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Full Length Research Paper

Molecular detection of *TasA* gene in endophytic *Bacillus* species and characterization of the gene in *Bacillus amyloliquefaciens*

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***TasA*, the gene which play an important role in bacteria development, physiology and bacteria biofilm formation in *Bacillus* species was detected in the endophytic bacteria by polymerase chain reaction (PCR) amplification. In ten endophytic *Bacillus* strains tested, *TasA* gene was readily detected in *Bacillus amyloliquefaciens* PEBA20 and 7 strains of *Bacillus subtilis*, whereas *Bacillus megaterium* and *Bacillus cereus* did not exhibit detectable gene of *TasA*. The *TasA* gene cloned from *B. amyloliquefaciens* consists of the intact open reading frame (ORF) of 786 base pairs encoding a protein of 261 amino acid residues including a conserved signal peptide consisting of 27 amino acid residues. Sequences analyses revealed *TasA* is highly conserved; however, there is certain difference in the nucleotide sequence and deduced amino acid sequence among the genes harbored in *B. amyloliquefaciens* and *B. subtilis*, implying the possible distinction of structure and function of the protein in different bacteria species. This study may be beneficial for the understanding of the genes harbored in *Bacillus* species in nature and may lead to further study on the gene function.**

Key words: *TasA* gene, detection, *Bacillus amyloliquefaciens*, *Bacillus* species, biofilm.

INTRODUCTION

TasA gene plays an important role in bacteria development, physiology and bacteria biofilm formation. The gene encoded *TasA*, known as translocated anti-bacterial spore-associated protein. TasA is thought to confer a competitive advantage to the spore from the onset of sporulation and later, during germination, by inhibiting the growth of other organisms (Stöver and Driks, 1999b, c). The protein has been suggested to be required for proper spore coat assembly (Serrano et al., 1999) and as a major protein component of the *B. subtilis* biofilm, TasA is located in the extracellular matrix of pellicles, required for structural integrity of the matrix as well as for the development of biofilm architecture (Kolter and Greenberg, 2006; Mah and O'Toole, 2001; Vu et al., 2009). Cells unable to properly synthesize TasA produce fragile pellicles and colonies with a smooth texture (Branda et al., 2001, 2005, 2006; Hamon et al., 2004). TasA is first detected in stationary phase and sporulating cultures in *Bacillus subtilis* (Antelmann et al., 2001; Serrano et al., 1999; Stöver and Driks, 1999a). Till now, only few bacteria species and strains have been verified possessing *TasA* gene (Chen et al., 2007; Gioia et al., 2007; Kunst et al., 1997; Mizuno et al., 1996; Rey et al., 2004). Some of the *TasA* gene sequences submitted in Genbank were incomplete gene sequence and some were direct submitted with non-experimental evidence. In addition, most of the *Bacillus* strains whose *TasA* genes have been submitted in Genbank have not been referred to endophytic bacteria (Chen et al., 2007; Gioia et al., 2007; Kunst et al., 1997; Mizuno et al., 1996; Rey et al., 2004; Veith et al., 2004).

TasA is the terminal member of the *yqxM-sipW-tasA* operon. The *yqxM* operon also encodes the signal peptidase SipW required for TasA secretion and the YqxM protein required for localization of TasA to the extracellular matrix (Branda et al., 2001, 2006; Stöver and Driks 1999a, b, c). Although, the regulatory circuitry

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Table 1. Strains and plasmids for gene clone and expression.

