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Molecular Cloning and Characterization of an Insecticidal Toxin from *Pseudomonas taiwanensis*

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An insecticidal toxin gene, *tccC*, was cloned from the recently discovered novel species *Pseudomonas taiwanensis* using degenerate PCR and genomic walking. The DNA sequence of the *tccC* gene (2,940 bp) has an open reading frame encoding 980 amino acids with a calculated molecular weight of 107.93 kDa. The amino acid sequence alignment showed the highest sequence identity (41.2%) with the insecticidal toxin from *Pseudomonas entomophila*. To examine the insecticidal functionality of the *tccC* gene product, TccC was heterologously expressed in *Escherichia coli* as a recombinant His_6 fusion protein and purified by immobilized metal ion-affinity chromatography. The recombinant TccC was fed to *Drosophila* larvae at a concentration of 350 ppm, which induced about 60% mortality within 72 h. The recombinant TccC was stable at pH 7.0 and at 37 °C. When the pH was less than 5.0 or greater than 9.0, or temperature was greater than 55 °C, less than 20% *Drosophila* larvae mortality was observed. These results prove that *Pseudomonas taiwanensis* could be used as a source for developing novel biopesticides.

KEYWORDS: Molecular cloning; insecticidal toxin; *Pseudomonas taiwanensis*

INTRODUCTION

Over the years it has been important for humans to control populations of harmful insects; thus insecticides have been used for this purpose in agriculture and horticultural. Synthetic insecticides, due to their various side effects, have been widely replaced by bioinsecticides. Most of the bioinsecticides used in agriculture come from a single bacterium, *Bacillus thuringiensis*, which accounts for 90% of the bioinsecticide market (*1*). Recently there have been increasing instances of insect resistance to *Bacillus thuringiensis* bioinsecticides, which have led researchers to seek suitable alternatives. *Photorhabdus* and *Xenorhabdus*, both symbiotic with entomopathogenic nematodes, produce insecticidal toxins, which have potential for use in agriculture as alternatives to *Bacillus thuringiensis*. Despite the fact that oral infection of an insect by *Photorhabdus* or *Xenorhabdus* is not thought to be important to the bacterial biology, the toxin complex (Tc) produced by *Photorhabdus* and *Xenorhabdus* has oral activity against some insect species (*2*). Tc toxins are high-molecular-weight, multisubunit toxins requiring three components (A, B, and C) for full toxicity (*2*, *3*). The *tc* genes encoding Tc toxins were first described in *Photorhabdus* and subsequently have been documented in a range of bacteria, some clearly insect-associated (such as *Serratia entomophila*) and others with no obvious link to insects (*4*).

Pseudomonas spp. are ubiquitous Gram-negative bacteria that colonize and survive in numerous ecological niches including soil, water, and plant surfaces. Genes with predicted amino acid similarity to *tc* genes are found in some species of *Pseudomonas*, including insect-associated *Pseudomonas entomophila* and non-insect-associated *Pseudomonas syringae* pv *tomato* and *Pseudomonas fluorescens*. *Pseudomonas entomophila*, the first known strain to be pathogenic in *Drosophila melanogaster*, triggered a systemic immune response and caused lethality in *Drosophila melanogaster* larvae and adults after ingestion (*5*). The *Pseudomonas entomophila* genome encodes three TccC-type toxins and one TcdB-type Tc toxin, which likely play a major role in the pathogenicity of *Pseudomonas entomophila*, as TccC and TcdB proteins have insecticidal activity (*6*). Some other *Pseudomonas* spp., such as *Pseudomonas syringae* pv *tomato* and *Pseudomonas fluorescens*, have no known association with insects, but their genomes also encode Tc toxins (*7*, *8*). Therefore, it is reasonable to speculate that the presence of *tc*-like genes infers that *Pseudomonas syringae* pv *tomato* interacts with an unknown insect (*2*).

Pseudomonas sp. TKU015 was first isolated from soils using shrimp shell as the sole carbon and nitrogen source. By using physiological, biochemical, cellular fatty acid, and 16S rRNA gene sequence analysis methods, *Pseudomonas* sp. TKU015 was classified as a novel species, *Pseudomonas taiwanensis* (*9*). *Pseudomonas taiwanensis* displayed high levels of extracellular chitinase, chitosanase, and nattokinase activities with shrimp shell wastes as the sole carbon and nitrogen source (*10*, *11*). To the best of our knowledge, the association between *Pseudomonas taiwanensis* and insects has not been previously reported.

In this study, we report the cloning and heterologous expression of an insecticidal gene *tccC* from *Pseudomonas taiwanensis*.

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The insecticidal activities of the recombinant TccC toward *Drosophila* larvae as well as the effect of pH and temperature on the insecticidal activities of the recombinant TccC were also studied.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. *Pseudomonas taiwanensis* BCRC 17751 was used for the preparation of genomic DNA. All PCR products intended for sequence analysis were cloned into the pGEM-T Easy vector (Promega, Medison, WI). *Escherichia coli* DH5α was used in all cloning experiments. The pET-29a vector and *E. coli* BL21 (DE3) (Novagen, Madison, WI) were used for the recombinant protein expression experiments.

Pseudomonas taiwanensis BCRC 17751 was cultured in medium containing 2% (w/v) shrimp shell powder (Shin-Ma Frozen Food Co., Ilan, Taiwan), 0.1% (w/v) K_2HPO_4, and 0.05% (w/v) $MgSO_4 \cdot 7H_2O$ (pH 7) at 37 °C in an orbital shaker at 150 rpm. *E. coli* were grown at 37 °C in Luria−Bertani (LB) broth (Difco Laboratories, Detroit, MI). Agar plates were prepared by adding agar (1.5% w/v) (Difco Laboratorie) to broth.

