production and purification of DNT [7,8] and analyses of its pathogenesis [9,10]. This was the first sequenced genome of a *B. bronchiseptica* pig isolate. To gain insight into genetic characteristics that may reflect the host tropism of *B. bronchiseptica*, we compared the S798 genome with previously sequenced genomes of rabbit, dog, and human isolates, but did not find any S798–specific genetic loci that could be related to the bacterial pathogenesis. Instead, four single-nucleotide polymorphisms (SNPs) were found in the region upstream of *dnt*, when compared with a rabbit isolate, RB50, which has been widely used as a reference strain [1,11]. In this study, we examined the effects of the SNPs on bacterial phenotype and found that they resulted in the increased promoter activity of *dnt*, leading to the high production of DNT, which is known to cause turbinate atrophy, by which atrophic rhinitis is characterized [8]. Further analyses revealed that 12 of 13 pig isolates carried 3 to 4 SNPs at the same sites and produced larger amounts of DNT than the other strains of *B. bronchiseptica* and *B. pertussis* having no nucleotide substitutions at the corresponding positions. These results might imply a correlation between genetic characteristics and pathogenesis to pigs in *B. bronchiseptica*.

**Materials and Methods**

**Bacterial strains and growth media**

The bacterial strains used in this study are listed in *S1 Table*. *B. bronchiseptica* pig isolates S798, R033, S807, AFUY13, KII13, KII24, KG19, KHI11, MH110, TAY12, TAY13, STY136, and STY137, which were field isolates from pigs, were provided by N. Terakado (National Institute of Animal Health, Ibaraki, Japan) and S. Nagai (Nippon Institute for Biological Science, Tokyo, Japan). Four strains of *B. bronchiseptica* 10177–36, 10255–6, 10519–15, and 10092–26 were isolated from mice in Japan. *B. bronchiseptica* RB50 was provided by P. A. Cotter (University of North Carolina, NC). *B. pertussis* Tohama was maintained in the laboratory. The *Bordetella* strains were grown at 37°C on a Bordet-Gengou (BG) agar plate (Becton Dickinson, Franklin Lakes, NJ) containing 0.8% (v/v) glycerol and 20% (v/v) defibrinated horse blood or in Stainer-Scholte (SS) liquid medium [12]. *Escherichia coli* strains were grown at 37°C on an LB agar plate or LB broth. The growth media were supplemented with antibiotics at the following concentrations when necessary: gentamicin, 10 μg/ml; cefituben, 10 μg/ml; streptomycin, 10 μg/ml; and ampicillin, 50 μg/ml. The concentration of bacterial cells was estimated from OD650 values according to the following equation: 1 OD650 = 4.5 x 10⁸ cells/ml.

Mutant strains of *B. bronchiseptica* were constructed as described previously by Sekiya et al. [13] with slight modifications. The plasmids and primers used in this study are listed in *S2 and S3 Tables*. A single-base substitution was introduced into the region upstream of *dnt* as follows. A 0.5-kbp DNA fragment to generate the single-base substitution was amplified by PCR with *B. bronchiseptica* S798 genomic DNA as a template and the combination of primers S798-DNTr-Up-S and S798-DNTr-Down-AS. The single-base substitution was generated by the underlined base of the primer (*S3 Table*). A 0.7-kbp DNA fragment was amplified by PCR with *B. bronchiseptica* S798 genomic DNA as a template and the combination of primers S798-DNTr-Up2-S and S798-DNTr-Down-AS. Both DNA fragments were joined by PCR with the combination of primers S798-DNTr-Up-S+ and S798-DNTr-Down-AS+. The resulting PCR product contained sequences corresponding to those of the terminal regions of *Sma*I-digested...
pABB-CRS2-GmA2, which was used as a suicide vector at each end. The PCR product was ligated with pABB-CRS2-GmA2 via the overlapping sequences using the In-Fusion HD cloning kit (Takara Biochemicals, Shiga, Japan) according to the manufacturer’s instructions. The resulting plasmid was designated pABB-CRS2-Pdnt-72C and introduced into E. coli DH5α λpir, and then transferred to B. bronchiseptica RB50 by triparental conjugation with a helper strain, HB101/pRK2013. The first recombinant strain that had integrated pABB-CRS2-Pdnt-72C into the chromosome was selected by growth on BG agar plates containing cefitabuten and gentamicin. Subsequently, the second recombinant strains, in which pABB-CRS2-Pdnt-72C was eliminated by homologous recombination, were isolated, after incubation on LB agar plates without NaCl and containing 10% sucrose, cefitabuten, and streptomycin. PCR with the combination of the genomic DNA of recombinant strains as templates and primers PstI-GmR-S and PstI-GmR-AS did not amplify any DNA fragments, confirming elimination of pABB-CRS2-Pdnt-72C.