Strain or plasmid	Relevant characteristics	Source
Bacteria		
Bacillusamyloliquefaciens PEBA20	AC: FJ685773	This study
B. subtilis PEBSB307040301	AC: FJ685766	This study
B. subtilis PEBS2501	AC: HM015507	This study
B. subtilis PEBSB807040302	AC: FJ685768	This study
B. subtilis PEBSB807040301	AC: FJ685767	This study
B. subtilis PEBS07040302	AC: HM015506	This study
B. subtilis PEBSB207040301	AC: FJ685765	This study
B. subtilis PEBS07032512	AC: HM015508	This study
B. cereus PEBC08010810	AC: FJ685763	This study
B. megaterium PEBM08010809	AC: FJ685762	This study
Escherichia coli DH5α	F^- φ80d *lacZ*Δ*M15 endA1 hsdR17* (r_k^- m_k^-) *supE44 thi-1 gyrA96* Δ (*lacZYA-argF*)	TransGen Biotech, China
E. coli BL21 (DE3) pLysS	F^- *ompT hsdSB (rB-mB-) gal dcm(DE3) pLysS* Cm^r	TransGen Biotech, China
Plasmid		
pMD-18T	Cloning vector, Ap^r	TaKaRa Bio, Japan
pEASY-E1	Expression vector, T- vector, Ap^r	TransGen Biotech, China
pMD-18T-*TasA*	pMD-18T with the *TasA* gene from *Bacillus* spp.; Ap^r	This study
pMD-18T-*TasA-BA*	pMD-18T with the *TasA* gene from *B. amyloliquefaciens* PEBA20; Ap^r	This study
pEASY-E1/*TasA-BA*	pEASY-E1 with the *TasA* gene from *B. amyloliquefaciens* PEBA20; Ap^r	This study

*Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; AC, GenBank accession no. of 16S rRNA.

that governs the expression of the *yqxM* operon has been elucidated with more and more evidences, the character of *TasA* itself seems to be lacking enough attention (Branda et al., 2001, 2005, 2006; Hamon et al., 2004).

Since the importance of *TasA* in bacteria development, physiology and bacteria biofilm formation, revealing the existence of *TasA* in bacteria of different taxonomic lineages and characterization of *TasA* may be beneficial for the understanding of the biofilm of *Bacillus* species in nature. Therefore, in the present study, the endophytic *Bacillus* strains, including *Bacillus amyloliquefaciens* were selected for the detection of *TasA* gene, and the characterization of the gene in *B. amyloliquefaciens* was analysed.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and primers

The bacterial strains, plasmids used in this study for gene clone and expression are listed in Table 1. Ten strains of endophytic bacteria used in the study for detection of *TasA* gene were isolated and identified from five 6-year-old poplar trees (*Populus tomentosa* Carr.), deposited in the Key Laboratory of Agromicobiology, Shandong Agricultural University. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB plates fortified with 1.5% Bacto agar at 37°C. When appropriate, 100 µg of ampicillin (Sigma) per ml was added to the medium as needed. X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) (Promega) was included in the medium at a concentration of 50 µg ml^{-1} for the detection of ß-galactosidase activity. Primers used in the study were listed as follows: TasA1:5′-GAATTCATGGGTATGAAAAAGAA -3′ (The *EcoR* Ⅰ site is underlined); TasA2: 5′-CTGCAGTTAATTTTTATCCTCGCT-3′ (Th-*Pst* Ⅰ site is underlined); TasA3: 5′-ATGGGT-ATGAAAAAGAA-3′; TasA4:5′-TTAATTTTTATCCTCGCT-3′; T7, promoter primer: 5′-TAATACGACTCAGTATA-3′; T7 terminator primer:5′-CTGCCACCGCTGAGCAATAACTAG-3′.

PCR amplification of *TasA* gene and gene sequencing

The bacteria were cultured in nutrient broth (NB) at 28°C. Genomic DNA was extracted by CTAB method and used for PCR templates. Detection of *TasA* gene in the species of *Bacillus* was performed with PCR amplification using primers TasA1 and TasA2. The primers were designed based on the sequences of gene homologies published in GenBank using DNAman 6.0. For *TasA* amplification, touchdown PCR was performed with annealing step of 56°C for 45 s and decreasing to 52°C at 2°C per 2 cycles, followed by 28 cycles at 50°C for 45 s, and elongation step of 10 min at 72°C. The amplification products were ligated to pMD-18T vector (TaKaRa) to obtain the recombinant plasmid pMD-18T-*TasA*, which was then transformed into *E. coli* DH5α competent cells. The selected clones were sequenced on an ABIPRISM 3730 DNA sequencer by the dideoxyl chain reaction termination method in Beijing Genomics Institute.