DNA Isolation and Manipulation. Genomic DNA was prepared from *Pseudomonas taiwanensis* using the DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA). Plasmid DNA was isolated from *E. coli* using the alkaline lysis method (*12*). Restriction enzymes and T4 DNA ligase (New England BioLabs Inc., Beverly, MA) were used according to the manufacturer's instructions. All other DNA manipulations were performed using established procedures (*13*). All DNA sequences were determined by an automatic sequencing service provided by Mission Biotech Inc. (Taipei, Taiwan).

Degenerate PCR and Genome Walking. Part of the *TccC* gene of *Pseudomonas taiwanensis* was amplified by degenerate PCR using the degenerated forward primer BF (5′ AYGAYCCNGTNGGNAAYRT 3′) and the reverse primer BR (5′ CGYTCYTTRCCNGARTA 3′) designed based on the conserved core region of the C component of the Tc proteins (TccC) from various bacteria as described by Bockett et al. (*14*). PCR amplification was done with an initial denaturation of 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min, and a final elongation at 72 °C for 7 min.

The unknown genomic DNA sequences upstream and downstream of the degenerate PCR fragment were obtained by using the GenomeWalker Universal kit (BD Bioscience, Palo Alto, CA) according to the manufacturer's instructions. In brief, genomic DNA of *Pseudomonas taiwanensis* was digested separately using each of four blunt-end-cutting restriction enzymes *Dra*I, *Eco*RV, *Pvu*II, and *Stu*I. These separate pools of DNA fragments were independently ligated to adaptors to create four different genomic DNA libraries. The adaptor contains regions for the annealing of two nested primers that served as anchors for PCR amplification with gene-specific primers. The short (~900 bp) DNA sequences obtained from degenerate PCR were used for primer design for the first walk. For subsequent walks, primers were designed from the sequence acquired by the previous walk. The sequences of all PCR primers used in this study are listed in Table S1 in the Supporting Information. Overlap between walks enabled the assembly of the sequences obtained from all walks possible using Sequencher software (Gene Codes, Corp., Ann Arbor, MI).

The nucleotide sequence obtained for the *tccC* gene from *Pseudomonas taiwanensis* and its flanking regions and deduced amino acid sequence were compared to sequences from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Sequences were aligned in the BioEdit Sequence Alignment Editor program (*15*), and a phylogenetic tree was constructed by the neighbor-joining method in ClustalW (*16*) and displayed with the TreeView program (*17*).

Subcloning of tccC. The complete *tccC* gene was amplified by PCR from genomic DNA of *Pseudomonas taiwanensis* using the oligonucleotide forward primer: 5′ AGATCTGTTGGGACACATAGACTTTTTCAC 3′ and reverse primer: 5′ CTCGAGAGTACGAAATTTTTTTTCG 3′ (the underlined sequences in the primers are additional sequences that represent the restriction sites for *Bgl*II and *Xho*I, respectively). PCR amplification was done with an initial denaturation of 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 1 min, and extension at 68 °C for 3 min, and a final elongation at 68 °C for 7 min.

The PCR fragments encoding TccC were digested with *Bgl*II and *Xho*I, and ligated with *Bgl*II-*Xho*I digested pET-29a (Novagen). The pET-29a vector was designed for the expression of the recombinant protein fused to the 15-amino-acid S-tag and 6-amino-acid His-tag sequences upstream and downstream of the cloning site respectively. The resultant plasmids, designated pET-tccC, were sequenced to ensure that no errors were introduced by PCR; they were used to transform *E. coli* BL21 (DE3) (Novagen) by standard techniques (*13*). Transformants were selected on LB agar plates containing kanamycin (30 μg/mL) (Sigma-Aldrich Co. St. Louis, MO).

Purification of the Recombinant TccC. *E. coli* BL21 transformed cells were cultured in LB broth, and cell growth was then measured turbidimetrically at 600 nm (OD600). To produce the recombinant protein, the overnight culture was prepared and subsequently seeded at a 1:100 dilution into 5 mL of fresh LB broth. The cell cultures were maintained at 37 °C and induced with 100 μM of isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich Co.) for protein production upon reaching an OD600 of 0.5. After 4 h of induction, the cells were harvested by centrifugation at 5000*g* for 20 min at 4 °C.

The cell pellet was resuspended in 1 mL of 0.1 M sodium phosphate buffer (pH 7.4), sonicated for 10 min with an ultrasonicator (Model XL, Misonix, Farmingdale, NY), and fractioned into supernatant and pellet parts by subsequent centrifugation at 13000*g* for 20 min at 4 °C. The recombinant proteins were present mainly in the supernatant and were then purified by immobilized metal ion-affinity chromatography using a prepacked HisTrap Ni-Sepharose column (GE Healthcare, Piscataway, NJ). The protein production in each step was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) according to the method described by Laemmli (*18*). Total protein concentration was measured using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) against a standard curve of bovine serum albumin (Sigma-Aldrich Co.).

Bioassay for Insecticidal Activity of TccC in *Drosophila* Larvae. *Drosophila* is a valuable model for the investigation of host−pathogen interactions (*5*). The Oregon R strain of *Drosophila melanogaster* was used as the standard wild-type fly strain. Flies were reared in plastic vials containing standard fly medium (yeast, corn syrup and agar) at 25 °C, 60% humidity with a 12 h light−dark cycle (*5*).