DNA sequencing

Genomic DNA was isolated using the Genomic DNA Buffer Set and Genomic-tip 100/G (Qiagen, Venlo, Netherlands). A DNA fragment containing dnt and its flanking regions was amplified by PCR with B. bronchiseptica genomic DNA as a template and the combination of primers BB3979-S and BB3975-AS. The resulting PCR product was isolated by agarose gel electrophoresis, purified using a QIAquick Gel Extraction Kit (Qiagen), and sequenced with the primers BB3979-AS_reverse and BB3979-S-2. Multilocus Sequence Typing (MLST) analysis on the basis of the sequence of seven housekeeping genes (adk, fumC, glyA, tyrB, icd, pepA, and pgm) was performed as previously described [4]. Sequence types of each strain were designated according to the Bordetella MLST database (http://pubmlst.org/bordetella).

Immunoblotting

B. bronchiseptica and B. pertussis grown in SS medium for 12 h and 24 h, respectively, were recovered by centrifugation at 12,000 rpm for 5 min, suspended in sodium dodecyl sulfate (SDS) gel-loading buffer (62.5 mM Tris-HCl, pH 6.8, containing 10% glycerol, 2% SDS, 25 mM dithiothreitol, and 0.005% bromophenol blue), sonicated, and subjected to SDS polyacrylamide gel electrophoresis (7.5% polyacrylamide) [14] after incubation at 98°C for 5 min, followed by electrotransfer to a polyvinylidene difluoride membrane (Millipore Billerica, MA). The membrane was probed with the combination of mouse monoclonal antibody against DNT [15] and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Cappel, MP Biomedicals, Santa Ana, CA), or another combination of rabbit antiserum against FtsZ, a bacterial cytoskeletal protein, and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The proteins on the membrane were detected with an ECL Western Blotting Detection System (GE Healthcare, Uppsala, Sweden). DNT purified from B. bronchiseptica S798 as described previously [16] was used as a positive control.

Quantitative RT-PCR

Bacterial RNA was isolated with TRIzol (Ambion, Life Technologies) according to the manufacturer’s instructions. Contaminating genomic DNA was digested by RNase-free DNase I (Takara). RNA was reverse-transcribed with SuperScript III Reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad, CA) with random primers. With the resulting cDNA as a template, dnt and recA were amplified using Power SYBR Green PCR Master Mix (Applied Biosystems, CA) with primer pairs RT-3978-F/RT-3978-R and RT-recA-F/RT-recA-R, respectively.
The relative levels of *dnt* mRNA in each strain were normalized to the corresponding *recA* levels by the comparative cycle threshold method [17].

### 5′-Rapid amplification of cDNA ends (RACE)

The transcriptional start site of *dnt* was determined by sequencing of the 5′ terminal region of mRNA, which was cloned using the 5′-Full RACE Core set (Takara) according to the manufacturer’s instructions. A DNA fragment complementary to the 5′ terminal region of *dnt* mRNA was obtained by reverse transcription with 5′ end-phosphorylated primer DNT-5RACE-RT and circularized by T4 RNA ligase. A 0.3-kbp DNA fragment containing the sequence corresponding to the 5′ terminal region of *dnt* mRNA was amplified by nested PCR with the circularized complementary DNA as a template and two combinations of the primers: DNT-5RACE-S1 and DNT-5RACE-A1, or DNT-5RACE-S2 and DNT-5RACE-A2. The resulting PCR product was cloned into pCR2.1-TOPO (Invitrogen) and sequenced.