Sequence analyses

The nucleotide sequences of the *TasA* gene were compared with homologous sequences in GenBank by the basic local alignment search tool (BLAST). Nucleotide sequence and deduced amino acid sequence alignment and analyses were performed using molecular evolutionary genetic analysis (MEGA) version 4.1 (Tamura et al., 2007). Phylogenetic trees were constructed using MEGA by the neighbor-joining (NJ), minimum evolution (ME) and maximum parsimony (MP) methods.

Construction of the *TasA* gene expression vector and expression of the *TasA* gene in *E. coli*

The endophytic bacterium, *B. amyloliquefaciens* PEBA20 was selected for the *TasA* expression analysis. The *TasA* gene was amplified with the genomic DNA of *B. amyloliquefaciens* PEBA20 as template using the primers TasA3 and TasA4, which were designed based on the sequence of the *TasA* gene cloned from *B. amyloliquefaciens* PEBA20. The ligation reactions were performed with the mixture of PCR products and expression vector pEASY-E1 (TransGen) at 28°C for 15 min. The recombinant DNA was then transformed into *E. coli* BL21 (DE3) pLysS (TransGen). Screening for recombinant DNA clones with correct insert orientation was performed by colony PCR amplification and gene sequencing. Colony PCR amplification was carried out using primer T7 promoter primer and primer TasA4, or using primer T7 terminator primer and primer TasA3. Gene sequencing was carried out with primerT7 promoter primer and primerT7 terminator primer. The recombinant clone verified was designated pEASY-E1/*TasA-BA*.

The transformant *E. coli* BL21 (DE3) pLysS /pEASY-E1/*TasA-BA* with the correct insert orientation was cultivated overnight at 37°C in LB broth supplemented with ampicillin (100 μg ml^{-1}). Overnight cultures (50 μl) were added to 5 ml fresh LB-Amp medium. The mixture was incubated at 37°C with 150 rpm shaking until OD_{600} reached 0.5. Subsequently, gene expression was induced with 1 mmol l^{-1} of isopropyl-β-D-thiogalactopyranoside (IPTG) (TakaRa) cultivated at 28°C for 1, 2, 3 and 4 h, respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

1 ml of each induced cell suspension was harvested by centrifugation at 8000 *g* for 5 min and washed with ddH_2O. The harvested cells were mixed with 15 μl loading buffer (100 mmol l^{-1} Tris–HCl, pH 6.8; 200 mmol l^{-1} DTT; 4% SDS; 0.2% BPB; 20% glycerin), then incubated at 100°C for 10 min. The heated mixture (25 μl) was loaded in sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) for detecting the TasA protein expression. Proteins were visualized with a 0.02% Coomassie-Blue® staining.

RESULTS

Detection of *TasA* gene in the endophytic *Bacillus* species

The DNA bands with expected size, that is about 780 bp were clearly produced from the genomic DNA of *B. amyloliquefaciens* PEBA20 and *B. subtilis* strains tested (Figures 1A and B) by PCR-based gene detection assay with specific primers TasA1 and TasA2, implying *TasA* gene exist in all of the strains of *B. subtilis* and *B. amyloliquefaciens* tested. Gene sequencing and BLAST analyses confirmed the genes were *TasA*, indicating the gene harbored in *B. subtilis* and *B. amyloliquefaciens.* However, faint DNA band was produced from the strain *B. cereus* PEBC08010810 with TasA1 and TasA2, but gene sequencing demonstrated was not *TasA* gene. *B. megaterium* PEBM08010809 failed to yield positive amplification of *TasA* gene by this primer set, inspite of two bands that have been produced(Figure 1A). Gene sequencing also demonstrated that they were not *TasA* gene. .

TasA gene cloning from *B. amyloliquefaciens* PEBA20 and gene expression of the gene in *E. coli*

TasA gene was amplified from genomic DNA of *B. amyloliquefaciens* PEBA20 (Figure 2). The gene was found to consist of the intact open reading frame (ORF) of 786 base pairs, encoding a protein of 261 amino acid residues, with predicated molecular mass of 28 kDa. The gene was submitted in Genbank with the accession number: FJ713580.