Eggs were collected from these flies during a 6 h period and remained at 25 °C and 60% humidity for the rest of their development. After 72 h, each larva was transferred into one well of a 96-well tissue culture plate containing 30 μL of standard fly medium that was covered with a paper filter disk, which was moistened with 10 μL of cell-free culture supernatant of *Pseudomonas taiwanensis* or the respective concentrations of the purified recombinant TccC (25, 100, 200, or 350 ppm). A well-known microbial insecticide, thuringiensin (Valent BioSciences, Libertyville, IL), at a concentration of 200 ppm, served as the positive control, while distilled water served as the negative control. The plates were maintained at 25 °C, and the larvae still alive were counted at 8, 12, 24, 36, 48, and 72 h. The survival rate of *Drosophila* larvae fed with the tested compounds was calculated as a percentage (%) = (the number of surviving larvae in the treated group/the number of surviving larvae in the negative control group) × 100.

Effect of pH and Temperature on TccC Insecticidal Activity. The effect of pH on the insecticidal activity of TccC was evaluated by incubating the purified recombinant TccC (350 ppm) at 37 °C over a pH range of 4.0−10.0, using the following buffers (100 mM): acetate buffer (pH 4.0 and 5.0), phosphate buffer (pH 6.0−8.0), Tris-HCl buffer (pH 9.0), and glycine-NaOH buffer (pH 10.0). Aliquots were withdrawn at 30 min, and the residual insecticidal activities were measured as described above.

The thermal stability of TccC was determined by incubating the purified recombinant TccC (350 ppm) in 100 mM sodium phosphate buffer (pH 7.0) at 25, 30, 37, 50, 60, or 70 °C. Aliquots were withdrawn at 30 min, and the residual insecticidal activities were measured accordingly.

The mortality of *Drosophila* larvae caused by TccC was calculated as a percentage (%) = (the number of surviving larvae in the negative control group − the number of surviving larvae in the treated group)/the number of surviving larvae in the negative control group × 100.

Nucleotide Sequence Accession Number. The nucleotide sequence of *Pseudomonas taiwanensis tccC* was submitted to the GenBank database under accession number HQ260745.

RESULTS AND DISCUSSION

Isolation of the *Pseudomonas taiwanensis tccC* gene. In a previous study, the *tccC* genes of several bacteria including *Photorhabdus luminescens*, *Yersinia* spp., *Serratia* spp., *Burkholderia pseudomallei*, and *Pseudomonas* spp. were compared in a multiple alignment analysis, which revealed three conserved regions corresponding to conserved amino acid sequences (*14*). Degenerate oligonucleotides BF and BR were designed on the basis of these highly conserved regions (**Figure 1**; nucleotide 1576−1594 and 2463−2479).

Degenerate PCR conducted with the primers BF and BR resulted in the appearance of several amplified products, probably due to the ability of the primers to anneal with various genome fragments encoding proteins with similar properties. However, on the basis of the *tccC* sequences of *Pseudomonas* spp., a fragment ranging from 862 to 901 bp was expected for the *tccC* gene of *Pseudomonas taiwanensis*. A 904-bp fragment was therefore isolated, and subsequent sequence analysis revealed 64% amino acid sequence identity with the SppC of *Serratia proteamaculans* (accession no. ABB69941).

Successive genome-walking steps were used to identify the regions adjacent to the obtained fragment. As a result, a DNA sequence of 3954 bp was obtained, and contained a complete open reading frame encoding the putative TccC sequence (2,940 bp long) with 5′ and 3′ flanking regions of 593 and 418 bp, respectively (**Figure 1**).