### β-galactosidase reporter assay

A reporter plasmid, pT1T2-SD-*lacZ*, was constructed and used for a β-galactosidase reporter assay (S1 Fig.). A 3.8-kbp DNA fragment containing genes required for plasmid replication (*rep*), resistance to gentamicin (GmR), and plasmid mobilization (*mob*) and an SD sequence, which is a ribosome-binding site in the mRNA, was amplified by inverse PCR with pBBR1MCS-5 as a template and the combination of primers XhoI-pBBR1MCS-AS+ and SD-SmaI-pBBR1MCS-S+. A 0.4-kbp DNA fragment containing the BBr01 promoter, for 16S ribosomal RNA, was amplified by PCR with *B. bronchiseptica* RB50 genomic DNA as a template and the combination of primers XhoI-PBBr01-SD-S+ and SmaI-PBBr01-SD-AS+. The DNA fragment contained sequences corresponding to those of the terminal regions of the fragment of the portion of pBBR1MCS-5 at each end. These corresponding sequences were generated from the underlined 15-base sequences of the primers (S3 Table). Both DNA fragments were ligated via the overlapping sequences using the In-Fusion HD cloning kit (Takara), and pBBr01-SD was obtained. A 3.1-kbp DNA fragment containing *lacZ* was amplified by PCR with E. coli W3110 genomic DNA as a template and the combination of primers Ecoli-*lacZ*-S+ and Ecoli-*lacZ*-AS+. The resulting PCR product was cloned into SmaI-digested pBBr01-SD using the In-Fusion HD cloning kit (Takara), and pBBr01-SD-*lacZ* was obtained. A 7.3-kbp DNA fragment was amplified by inverse PCR with pBBr01-SD-*lacZ* as a template and the combination of primers Promoter-T1-S and Promoter-T1-AS, and linearized pBBr01-SD-*lacZ* was obtained. A 0.3-kbp DNA fragment containing *rrnB* terminator was amplified by PCR with pKK232–8 (Amersham, GE Healthcare, Uppsala, Sweden) as a template and the combination of primers rrnB-T1-termi-S2+ and rrnB-T2-termi-AS2+. The resulting PCR product was joined with linearized pBBr01-SD-*lacZ* using the In-Fusion HD cloning kit (Takara), and pT1T2-BBr01-SD-*lacZ* was obtained. A 7.3-kbp DNA fragment without a BBr01 promoter was amplified by inverse PCR with pT1T2-BBr01-SD-*lacZ* as a template and the combination of primers SD-*lacZ*-S and SD-*lacZ*-AS, and linearized pT1T2-BBr01-SD-*lacZ* without a BBr01 promoter was obtained. The resulting PCR product was phosphorylated by T4 polynucleotide kinase (Takara) and circularized by Ligaction high Ver.2 (TOYOBO, Osaka, Japan), and pT1T2-SD-*lacZ* was obtained. For the construction of the *lacZ* fusion with the *dnt* promoter, a 0.2-kbp DNA fragment containing the sequence upstream of *dnt* was amplified by PCR with *B. bronchiseptica* genomic DNA as a template and two combinations of primers: DNT-P-SD-S+ and RB50-DNT-P-SD-AS+, or DNT-P-SD-S+ and S798-DNT-P-SD-AS+. The resulting PCR products were independently cloned into linearized pT1T2-BBr01-SD-*lacZ* without a BBr01 promoter using the In-Fusion HD cloning kit (Takara), and pT1T2-RB50, pT1T2-S798, and pT1T2-AFUY13
were obtained. Single-base substitutions were introduced into the upstream region of *dnt* following a standard site-directed mutagenesis protocol (QuickChange, Stratagene, Agilent Technologies, Inc., Santa Clara, CA) with pT1T2-RB50 as a template and four combinations of primers: RB50-DNT-P-72T-S and RB50-DNT-P-72T-AS, RB50-DNT-P-129C-S and RB50-DNT-P-129C-AS, RB50-DNT-P+22T-S and RB50-DNT-P+22T-AS, or RB50-DNT-P+38T-S and RB50-DNT-P+38T-AS, and nine plasmids, pT1T2-RB50-1 to pT1T2-RB50-9, were obtained. Single-base substitutions were generated from the underlined one-base sequence of the primers (S3 Table). *B. bronchiseptica* RB50 strains carrying the reporter plasmids were grown in SS medium. One hundred microliters of the bacterial culture was added to one milliliter of 60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, 50 mM β-mercaptoethanol, 0.001% SDS, and 5% chloroform in a test tube and lysed by thorough shaking. β-galactosidase activity in the bacterial lysate was determined as described previously [18] and obtained values were normalized to OD<sub>650</sub> values representing bacterial numbers of the respective cultures.

**Results**

SNPs influencing the expression of *dnt* in *B. bronchiseptica*

When compared with *B. bronchiseptica* RB50, a pig isolate S798 was found to contain four SNPs at positions -129, -72, +22, and +38 in the region upstream of *dnt*, where position +1 is assigned for the transcriptional start site that was determined by the 5'-RACE method (Fig. 1A). Similar SNPs were found in the corresponding regions of the other *B. bronchiseptica* strains that were kept in our laboratory. Some strains have the same four SNPs, and the others have three of four at positions -129, +22, and +38. We classified the strains into three groups according to the pattern of SNPs, and designated them as D1 for the strains without the nucleotide substitutions, compared with RB50, D2 for those with the three SNPs, and D3 for the remainder with the four SNPs like S798. S798 strain has been used for purification of DNT because of its high ability to produce the toxin [19]. Therefore, we examined whether the SNPs upstream of *dnt* influence the expression level of the toxin by immunoblotting and quantitative RT-PCR (Fig. 1, B and C). The immunoblotting revealed that the level of DNT produced by the bacteria was increased in the order of D1, D2, and D3 SNP types. The results of the quantitative RT-PCR were consistent with those of the immunoblotting. The level of *dnt* mRNA in S798 (D3 type) was 13.8 times that in RB50 (D1 type) and 6.3 times that in AFUY13 (D2 type). These results indicate that the strains of D3 type have the highest activity of *dnt* transcription, while those of D1 and D2 have the lowest and intermediate activities, respectively. *B. pertussis* Tohama strain belonging to D1 type was found to produce as a small amount of DNT as *B. bronchiseptica* strains of D1 type (Fig. 1, A and B).