For the gene expression, the gene *TasA-BA* was amplified and the expression vector pEASY-E1/*TasA-BA* was constructed and transformed into *E. coli* BL21 (DE3) pLysS. The apparent bands with molecular weight of about 28 kDa were detected on the SDS-PAGE gel in the cell lysates of *E. coli* BL21 (DE3)/pEASY-E1/*TasA-BA* after incubation for 1 to 4 h induced with IPTG, but not in those controls (Figure 3), suggesting the *TasA-BA* being expressed in *E. coli* BL21 (DE3) and verifying the correct predicated molecular mass of 28 kDa of the gene.

The signal peptide of TasA protein of *B. subtilis* and *B. amyloliquefaciens*

The analyses of the deduced amino acid sequence revealed the TasA protein consisted of the signal peptide locating at the N terminal of amino acid sequence of TasA. The signal peptide was determined by SignalP3.0

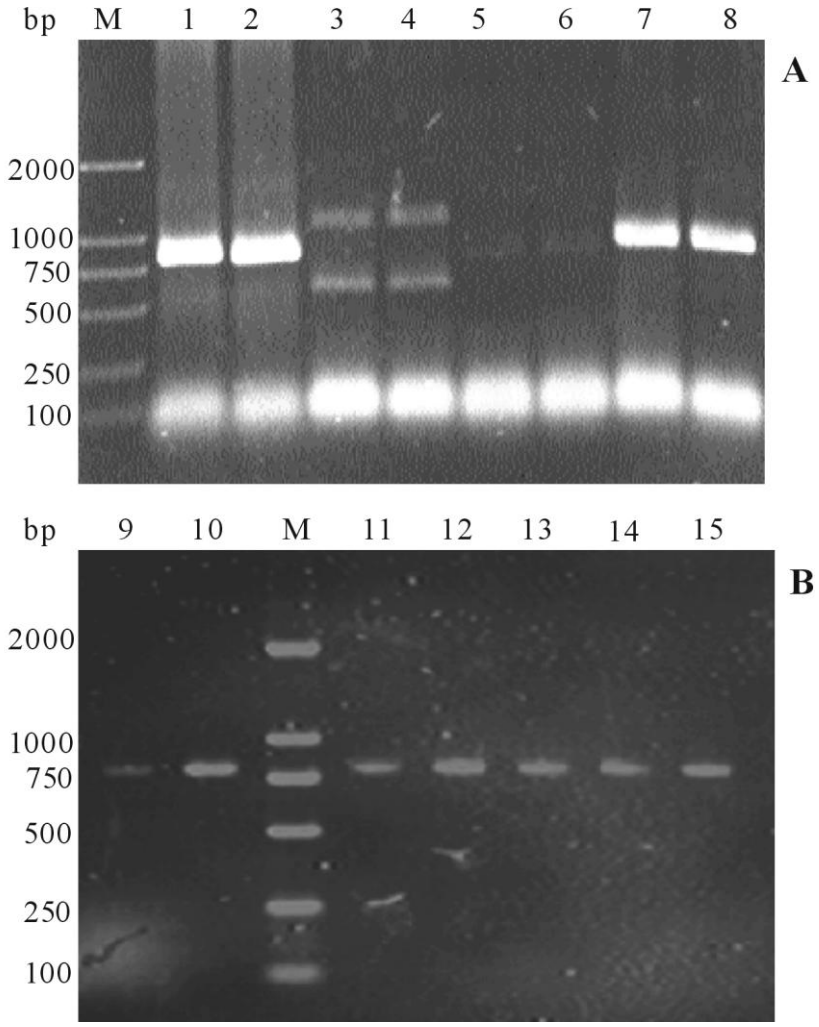

Figure 1. Detection of *TasA* gene in the endophytic *Bacillus* spp. from poplar by PCR amplification with the genomic DNA of 4 species of *Bacillus* (A) and 7 strains of *Bacillus subtilis* (B). The species and strains used for the detection of *TasA* gene were as follows (the PCR product detected in different lane for the same strain was PCR amplified independently): Lanes 1 and 2: PEBSB307040301; lanes 3 and 4: PEBM08010809; lanes 5 and 6: PEBC08010810; lanes 7 and 8: PEBA20; lane 9-15: PEBS2501, PEBSB307040301, PEBSB807040301, PEBSB807040302, PEBS07040302, PEBSB207040301, PEBS07032512; lane M: DL2000 DNA Marker.