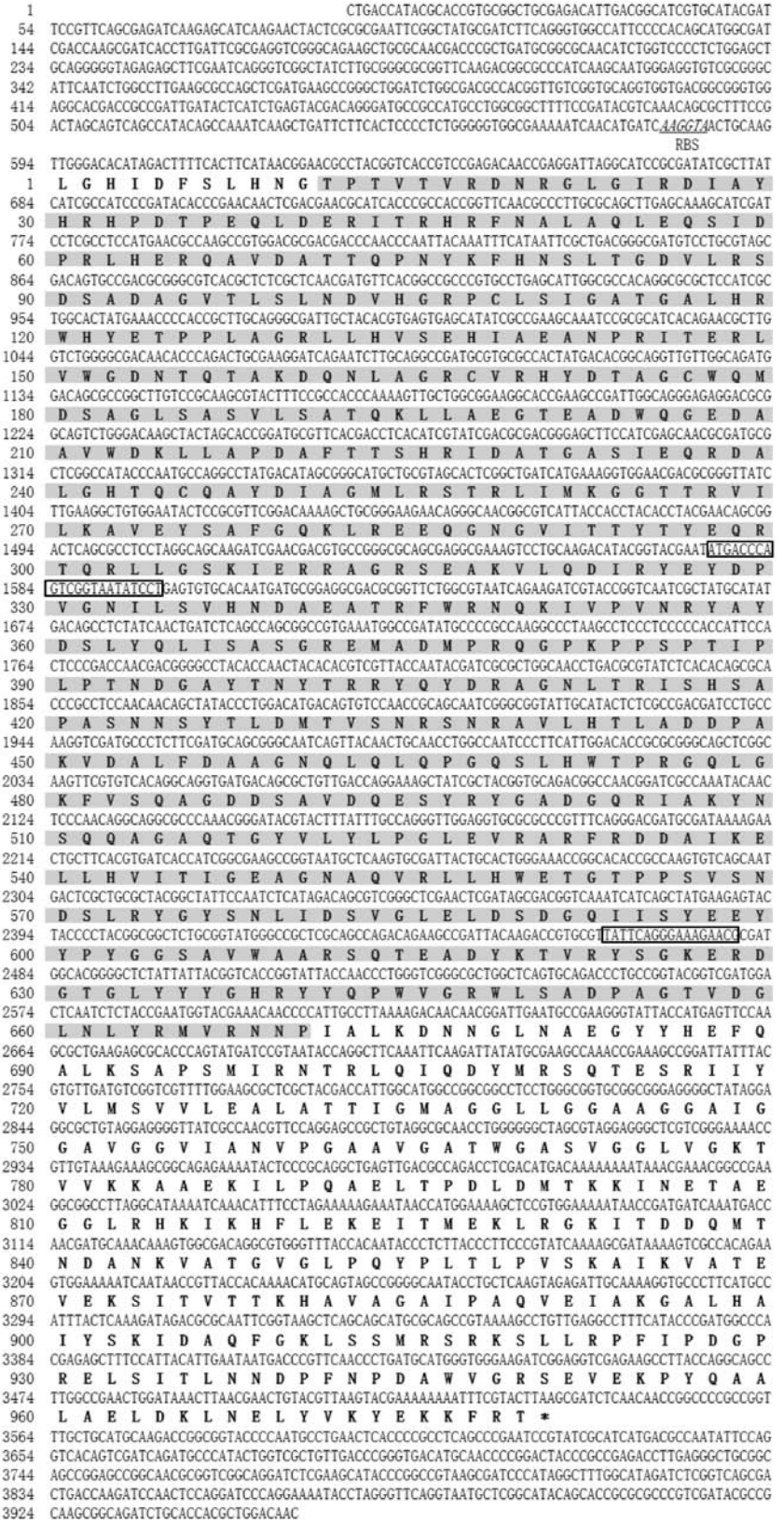
Characterization of the tccC Sequence. Translation of the open reading frame of *Pseudomonas taiwanensis tccC* revealed a protein of 980 amino acids with a calculated molecular weight of 107.93 kDa and an estimated p*I* of 7.49. Database searches of the deduced amino acid sequence were performed using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST). The deduced amino acid sequence matched several insecticidal toxin complex proteins TccC. The amino acid sequence alignment of *Pseudomonas taiwanensis* TccC and other insecticidal toxin complex proteins available from GenBank showed that the highest identity was with an insecticidal toxin from *Pseudomonas entomophila* (41.2%, accession no. YP_608362), followed by the other known and putative insecticidal toxins from *Serratia entomophila* (41.0%, accession no. NP_065279), *Yersinia pseudotuberculosis* (40.6%, accession no. YP_070748), *Serratia proteamaculans* (40.5%, accession no. ABB69941), *Photorhabdus luminescens* (40.4%, accession no. NP_931365), *Yersinia pestis* (40.3%, accession no. NP_669335), and *Photorhabdus luminescens* (40.2%, accession no. AAO17196).

Bockett et al. (*14*) indicated that TccC-like proteins are related in structure to the recombinational hot spot (Rhs) elements, which were described originally in *E. coli* and are thought to be regions of similar nucleotide sequences that promote frequent recombination (*19*, *20*). The Rhs elements are thought to be part of a family similar to core-extension proteins. Rhs elements are typified by a GC-rich (62%) core common to all members, followed by an AT-rich (60%) core extension region that is unique to each member of the family (*20*). In the present study, a putative conserved domain of Rhs-like element was detected at the N-terminus of *Pseudomonas taiwanensis* TccC (position 12−670; **Figure 1**) and was further compared to the amino acid sequences of numerous other Rhs-like elements predicted in the other bacteria by phylogenetic analysis. As shown in **Figure 2**, the phylogenetic tree shows that these Rhs-like elements form four distinct families: (1) a clade representing homologues from *Salmonella*; (2) a Tcc-like clade containing *Pseudomonas taiwanensis* TccC and the other TccC-like proteins from *Xenorhabdus nematophila*, *Serratia proteamaculans*, *Serratia entomophila*, *Photorhabdus luminescens*, and *Pseudomonas entomophila*; (3) a clade of sequences typified by homologues within Gram-positive bacteria; (4) a Rhs-like clade typified by the Rhs elements of *E. coli*. As the TccC-type proteins show a very similar structure to the Rhs elements with a highly conserved core of amino acids, Waterfield et al. (*21*) speculated that the TccC homologues may be involved in the recombinational insertion or deletion of *tc* loci or parts of *tc* loci. However, Lee et al. (*22*) demonstrated that TccC1 from *Xenorhabdus nematophilus* caused a rapid cessation in mortality of *Galleria mellonella* larvae. In order to verify the functionality of the *Pseudomonas taiwanensis* TccC, the *tccC* gene encoding the TccC-like protein was expressed in *E. coli* and purified for further analysis.

Heterologous Expression and Purification of TccC. A band of about 110 kDa corresponding to the S-tag-TccC-His_6 fusion proteins was observed in the induced recombinant bacteria (**Figure 3**, lane 2). After centrifugation, the expressed recombinant proteins were predominately found in the supernatant fraction of cell lysate (**Figure 3**, lane 4). The purified S-tag-TccC-His_6 fusion proteins were obtained after purification by affinity chromatography (**Figure 3**, lane 5). The yield of the purified recombinant TccC was 45.21 ± 3.65 μg, starting from 120 mg (wet weight) of *E. coli* cells.