To examine the time course of DNT production, we introduced the reporter plasmids carrying D1-, D2-, and D3-type promoter regions into RB50 strain and carried out the β-galactosidase reporter assay to monitor the transcription activity of each promoter (Fig. 1D). The activity of the D3-type promoter was much higher than those of D1- and D2-type promoters, increased in the early period of incubation to a peak at the mid-log growth phase, and then gradually decreased (Fig. 1D). This time course was in agreement with those described in previous studies that detected DNT activity of cultured *B. pertussis* [20] and *B. bronchiseptica* [21]. The D1- and D2-type promoter activities were slightly higher than that of the negative control without the reporter plasmids, but did not show any increase over the entire period of incubation. The reporter plasmid without any promoter sequence (promoterless) showed higher activity than those with D1- and D2-type promoters, implying that these promoters might be consistently repressed.
Fig 1. SNPs upstream of dnt influence the expression level of DNT. (A) Alignments of SNPs upstream of dnt. The positions +1 and +47 indicate the start sites of transcription and translation, respectively. The strains indicated by boldface are hereafter used as representatives for each SNP type. BP and BB indicate B. pertussis and B. bronchiseptica, respectively. (B) Detection of DNT in whole-cell lysates by immunoblotting. Whole-cell lysates of indicated strains that were cultivated for 12 h were prepared and subjected to SDS-PAGE, followed by immunoblotting, as described in Materials and Methods. The intensity of each band was measured using Image Gauge Version 4.1 (FUJIFILM Corporation, Tokyo, Japan) and the obtained values of

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Base changes at -72 and +22 are crucial for high-level expression of dnt

The results shown in Fig. 1 indicate that the SNPs at positions -129, -72, +22, and +38 in the region upstream of dnt influence the transcriptional level of the gene. To determine which base changes are crucial, we carried out the β-galactosidase reporter assay by using the reporter plasmid including the dnt promoter regions that harbor nucleotide substitutions at the positions in question, and examined their promoter activities (Fig. 2A). In these assays, the promoters with DNT signals were normalized by those of FtsZ signals in each lane, and are expressed below the upper panel as relative intensities when DNT in the sample from RB50 is 1.0. Purified DNT was subjected to SDS-PAGE at 50 ng/lane to indicate the position of the toxin. The results of strains R033, TYA13, and STY137 were omitted but showed similar amounts of DNT as other D2-type strains. (C) Measurement of dnt mRNA level by quantitative RT-PCR. Total bacterial RNA was prepared from S798 with D3-type promoter, RB50 with D1-type promoter, AFUY13 with D2-type promoter, and S798Pdnt-72C, three independent derivatives of S798 having a nucleotide substitution of C for T at position -72 and subjected to quantitative RT-PCR, as described in Materials and Methods. The relative levels of dnt mRNA normalized to recA mRNA are shown. Each bar represents the mean ± standard deviation of three samples. (D) The reporter plasmids harboring a promoter region of D1, D2, or D3 type were introduced into B. bronchiseptica RB50 and the β-galactosidase reporter assay was carried out after incubation of the bacteria for the indicated periods. Dashed black lines represent growth curves of host strain (No-plasmid) as representative data monitored by OD650. Each plot represents the mean ± standard deviation of four samples.

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Fig 2. Activities of dnt promoters, in which nucleotides are substituted at positions -129, -72, +22, and/or +38. (A) Transcriptional activities of the promoter regions of dnt with or without nucleotide substitutions as indicated in the left column were estimated by the β-galactosidase reporter assay. The nucleotide substitutions were made on the basis of three typical promoter sequences, D1, D2, and D3, which are exemplified by RB50, AFUY13, and S798 strains, respectively. *, P < 0.01; **, P < 0.001; ***, P < 0.0001, compared between indicated samples. Each bar represents the mean ± standard deviation of six samples. (B) Detection of DNT in whole-cell lysates by immunoblotting. RB50 (D1 type), AFUY13 (D2), S798 (D3), and an S798 derivative S798Pdnt-72C having a nucleotide substitution of C for T at position -72 were cultivated for 12 h and whole-cell lysates were prepared and subjected to SDS-PAGE, followed by immunoblotting, as described in Materials and Methods. The amount of DNT in the samples was estimated as mentioned in the legend for Fig. 1B. Purified DNT was subjected to SDS-PAGE at 50 ng/lane to indicate the position of the toxin.