(http://www.cbs.dtu.dk/services/SignalP/) search of signal peptide. The alignment of the signal peptide sequences showed 100% similarity among all the signal peptide sequences of TasA deduced from *TasA*, including the sequences of *B. amyloliquefaciens* PEBA20 and all of the *B. subtilis* strains in the study, and the others of *B. amyloliquefaciens* and *B. subtilis* in genbank database, implying the highly conservation of the signal peptide sequences. The signal peptide consisted of 27 amino acid residues, that is, MGMKKKLSLGVASAA-LGLALVGGGTWA, and the mature protein starting at amino acid residue 28 in the deduced amino acid sequence of TasA.

Sequence conservation and variability of *TasA* gene

TasA gene is highly conserved in the strains of *B. amyloliquefaciens*. The *TasA* gene cloned from *B. amyloliquefaciens* PEBA20 shared identities of 97.8 and 98.6% at nucleotide level, 99.3 and 100% at amino acid level with the *TasA* from *B. amyloliquefaciens* FZB42 (Ac.: CP000560) and TB2 (Ac.: EU359776), respectively.

However, *TasA* gene showed obvious variability between the gene cloned from *B. amyloliquefaciens* and the genes from *B. subtilis*. Nucleotide sequence analyses revealed that the *TasA* cloned from *B. amyloliquefaciens* PEBA20 shared the identities of 75.8% to the *TasA* genes

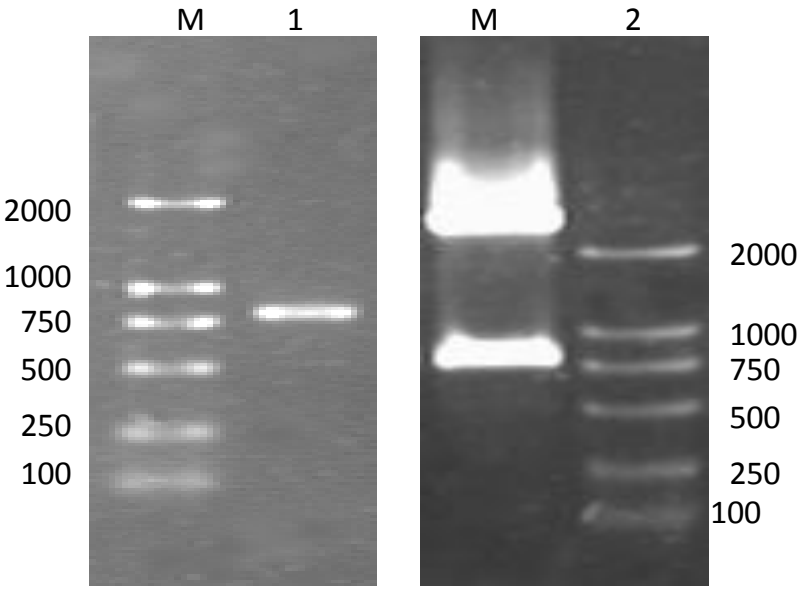

Figure 2. Cloning of *TasA* from *Bacillus amyloliquefaciens* PEBA20. Lane 1: Products of PCR, lane 2: Recombinant plasmid pMD18-T/*TasA* digested by *EcoR* Ⅰ and *Pst* Ⅰ, lane M: Molecular weight marker.