Insecticidal Activity of *Pseudomonas taiwanensis* and Recombinant TccC in *Drosophila* Larvae. As shown in **Figure 4**, the average survival rate of larvae in the control experiments (standard fly medium) was 100% at the end of the 72 h feeding period. The average survival rate of larvae fed with the overnight culture supernatant of *Pseudomonas taiwanensis* was 41.7%, whereas average survival rate of larvae fed with the well-known microbial insecticide thuringiensin was 0% at the end of the 72 h feeding period. To determine the insecticidal potency, the purified recombinant TccC was fed to the *Drosophila* larvae at a concentration ranging from 25 to 350 ppm. At the end of the 72 h feeding period, the survival rate of larvae fed with the recombinant TccC at a concentration of 25 ppm was 92%. After larvae were fed with the recombinant TccC at a concentration of 350 ppm, the survival rate was higher than 80% at 8 h, and decreased from 62.5% at 24 h to 41.7% at 72 h (**Figure 4**). The lethal concentration causing 50% larval mortality (LC_{50}) of the recombinant TccC at 72 h exposure periods was 285.3 ppm. Therefore, we concluded that the heterologous expression of the cloned *tccC* genes in the recombinant *E. coli* resulted in production of recombinant toxins with insecticidal activity against *Drosophila* larvae.

Most studies investigating the host−pathogen interactions were analyses of host reactions after direct injection of bacteria into the body cavity of *Drosophila* larvae or adults, bypassing the entry of microbes through natural routes of infection (e.g., orally or through the trachea). In this study, the overnight culture supernatant of a previously uncharacterized bacterial species, *Pseudomonas taiwanensis*, was fed to the *Drosophila* larvae and induced about 60% mortality in larvae within 72 h. This result demonstrates that *Pseudomonas taiwanensis* has some insecticidal properties that could be useful for developing novel biopesticides. In a previous study, Vodovar et al. (*5*) found that *Pseudomonas entomophila* was highly pathogenic to both *Drosophila* larvae and adults, and persisted in the larvae leading to massive destruction of gut cells. The mechanisms by which *Pseudomonas entomophila* kills *Drosophila* larvae remains to be investigated; however, it was suggested that *Pseudomonas entomophila* virulence is mediated by a toxin, as described in other entomophatogenic bacteria such as *Photorhabdus luminescens* or *Bacillus thuringiensis* (*5*). In the present study, the purified recombinant TccC was fed to the *Drosophila* larvae and induced about 60% mortality in larvae within 72 h (**Figure 4**). Thus, we suggest that the insecticidal