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D2- and D3-type sequences showed 3.0 times and 22.6 times higher activity than the D1 type, respectively. The promoters having C at -72 and G at +22 were less active than a reporter plasmid without a promoter, suggesting that transcription is repressed in these promoters. The substitution between G and C at -129 did not affect the transcription activity (compare the results of the sequences “G C G C (D1 type)” and “C C G C”, “C C T T (D2 type)” and “G C T T” or “G T T T” and “C T T T”). Similarly, change from C to T at +38 did not enhance the promoter activity (see the results of the sequences “G C G C (D1 type)” and “GCGT”, “GCTC” and “GCTT”, or “GTTT” and “CTTT”). In contrast, substitutions of T for C at -72 and/or for G at +22 resulted in an increase of the transcription level of the reporter. The promoters carrying T at both -72 and +22 showed the same level of the promoter activity as that of D3 type. These results imply that the base changes from C to T at -72 and from G to T at +22 are crucial for high-level expression of dnt. Notably, when comparing the sequences of D2 and D3 types, substitution of T for C at -72 appeared to result in a marked increase of transcription activity, as judged by the β-galactosidase reporter assay. To confirm this, we generated a derivative of D3-type S798 strain (S798Pdnt-72C) by substituting C for T at -72 in the region upstream of dnt on the chromosome (as a result, its SNP type becomes D2), and estimated the level of DNT production by immunoblotting (Fig. 2B). As expected, the amount of DNT produced by S798Pdnt-72C was reduced to about 40% of that of wild-type S798, the level that corresponds to that of D2-type strain AFUY13. Quantitative RT-PCR also demonstrated that the dnt mRNA level of S798Pdnt-72C was comparable to that of AFUY13 (Fig. 1C). These results imply that T at -72 is a key base to confer the highest transcriptional activity of dnt on D3-type strains like S798 and factors other than SNP at -72 are not involved in the difference in the level of DNT production between D2-type and D3-type bordetellae.

DNT is not expressed by B. bronchiseptica strain isolated from mice

In the course of study to compare the ability to produce DNT among B. bronchiseptica strains, we found that mouse isolates produced no DNT (Fig. 3A). PCR to amplify a locus including dnt revealed that the amplified fragments from the mouse isolates were about 5 kbp shorter than those from RB50 and S798, rabbit and pig isolates, respectively, whereas genes located in the vicinity of dnt, BB3973, BB3975, BB3979, and BB3983, were amplified at the same sizes among all the strains (Fig. 3B). DNA sequencing of the short fragment amplified by PCR demonstrated that the mouse isolates lacked a region from 200 bp upstream to 93 bp downstream of dnt, and instead, a 63-bp fragment including a GC-rich palindrome of 14 bp was inserted (Fig. 3C).

Distribution of promoter types of dnt in Bordetella spp.

Of 13 pig isolates of B. bronchiseptica that were examined in this study, nine carried a D2-type promoter region, three carried D3, and only one carried D1 (Fig. 1A and Table 1), implying that most B. bronchiseptica strains isolated from pigs may carry a D2 or D3 type. It is widely accepted that DNT is involved in the pathogenesis of B. bronchiseptica infection in pigs [22]. Therefore, there could be a correlation between the abilities of the bacteria to infect pigs and to produce a larger amount of DNT. To verify this, we examined the sequences upstream of dnt of strains that were deposited in the Genome database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) (Table 1). Of 60 strains found in the database besides RB50 and S798, three were pig isolates, all of which carried a D2-type promoter. D2-type promoters were also found in three other strains that were isolated from monkey, guinea pig, and dog. D1 type was found in 27 strains. Eleven strains showed sequences distinct
from D1, D2, and D3 types, but could be divided into three groups, which were tentatively designated as undefined type 1 (UT1) for seven strains, UT2 for three, and UT3 for one (S2 Fig.). For the remaining 16 strains, no dnt but sequences including the GC-rich palindrome reported in this study were found in the database. We next extended examples to *B. pertussis* and *B. parapertussis*, the sequences of which were obtained from the same database (S4 Table). All of these strains but one that was designated as UT4 were found to have a D1-type promoter. No strains harboring a D3-type promoter were found in the database of the three *Bordetella* species.

**Discussion**

DNT is known as the major virulence factor of *B. bronchiseptica* causing pig atrophic rhinitis [23]. The toxin deamidates or polyaminates, and activates the members of the small GTPase Rho family, which results in the inhibition of osteoblastic differentiation, leading to atrophy of turbinate bone, the characteristic pathological lesion of atrophic rhinitis [7,8,23–26]. While it is
Table 1. SNP type of the dnt promoter in *Bordetella bronchiseptica*.