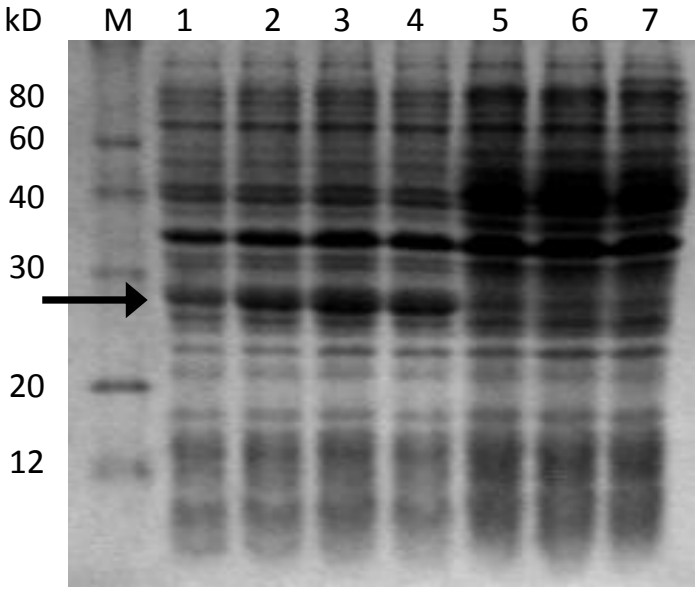

Figure 3. SDS-PAGE analysis of the TasA produced in *Escherichia coli* BL21 (DE3) pLysS/*TasA-BA*. Lane M: Protein molecular weight markers; lanes 1-5: The supernatants from the cells of *E. coli* BL21(DE3)/pEASY-E1/*TasA-BA* cultured in LB and induced with IPTG for 1 h to 4 h (lanes 1-4), and cultured in LB for 4 h and induced without IPTG (Lane 5); lanes 6-7: the supernatants from the cells of *E. coli* BL21(DE3) pLysS cultured in LB for 4 hours induced with IPTG (lane 7) or without (lane 6). The arrowhead indicated the position of the 28 kDa TasA expressed.

in *B. subtilis* in this study, and from 75.7 to 76.0 % to the known *TasA* genes in *B. subtilis* submitted in Genbank. Correspondingly, the identities were 83.1, 82.8 and 83.5% at amino acid level, respectively. Compared with

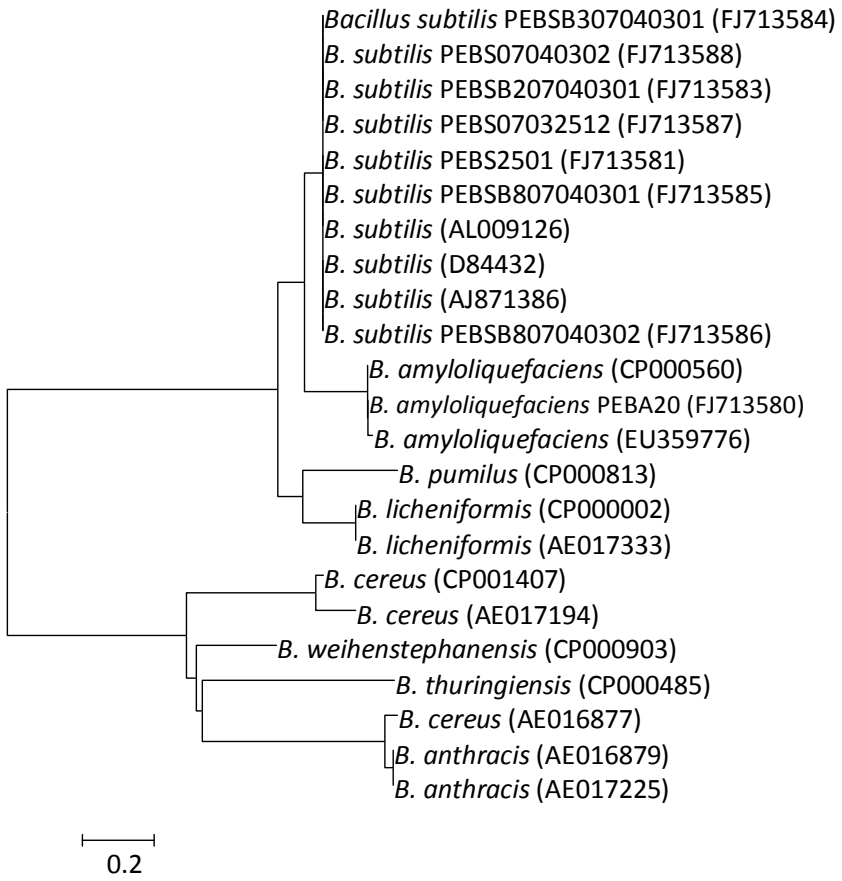

Figure 4. Phylogenetic trees of *Bacillus* spp. based on the nucleotide sequences of *TasA* gene. Bootstrap analysis was done with 1000 cycles. The numbers at the nodes represent percent of bootstrap values obtained from 1000 samplings. The scale bar indicates 0.2 nucleotide substitutions per nucleotide position. Numbers in brackets refer to accession number in Genbank.

the genes obtained from *Bacillus licheniformis* and *Bacillus pumilus*, the identities were 69.5 and 65.1% at nucleotide level, 68.2 and 61.1% at amino acid level, respectively.