1 CTGACCATACGCACCGTGCGGCTGCGAGACATTGACGGCATCGTGCATACGAT
54 TCCGTTCAGCGAGATCAAGAGCATCAAGAACTACTCGCGCGAATTCGGCTATGCGATCTTCAGGGTGGCCATTCCCCACAGCATGGCGAT
144 CGACCAAGCGATCACCTTGATTCGCGAGGTCGGGCAGAAGCTGCGCAACGACCCGCTGATGCGGCGCAACATCTGGTCCCCTCTGGAGCT
234 GCAGGGGGTAGAGAGCTTCGAATCAGGGTCGGCTATCTTGCGGGCGCGGTTCAAGACGGCGCCCATCAAGCAATGGGAGGTGTCGCGGGC
342 ATTCAATCTGGCCTTGAAGCGCCAGCTCGATGAAGCCGGGCTGGATCTGGCGACGCCACGGTTGTCGGTGCAGGTGGTGACGGCGGGTGG
414 AGGCACGACCGCCGATTGATACTCATCTGAGTACGACAGGGATGCCGCCATGCCTGGCGGCTTTTCCGATACGTCAAACAGCGCTTTCCG
504 ACTAGCAGTCAGCCATACAGCCAAATCAAGCTGATTCTTCACTCCCCTCTGGGGGTGGCGAAAAATCAACATGATC*AAGGTA*ACTGCAAG
RBS
594 TTGGGACACATAGACTTTTCACTTCATAACGGAACGCCTACGGTCACCGTCCGAGACAACCGAGGATTAGGCATCCGCGATATCGCTTAT
1 L G H I D F S L H N G T P T V T V R D N R G L G I R D I A Y
684 CATCGCCATCCCGATACACCCGAACAACTCGACGAACGCATCACCCGCCACCGGTTCAACGCCCTTGCGCAGCTTGAGCAAAGCATCGAT
30 H R H P D T P E Q L D E R I T R H R F N A L A Q L E Q S I D
774 CCTCGCCTCCATGAACGCCAAGCCGTGGACGCGACGACCCAACCCAATTACAAATTTCATAATTCGCTGACGGGCGATGTCCTGCGTAGC
60 P R L H E R Q A V D A T T Q P N Y K F H N S L T G D V L R S
864 GACAGTGCCGACGCGGGCGTCACGCTCTCGCTCAACGATGTTCACGGCCGCCCGTGCCTGAGCATTGGCGCCACAGGCGCGCTCCATCGC
90 D S A D A G V T L S L N D V H G R P C L S I G A T G A L H R
954 TGGCACTATGAAACCCCACCGCTTGCAGGGCGATTGCTACACGTGAGTGAGCATATCGCCGAAGCAAATCCGCGCATCACAGAACGCTTG
120 W H Y E T P P L A G R L L H V S E H I A E A N P R I T E R L
1044 GTCTGGGGCGACAACACCCAGACTGCGAAGGATCAGAATCTTGCAGGCCGATGCGTGCGCCACTATGACACGGCAGGTTGTTGGCAGATG
150 V W G D N T Q T A K D Q N L A G R C V R H Y D T A G C W Q M
1134 GACAGCGCCGGCTTGTCCGCAAGCGTACTTTCCGCCACCCAAAAGTTGCTGGCGGAAGGCACCGAAGCCGATTGGCAGGGAGAGGACGCG
180 D S A G L S A S V L S A T Q K L L A E G T E A D W Q G E D A
1224 GCAGTCTGGGACAAGCTACTAGCACCGGATGCGTTCACGACCTCACATCGTATCGACGCGACGGGAGCTTCCATCGAGCAACGCGATGCG
210 A V W D K L L A P D A F T T S H R I D A T G A S I E Q R D A
1314 CTCGGCCATACCCAATGCCAGGCCTATGACATAGCGGGCATGCTGCGTAGCACTCGGCTGATCATGAAAGGTGGAACGACGCGGGTTATC
240 L G H T Q C Q A Y D I A G M L R S T R L I M K G G T T R V I
1404 TTGAAGGCTGTGGAATACTCCGCGTTCGGACAAAAGCTGCGGGAAGAACAGGGCAACGGCGTCATTACCACCTACACCTACGAACAGCGG
270 L K A V E Y S A F G Q K L R E E Q G N G V I T T Y T Y E Q R
1494 ACTCAGCGCCTCCTAGGCAGCAAGATCGAACGACGTGCCGGGCGCAGCGAGGCGAAAGTCCTGCAAGACATACGGTACGAATATGACCCA
300 T Q R L L G S K I E R R A G R S E A K V L Q D I R Y E Y D P
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330 V G N I L S V H N D A E A T R F W R N Q K I V P V N R Y A Y
1674 GACAGCCTCTATCAACTGATCTCAGCCAGCGGCCGTGAAATGGCCGATATGCCCCGCCAAGGCCCTAAGCCTCCCTCCCCCACCATTCCA
360 D S L Y Q L I S A S G R E M A D M P R Q G P K P P S P T I P
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390 L P T N D G A Y T N Y T R R Y Q Y D R A G N L T R I S H S A
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420 P A S N N S Y T L D M T V S N R S N R A V L H T L A D D P A
1944 AAGGTCGATGCCCTCTTCGATGCAGCGGGCAATCAGTTACAACTGCAACCTGGCCAATCCCTTCATTGGACACCGCGCGGGCAGCTCGGC
450 K V D A L F D A A G N Q L Q L Q P G Q S L H W T P R G Q L G
2034 AAGTTCGTGTCACAGGCAGGTGATGACAGCGCTGTTGACCAGGAAAGCTATCGCTACGGTGCAGACGGCCAACGGATCGCCAAATACAAC
480 K F V S Q A G D D S A V D Q E S Y R Y G A D G Q R I A K Y N
2124 TCCCAACAGGCAGGCGCCCAAACGGGATACGTACTTTATTTGCCAGGGTTGGAGGTGCGCGCCCGTTTCAGGGACGATGCGATAAAAGAA
510 S Q Q A G A Q T G Y V L Y L P G L E V R A R F R D D A I K E
2214 CTGCTTCACGTGATCACCATCGGCGAAGCCGGTAATGCTCAAGTGCGATTACTGCACTGGGAAACCGGCACACCGCCAAGTGTCAGCAAT
540 L L H V I T I G E A G N A Q V R L L H W E T G T P P S V S N
2304 GACTCGCTGCGCTACGGCTATTCCAATCTCATAGACAGCGTCGGGCTCGAACTCGATAGCGACGGTCAAATCATCAGCTATGAAGAGTAC
570 D S L R Y G Y S N L I D S V G L E L D S D G Q I I S Y E E Y
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600 Y P Y G G S A V W A A R S Q T E A D Y K T V R Y S G K E R D
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630 G T G L Y Y Y G H R Y Y Q P W V G R W L S A D P A G T V D G
2574 CTCAATCTCTACCGAATGGTACGAAACAACCCCATTGCCTTAAAAGACAACAACGGATTGAATGCCGAAGGGTATTACCATGAGTTCCAA
660 L N L Y R M V R N N P I A L K D N N G L N A E G Y Y H E F Q
2664 GCGCTGAAGAGCGCACCCAGTATGATCCGTAATACCAGGCTTCAAATTCAAGATTATATGCGAAGCCAAACCGAAAGCCGGATTATTTAC
690 A L K S A P S M I R N T R L Q I Q D Y M R S Q T E S R I I Y
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750 G A V G G V I A N V P G A A V G A T W G A S V G G L V G K T
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810 G G L R H K I K H F L E K E I T M E K L R G K I T D D Q M T
3114 AACGATGCAAACAAAGTGGCGACAGGCGTGGGTTTACCACAATACCCTCTTACCCTTCCCGTATCAAAAGCGATAAAAGTCGCCACAGAA
840 N D A N K V A T G V G L P Q Y P L T L P V S K A I K V A T E
3204 GTGGAAAAATCAATAACCGTTACCACAAAACATGCAGTAGCCGGGGCAATACCTGCTCAAGTAGAGATTGCAAAAGGTGCCCTTCATGCC
870 V E K S I T V T T K H A V A G A I P A Q V E I A K G A L H A
3294 ATTTACTCAAAGATAGACGCGCAATTCGGTAAGCTCAGCAGCATGCGCAGCCGTAAAAGCCTGTTGAGGCCTTTCATACCCGATGGCCCA
900 I Y S K I D A Q F G K L S S M R S R K S L L R P F I P D G P
3384 CGAGAGCTTTCCATTACATTGAATAATGACCCGTTCAACCCTGATGCATGGGTGGGAAGATCGGAGGTCGAGAAGCCTTACCAGGCAGCC
930 R E L S I T L N N D P F N P D A W V G R S E V E K P Y Q A A
3474 TTGGCCGAACTGGATAAACTTAACGAACTGTACGTTAAGTACGAAAAAAAATTTCGTACTTAAGCGATCTCAACAACCGGCCCCGCCGGT
960 L A E L D K L N E L Y V K Y E K K F R T *
3564 TTGCTGCATGCAAGACCGGCGGTACCCCAATGCCTGAACTCACCCCGCCTCAGCCCGAATCCGTATCGCATCATGACGCCAATATTCCAG
3654 GTCACAGTCGATCAGATGCCCATACTGGTCGCTGTTGACCCGGGTGACATGCAACCCCGGACTACCCGCCGAGACCTTGAGGGCTGCGGC
3744 AGCCGGAGCCGGCAACGCGGTCGGCAGGATCTCGAAGCATACCCGGCCGTAAGCGATCCCATAGGCTTTGGCATAGATCTCGGTCAGCGA
3834 CTGACCAAGATCCAACTCCAGGATCCCAGGAAAATACCTAGGGTTCAGGTAATGCTCGGCATACAGCACCGCGCGCCCGTCGATACGCCG
3924 CAAGCGGCAGATCTGCACCACGCTGGACAAC