| Strain   | Origin | Geographic location | SNP<sup>a</sup> | ST<sup>d</sup> | Accession ID<sup>f</sup> |
|----------|--------|---------------------|----------------|------------|------------------------|
| RB50     | Rabbit | USA                 | D1             | 12         | NC_002027.3            |
| S798     | Pig    | Japan               | D3             | 6          | APO14582.1(This study) |
| AFUY13   | Pig    | Japan               | D2             | 7          | This study             |
| KG19     | Pig    | Japan               | D2             | 7          | This study             |
| KHI11    | Pig    | Japan               | D2             | 7          | This study             |
| KII13    | Pig    | Japan               | D3             | 6          | This study             |
| KII24    | Pig    | Japan               | D3             | 6          | This study             |
| MH110    | Pig    | Japan               | D2             | 7          | This study             |
| R033     | Pig    | Japan               | D2             | 7          | This study             |
| S807     | Pig    | Japan               | D1             | ND<sup>e</sup> | This study            |
| STY136   | Pig    | Japan               | D2             | 7          | This study             |
| STY137   | Pig    | Japan               | D2             | 7          | This study             |
| TYA12    | Pig    | Japan               | D2             | 7          | This study             |
| TYA13    | Pig    | Japan               | D2             | 7          | This study             |
| 10177–36 | Mouse  | Japan               | .<sup>b</sup>  | 8          | This study             |
| 10255–6  | Mouse  | Japan               | -              | 8          | This study             |
| 10519–15 | Mouse  | Japan               | -              | 8          | This study             |
| 10092–26 | Mouse  | Japan               | -              | 8          | This study             |
| 1289     | Monkey | South America       | D2             | 32         | NZ_HE983626.1          |
| 253      | Dog    | USA                 | D1             | 27         | NC_019382.1            |
| MO149    | Human  | USA                 | UT1<sup>c</sup>| 15         | NC_018829.1            |
| D445     | Human  | USA                 | -              | 17         | NZ_HE983627.1          |
| Bbr77    | Human  | Germany             | -              | 18         | NZ_HE983628.1          |
| 345      | Human  | USA                 | -              | 21         | JGWH00000000.1         |
| 00-P-2730| Human  | USA                 | UT1            | 34         | JGWH00000000.1         |
| 09-P-2796| Human  | USA                 | D1             | 27         | JGWH00000000.1         |
| 3E44     | Rabbit | USA                 | D1             | 10         | JGKW00000000.1         |
| 7E71     | Horse  | USA                 | -              | 13         | JGWL00000000.1         |
| 980      | Unknown| Unknown             | D1             | 10         | JGWM00000000.1         |
| 99-R-0433| Human  | USA                 | -              | 60         | JGWN00000000.1         |
| A1–7     | Unknown| Unknown             | D1             | 38         | JGWO00000000.1         |
| B18–5 (C3)| Unknown| Unknown             | D1             | 14         | JGPW00000000.1         |
| C4       | Rabbit | USA                 | D1             | 38         | JGWO00000000.1         |
| CA90 BB02| Turkey | USA                 | -              | 8          | JHBU00000000.1         |
| CA90 BB1334| Turkey| USA                | -              | 28         | JGWP00000000.1         |
| CARE970018BB| Pig  | USA                | D2             | 7          | JGWS00000000.1         |
| D756     | Unknown| Unknown             | D1             | 12         | JGWT00000000.1         |
| D989     | Human  | Unknown             | D1             | 12         | JGWW00000000.1         |
| D993     | Human  | Unknown             | D1             | 31         | JGWW00000000.1         |
| E010     | Human  | Unknown             | D1             | 31         | JGWW00000000.1         |
| E012     | Human  | Unknown             | D1             | 27         | JGWW00000000.1         |
| E013     | Human  | Unknown             | D1             | 27         | JGWW00000000.1         |
| E014     | Human  | Unknown             | -              | ND         | JGWW00000000.1         |
| F1       | Turkey | USA                 | UT1            | 65         | JGX00000000.1          |
| F2       | Turkey | USA                 | UT1            | 65         | JGXB00000000.1         |
| F4563    | Human  | USA                 | -              | 8          | JGXC00000000.1         |

(Continued)
well accepted that DNT is involved in the establishment of pig disease, the role of the toxin in diseases of mammals other than pigs is unknown. In this study, we demonstrated that pig isolates of *B. bronchiseptica* produced more DNT than *B. pertussis* Tohama and *B. bronchiseptica* RB50, both of which are widely used as typical reference strains. Quantitative RT-PCR and β-galactosidase reporter assays revealed that the difference in DNT production reflected the difference in its transcription. Furthermore, we

Table 1. (Continued)