The dendrogram based on the nucleotide sequences of *TasA* and *calY*

The BLAST result showed *TasA* has regions of sequence identity with the gene *calY*, which encode Camelysin, a membrane proteinase, called casein-cleaving membrane proteinase (Challacombe et al., 2007; Ivanova et al., 2003; Lapidus et al., 2008; Rasko et al., 2004; Read et al., 2003). The sequence of the *TasA* gene cloned from *B. amyloliquefaciens* PEBA20 showed 65.1 to 98.6% identities with the *TasA* gene sequences submitted in Genbank, whereas it showed 69.0 to 78.7% identities with the *calY* gene sequences. The deduced amino acid sequence showed 61.1 to 100% identities with the TasA amino acid sequences submitted in Genbank, whereas it showed only32.08 to 34.7% identities with most of the Camelysin sequences. The typical sequences of *TasA* and *calY* of *Bacillus* species, including all the sequences obtained in the study were subjected to phylogenetic analyses. The dendrogram based on the nucleotide sequences of *TasA* and *calY* (Figure 4) showed that all the *TasA* gene sequences of *Bacillus* species clustered together and yielded a group, while *calY* gene sequences formed another cluster group. The *TasA* gene sequences obtained from *B. subtilis* and *B. amyloliquefaciens* clustered closely and yielded a distinctive subgroup, while the gene sequences from *B. licheniformis* and *B. pumilus* yielded another subgroup. All the sequences from the same species cluster together besides *Bacillus cereus* (Ac: AE016877), which was cluster together with *Bacillus anthracis*.

DISCUSSION

Given the importance of *TasA* in formation of bacteria

biofilm, the character of *TasA* itself is not symmetrical to the function of the gene and to the information of regulatory circuitry that governs the expression of the *yqxM* operon. Information on biofilm of *Bacillus* species derived basically from *B. subtilis* as a model organism by now. In this study, *TasA* gene was readily detected in the strain *B. amyloliquefaciens* PEBA20 and the 7 strains of *B. subtilis*, whereas *B. megaterium* and *B. cereus* did not exhibit detectable *TasA* gene.

Although, *B. cereus* did not exhibit detectable *TasA* genes, we noted that *TasA* has regions of sequence identity with the gene *calY* (Challacombe et al., 2007; Ivanova et al., 2003; Lapidus et al., 2008; Rasko et al., 2004; Read et al., 2003). The *calY* exist in *B. anthracis, B. cereus, B. thuringiensis,* which belong to *B. cereus* group (Helgason et al., 2000; Manzano et al., 2003). With a bold speculation, revealing the relationship between the *calY* and biofilm in the *Bacillus* species belong to *B. cereus* group may also be valuable for elucidating the function of bacteria biofilm.

Moreover, the strains used for the detection of *TasA* gene in the study were endophytic bacteria of poplar, which is different from the domesticated model organism *B. subtilis* 168 (Kunst et al., 1997) and other strains whose *TasA* genes have been submitted in Genbank (Chen et al., 2007; Gioia et al., 2007; Mizuno et al., 1996; Rey et al., 2004; Veith et al., 2004). *TasA* were verified existing in *B. amyloliquefaciens* PEBA20 and the *B. subtilis* strains tested, implying the potential state and function of the biofilm in endophytic bacteria. This would lead to further study on the biofilm of bacteria in endophytic habitat.

Despite *B. amyloliquefaciens* being closely related to *B. subtilis*, *TasA* gene in *B. amyloliquefaciens* remains to be characterized. Detailed characterization of the sequences of *TasA* genes in different species of *Bacillus*, and further information on the structure of TasA protein based on the amino acid sequence would be useful for elucidation of the function of *TasA*. Sequence analyses revealed certain differences between the *Tas*A genes in the two related species, suggesting the possible nice distinction of the role and function of the protein. We are interested with expression of the gene, purification and demonstrating the dimensional structure of the protein. Some of the work is being carried out.

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