Figure 1. Nucleotide sequence of *tccC* gene and its flanking regions and the deduced amino acid sequence. The deduced amino acid sequence of the *tccC* gene product is indicated in boldface. The putative Shine−Dalgarno-type ribosome binding sequences (RBS) are capital italics and are underlined. The translational stop codon is indicated by an asterisk (*). The highly conserved Rhs-like element is shaded. The degenerate oligonucleotide primer sequences are boxed.

activity of the overnight culture supernatant of *Pseudomonas taiwanensis* is attributable, in part, to the TccC protein.

Effect of pH and Temperature on TccC. The toxicity of *Bacillus thuringiensis* insecticidal toxin protein may decrease under improper pH conditions. Significant conformational differences are observed between the secondary structures of the *Bacillus thuringiensis* insecticidal toxin protein molecules at different pH values (*23*). To verify the effect of pH on the insecticidal activity of TccC,

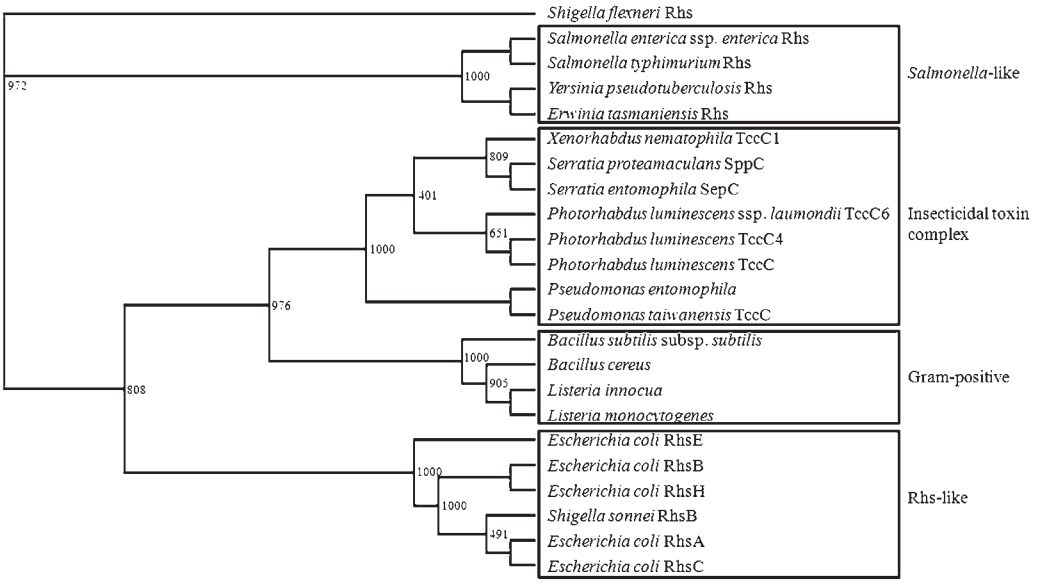

Figure 2. Phylogenetic tree of the Rhs-like sequences generated by the neighbor-joining method. At major nodes, bootstrap percentages for 1,000 resamplings are shown. The Rhs domain discovered in this study (*Pseudomonas taiwanensis* TccC) branches with the Rhs domain of Tc from *Pseudomonas entomophila* (accession no. YP_608362) and forms a clade with the Rhs domains of Tc from *Xenorhabdus nematophila* (accession no. AAS45281), *Serratia proteamaculans* (accession no. ABB69941), *Serratia entomophila* (accession no. NP_065279), *Photorhabdus luminescens* subsp. *laumondii* (accession no. NP_931365), *Photorhabdus luminescens* (accession no. AO17196 and AAC38630). The Rhs domains from *Salmonella enterica* ssp. *enterica* (accession no. NP_454898), *Salmonella typhimurium* (accession no. NP_459289), *Yersinia pseudotuberculosis* (accession no. YP_001399324), and *Erwinia tasmaniensis* (accession no. YP_001906810) form a group. The Gram-positive group contains *Bacillus subtilis* (accession no. NP_391802), *Bacillus cereus* (accession no. NP_977523), *Listeria innocua* (accession no. NP_469798), and *Listeria monocytogenes* (accession no. YP_002351156). The Rhs-like domains from *Escherichia coli* (accession no. YP_003229073, accession no. YP_003231479, accession no. YP_003231553, accession no. NP_209173, and accession no. NP_286413) and *Shigella sonnei* (accession no. YP_312590) also form a group.