| Strain   | Origin      | Geographic location | SNP<sup>a</sup> | ST<sup>d</sup> | Accession ID<sup>f</sup> |
|----------|-------------|---------------------|-----------------|---------------|--------------------------|
| GA96–01  | Human       | USA                 | -               | 21            | JGXD00000000.1          |
| KM22     | Pig         | Hungary             | D2              | 7             | JNHR00000000.1          |
| M435/02/3| Seal        | Scotland            | UT2<sup>b</sup> | 11            | JGXE00000000.1          |
| M85/00/2 | Seal        | Caspian Sea         | D1              | 33            | JGXF00000000.1          |
| MBORD591 | Dog         | USA                 | -               | 22            | JGXX00000000.1          |
| MBORD595 | Dog         | USA                 | D1              | 33            | JGXX00000000.1          |
| MBORD624 | Horse       | USA                 | D1              | 23            | JGXX00000000.1          |
| MBORD632 | Horse       | USA                 | D1              | 33            | JGJX00000000.1          |
| MBORD635 | Cat         | USA                 | D1              | 62            | JGXX00000000.1          |
| MBORD665 | Guinea Pig  | USA                 | UT2             | 7             | JGXL00000000.1          |
| MBORD668 | Guinea Pig  | USA                 | UT2             | 7             | JGXM00000000.1          |
| MBORD670 | Guinea Pig  | USA                 | D2              | 7             | JGXX00000000.1          |
| MBORD675 | Human       | Germany             | -               | 18            | JGXX00000000.1          |
| MBORD678 | Guinea Pig  | Australia           | UT3<sup>c</sup> | 14            | JHBQ00000000.1          |
| MBORD681 | Koala       | Australia           | D1              | ND            | JGXP00000000.1          |
| MBORD698 | Koala       | Australia           | D1              | ND            | JGXX00000000.1          |
| MBORD707 | Turkey      | USA                 | UT1             | 29            | JGRX00000000.1          |
| MBORD731 | Horse       | Denmark             | D1              | 6             | JGXS00000000.1          |
| MBORD762 | Guinea Pig  | Ireland             | D1              | 14            | JHBV00000000.1          |
| MBORD782 | Cat         | Netherlands         | D1              | 5             | JGXT00000000.1          |
| MBORD785 | Dog         | Netherlands         | D2              | 7             | JGXX00000000.1          |
| MBORD839 | Dog         | Switzerland         | D1              | 31            | JGXX00000000.1          |
| MBORD849 | Pig         | Netherlands         | D2              | 7             | JGXX00000000.1          |
| MBORD901 | Turkey      | Germany             | -               | 64            | JGXX00000000.1          |
| MO211    | Human       | USA                 | -               | 17            | CAKT00000000.1          |
| MO275    | Human       | USA                 | UT1             | 3             | JHBS00000000.1          |
| OSU054   | Human       | USA                 | -               | 28            | JHBS00000000.1          |
| OSU095   | Turkey      | USA                 | -               | 28            | JGXY00000000.1          |
| OSU553   | Turkey      | USA                 | D1              | 35            | JGXX00000000.1          |
| RB630    | Rabbit      | Hungary             | D1              | 12            | JGYA00000000.1          |
| SBL-F6116| Human       | USA                 | UT1             | 9             | JHBV00000000.1          |
| SO10328  | Sea otter   | USA                 | D1              | 23            | JGXB00000000.1          |

<sup>a</sup> SNP types of the region upstream of *dnt*.  
<sup>b</sup> no *dnt* in genomic DNA or shotgun DNA sequences.  
<sup>c</sup> undefined sequence types as shown in S2 Fig.  
<sup>d</sup> sequence types according to the Bordetella MLST database (http://pubmlst.org/bordetella).  
<sup>e</sup> not designated.  
<sup>f</sup> Four complete genome sequences and 58 whole-genome shotgun sequences were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/nuccore).  

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found the characteristic four SNPs at the promoter region of dnt and categorized them into three types, D1, D2, and D3, of which the ability to produce DNT increases in this order. Of the four SNPs, the two at positions -72 and +22 from the transcriptional start site were found to make synergistic and marked effects on the activity of dnt transcription (Fig. 2A): The transcriptional activities were highest and moderate when T was placed at both positions, like in D3 type, and at either position like in D2 type, respectively.

The transcription of dnt is known to be regulated by the BvgAS two-component system, in which BvgS phosphorylates the downstream transcriptional activator BvgA in response to environmental signals. Phosphorylated BvgA in turn binds to the promoter regions and activates transcription of virulence genes known as vagS (vir-activated genes) that, for example, encode filamentous hemagglutinin (fhaB), adenylate cyclase toxin (cyaA), pertussis toxin (ptx), and BvgAS per se (bvgAS), in addition to dnt. Many attempts to examine the nature of the BvgA-recognition sites upstream of vagS have proposed consensus sequences [27–30]. Notably, inverted heptameric sequences composed of TTTCCTA, which are found in the promoter region of fhaB, are widely accepted as typical high-affinity binding sites for BvgA [30,31]. Merkel et al. created an algorithm to predict BvgA-binding sites by using the data of a mutational analysis of heptameric sequences in the fhaB promoter [32,33]. The algorithm enables us to estimate the fitness of 7-bp sequences as BvgA-recognition sites and to express it as a numerical score, where the fhaB binding site TTTCCTA has a score of 0, which is decreased by penalty values assigned to each base-substitution affecting BvgA-binding and/or the fhaB promoter activity (Fig. 4A). At the region upstream of dnt, there was a direct repeat predicted to be a BvgA-binding site, TTTCCTGGTTCGG, at positions from +17 to +30, as reported previously [34]. One of the four SNPs that we found in this study is located in this direct repeat: D1 type of dnt promoter has G at position +22, whereas D2 and D3 types have T in place of G. In addition, we
found two additional possible BvgA-binding sites at positions -18 to -12 and -74 to -68. D3 type possesses an SNP substituting T for C at position -72. Thus, in total, four BvgA-binding sites could be predicted upstream of \( dnt \) (Fig. 4B). The scores of possible BvgA-binding sites that were calculated according to the algorithm by Merkel et al. revealed that SNPs found in D2 and D3 at +22 and in D3 at -72 made the heptad sequences more suitable as BvgA-recognition sites, which is consistent with our results showing that the promoter activities become higher in the order of D1, D2, and D3. Although further analysis should be undertaken to delimit the actual BvgA-binding regions and examine the effects of the SNPs on \( dnt \) transcription activity, the scores of Merkel et al. suggest that the SNPs of D2 and D3 result in better matches to the BvgA-recognition site.