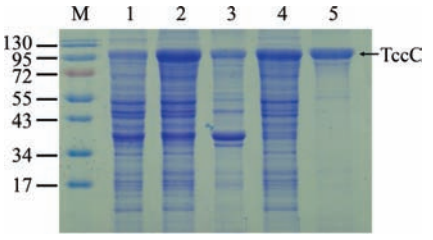

Figure 3. SDS−PAGE analysis of the expressed and purified proteins encoded by *tccC* gene. Lane M, molecular weight marker; lane 1, cell lysate of the recombinant *E. coli* before IPTG induction; lane 2, cell lysate of the recombinant *E. coli* after IPTG induction; lane 3, pellet fraction of the cell lysate after centrifugation; lane 4, supernatant fraction of the cell lysate after centrifugation; lane 5, purified S-tag-TccC-His_6 after HisTrap chromatography (2 μg of protein in each lane).

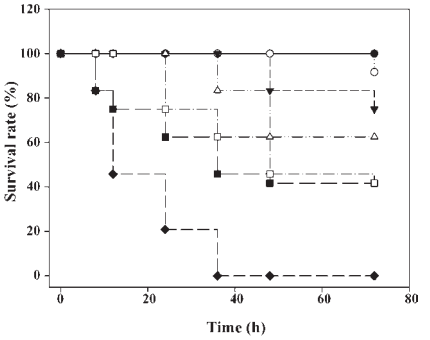

Figure 4. The insecticidal activity of *Pseudomonas taiwanensis* and the recombinant TccC to the *Drosophila* larvae. Ten microliters of the cell-free culture supernatant of *Pseudomonas taiwanensis* at a concentration of 200 ppm (open squares, □) or the purified recombinant TccC at a concentration of 25 (open circles, ○), 100 (filled triangles, ▼), 200 (open triangles, △), or 350 ppm (filled squares, ■) was added into 30 μL of standard fly medium. Positive and negative controls received the same quantities of thuringiensin at a concentration of 200 ppm (filled rhombi, ♦) or distilled water (filled circles, ●) respectively. Ten *Drosophila* larvae were used for each treatment. The experiment was repeated once with highly similar results.

the purified recombinant TccC was incubated at various pH values and then its insecticidal activity toward *Drosophila* larvae was assayed. As shown in **Figure 5A**, TccC was activity at pH 7.0. When the pH was less than 5.0 or greater than 9.0, the *Drosophila* larvae mortality was less than 20%.

A thermal-stable protein is resistant to breakdown and inactivation by heat. Therefore, the thermal stability of recombinant proteins is very important for practical applications. The thermal stability of TccC was evaluated by incubating the purified recombinant TccC at various temperatures. TccC was stable at 37 °C and induced about 60% mortality in the *Drosophila* larvae; at temperatures greater than 55 °C, less than 20% mortality in *Drosophila* larvae was observed (**Figure 5B**). Interestingly, the mortality in *Drosophila* larvae was reduced to about 40% at temperatures less than 30 °C. Maddrell et al. (*24*) indicated that the conformation of the *Bacillus thuringiensis* insecticidal toxin proteins could have changed at low temperatures, resulting in a lower pore formation rate in the insect cell membrane by the toxin. Further investigation is required to verify the effect of temperature on the conformation of *Pseudomonas taiwanensis* TccC and its effect on the insecticidal activity.

In conclusion, this study demonstrates that the recently discovered novel species *Pseudomonas taiwanensis* has insecticidal properties. An insecticidal toxin gene *tccC* was isolated from

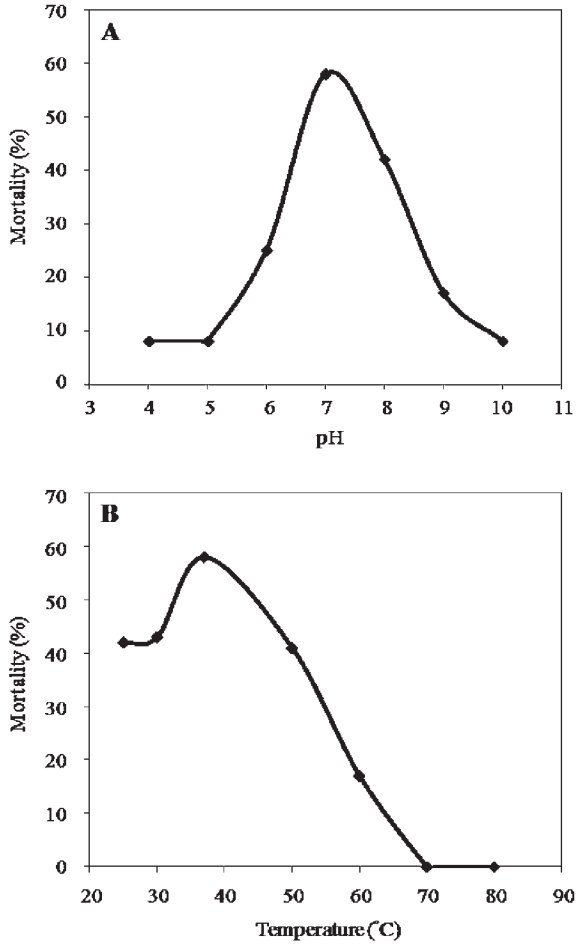

Figure 5. Effect of pH (**A**) and temperature (**B**) on the insecticidal activity of the recombinant TccC to the *Drosophila* larvae. Ten *Drosophila* larvae were used for each treatment. The experiment was repeated once with highly similar results.

Pseudomonas taiwanensis using degenerate PCR and genomic walking. The functionality of TccC was proven by heterologous expression in *E. coli*. The recombinant TccC exhibited insecticidal activity toward *Drosophila* larvae. These results prove that *Pseudomonas taiwanensis* could be used as a source for developing novel biopesticides.

Supporting Information Available: Table of primers used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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