A previous study to analyze the genetic diversity of \( B. \) \textit{pertussis}, \( B. \) \textit{parapertussis}, and \( B. \) \textit{bronchiseptica} by multilocus sequence typing (MLST) defined 32 sequence types (STs), which were categorized into four distinct lineages designated complexes I to IV \cite{4}. \( B. \) \textit{bronchiseptica} was divided into complexes I and IV, whereas \( B. \) \textit{pertussis} and \( B. \) \textit{parapertussis} human isolates were categorized as complexes II and III, respectively. \( B. \) \textit{bronchiseptica} of complex IV mostly contained human isolates, and was more closely related to \( B. \) \textit{pertussis} than those of complex I. \( B. \) \textit{bronchiseptica} pig isolates examined for DNT production in this study were categorized as complex I, except S807 that was untyped by MLST. In addition, we found that all of the D3-type strains belong to ST6 and all of the D2 strains to ST7. This was the case even when strains on the database were examined (Table 1): all the pig isolates belong to D2 type and ST7. Although these results do not indicate that D2 and D3 types fully correspond to ST7 and ST6, respectively, because strains of ST7 but not D2 and ones of ST6 but not D3 were found in the database, the fact that D2- and D3-type strains all belong to ST7 and ST6, respectively, implies that the SNPs upstream of \( dnt \) are the result of evolutionary processes, not sporadic mutations. Similarly, all of the mouse isolates, which were found not to possess \( dnt \), belong to ST8 of complex IV. This observation is consistent with previous studies that pointed out that \( dnt \) was missing or divergent in complex IV strains \cite{3,4}.

The distribution of the \( dnt \) promoter types in mammal isolates of bordetellae might reflect the importance of DNT in the pathogenesis of disease of each animal. \( B. \) \textit{pertussis} and \( B. \) \textit{parapertussis} human isolates mostly have D1-type promoter, which produces the least DNT, the role of which in whooping cough, a human disease of bordetellae, is unknown or limited, if any. In contrast, it is conceivable that almost all pig isolates belong to D2 and D3 types, which produce DNT at moderate and extreme levels, respectively, because the toxin causes turbinate bone atrophy, the characteristic lesion seen in atrophic rhinitis, pig infection by \( B. \) \textit{bronchiseptica}. In addition, our results raise the possibility that DNT is not only responsible for causing the characteristic bone atrophy but also necessary for the bacteria to colonize the pig respiratory tract. This might be supported by a previous report showing that \( dnt \) mutants of \( B. \) \textit{bronchiseptica} colonized the upper respiratory tract of pigs less than the parent strains \cite{22}.

It is known that \( B. \) \textit{bronchiseptica}, \( B. \) \textit{pertussis}, and \( B. \) \textit{parapertussis}, despite sharing many virulence factors, vary in the pathogenic phenotypes including disease severity and host specificity. This diverse bacterial pathogenesis should have resulted from gene deletion and inactivation, as well as genomic translocation, during evolution of the subspecies. Our results shown in this study might indicate the fact that the difference in promoter activities of virulence genes caused by SNPs contributes, at least partly, to the determination of host specificity of \( B. \) \textit{bronchiseptica}.

**Supporting Information**

**S1 Fig.** Schematic diagram of plasmid pT1T2-SD-\( \beta \)-galactosidase reporter assay. Genes required for plasmid replication (\( rep \)), resistance to gentamicin (GmR) and plasmid
mobilization (mob) are symbolized by solid arrows. β-galactosidase gene is symbolized by an open arrow. The promoter region of dnt is symbolized by an open box. The rrnB terminator region is indicated by a gray box.

S2 Fig. Alignments of nucleotide substitutions upstream of dnt. Positions +1 and +47 indicate the start sites of transcription and translation, respectively. Positions -129, -72, +22, and +38 indicate the SNP positions among the D1, D2, and D3 types. BB and BP indicate B. bronchiseptica and B. pertussis, respectively. Four undefined types (UT) were found in the strains, whose sequences were deposited in the Genome database of NCBI.

S1 Table. Bacterial strains used in this study.

S2 Table. Plasmids used in this study.

S3 Table. Primers used in this study.

S4 Table. Distribution of sequence type upstream of dnt in Bordetella spp.

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Author Contributions
Conceived and designed the experiments: KO HA YH. Performed the experiments: KO HA AFM KN NS. Analyzed the data: KO HA YH. Contributed reagents/materials/analysis tools: KO HA FI YO TH AFM KN NS. Wrote the paper: KO HA YH.

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