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Isolation, Cloning, DNA Sequencing and Bioinformatics Analysis of the Parasporin – 1 Gene of *Bacillus thuringiensis*

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Abstract

Native strains of *Bacillus thuringiensis* were isolated and screened for the presence of parasporin–1 gene. Polymerase Chain Reaction (PCR) based DNA amplification using the gene-specific primers was used for the purpose. Of all the isolated strains, only four exhibited amplicons of a size analogous to that of the known parasporin–1 gene. The amplified fragments were cloned into the pGEM-vector and then were sequenced and analyzed. Bioinformatics analysis revealed that the nucleotide sequences obtained from the isolates were 99% homologous to the known DNA sequence of the parasporin gene (Blast software) and 98% homologous to the known amino acid sequence. The parasporin gene sequence obtained from this study was submitted to GenBank (Accession no. KJ576792). The 2371 nucleotides long gene was found to encode for a protein composed of 789 amino acids that had an estimated molecular weight of 84 kDa and a calculated isoelectric point (pI) of 6.7.

Keywords: Parasporin–1; *Bacillus thuringiensis*; Cloning; DNA sequencing

Introduction

Bacillus thuringiensis is a spore-forming aerobic Gram-positive bacterium that belongs to the *Bacillus cereus* group. *B. thuringiensis* is known to produce unique proteinaceous crystalline paraspora–l inclusions in its sporangia during the sporulation process [1]. This unique characteristic is the only factor that discriminates between the two taxonomically close and related species of *B. thuringiensis* and B. cereus [2]. *B. thuringiensis* was first isolated in Japan as an entomopathogenic bacterium found in diseased larvae of the silkworm, *Bombyx mori* [3]. The extremely high pathogenicity of this organism can be attributed to the action of crystal (Cry) proteins contained within the crystalline parasporal inclusions. These proteins induce a form of oral toxicity that is highly specific towards insects and nematodes. It is now well accepted that the insecticidal activity of these Cry proteins is based upon their specific interaction with a receptor located on the plasma membrane of the midgut epithelial cells of susceptible insects [4,5]. This property makes *B. thuringiensis* an environmentally safe and ecologically sound microbial agent with respect to the control of agricultural insects and pests [6-8]. This organism has also been successfully used for suppressing the population levels of medically relevant dipteran pests. The examples of such pests include mosquitoes that serve as vectors for malaria, viral diseases (including dengue, hemorrhagic fever and West Nile fever), and lymphatic filariasis, as well as the black-fly that is responsible for transmitting onchocerciasis [9]. Of great interest is a recent finding that claims that activity of *B. thuringiensis* against a common human and animal hookworm parasite can be attributed to the activity of a unique nematode-killing Cry protein [10]. Historically, it was believed that *B. thuringiensis* acquired insecticidal activity through a host-parasite relationship born during the course of co-evolution with insects. Although this hypothesis is attractive for many investigators working in this field, circumstantial evidence has led to the belief that *B. thuringiensis*, as a species, is merely an environmental saprophyte and not an obligate pathogen of insects. This theory is supported by the fact that in normal natural environments *B. thuringiensis* isolates with non-insecticidal Cry proteins outnumber the ones that are insecticidal in nature [11]. It is noteworthy that the non-insecticidal isolates often account for >90% of the natural populations obtained from soils [12] and phylloplane [13]. An important question that arises from the above-mentioned information is whether Cry proteins that are synthesized in non-insecticidal *B. thuringiensis* isolates have any biological activity at all; this is an important piece of information that is yet to be uncovered [14]. Based on the above historical background, an extensive screening of *B. thuringiensis* Cry proteins that have novel biological activities other than insect toxicity was commenced in 1996. This effort has led to the discovery of unique proteins that target human cancer cells [13] as well as a human-pathogenic protozoan [15].

Discovery of parasporin

Mizuki et al. were the first to attempt a large-scale screening of *B. thuringiensis* strains whose parasporal inclusion proteins were non-hemolytic but cytocidal to human cancer cells [16]. The screening process involved protease-digestion of parasporal proteins belonging to 1,744 *B. thuringiensis* strains including 1,700 Japanese isolates obtained from the *B. thuringiensis* collection of the Kyushu University and 44 reference type strains of the then-existing *B. thuringiensis serovars* obtained from the Institute Pasteur, Paris. In preliminary screening test using sheep erythrocytes, it was observed that parasporal proteins obtained from the 60 strains were capable of inducing a strong hemolytic response. It is now known that such hemolysis is affected by broad-spectrum cytolysins (Cyt proteins) that are active on a wide range of invertebrate and vertebrate cells [17]. A total of 1,684 strains that lacked obvious hemolytic parasporal proteins were then examined for *in vitro* cytocidal activity against MOLT–4 cells (human leukemic T-cells). Of them, 42 strains tested positive for activity against leukemic

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cancer cells. It should be noted that parasporal proteins from these cytotoxic strains had not retained any insecticidal activities. Three of the 46 strains were chosen for further characterization of the parasporal protein associated anticancer activity. When tested on MOLT–4, A549 (human lung cancer) and HeLa cells (human uterus cervix cancer), parasporal proteins from the three strains exhibited strong cytocidal activities albeit with different toxicity spectra and varied activity levels. Of particular interest was the observation that proteins belonging to the 84-HS–1–11 (later designated A1190) and 89-T–26–17 (A1462) strains were capable of discriminating between leukemic and normal T-cells by preferentially killing the former. In 2000, a study conducted by Mizuk et al. reported the isolation of an anticancer Cry protein from the A1190 strain [16]. Subsequent to this report a novel category of proteins, the parasporins, was created. Parasporins are defined as bacterial parasporal proteins that are capable of discriminately killing cancer cells [16]. According to the Committee of Parasporin Classification and Nomenclature (website: http://parasporin.fitc.pref.fukuoka.jp/), this protein is a member of the parasporin–1Aa (PS1Aa) family. Subsequent investigations have further established 5 additional parasporin families: parasporin–2 (PS2) [18], parasporin–3 (PS3) [19], parasporin–4 (PS4), parasporin–5 (PS5) [20], and parasporin–6 (PS6) [21]. In addition, several recent studies have shown that organisms with parasporin activities are common among the members of naturally occurring *B. thuringiensis* populations in Japan [22], Vietnam [12], and Malaysia [23,24]. The objective and the importance of the current research study were to isolate, clone and undertake bioinformatics analysis for the parasporin–1 gene that showed anticancer activity against human cancer cells. This study will open new avenues to isolate and characterize more parasproin genes.

Materials and Methods

Preparation of chromosomal DNA from bacterial isolates

Genomic DNA was prepared as per the specifications provided by the manufacturer (Wizard DNA purification Kit, Promega, USA). A single bacterial colony was transferred to a 5-mL LB media contained in a loosely capped 10 mL tube. The culture was incubated overnight at 37°C with vigorous shaking. Of the culture, 1 mL was transferred to a micro centrifuge tube and centrifuged at 13,000–16,000 × g for 2 min in order to pellet the cells. Of a lysozyme solution (10 mg/mL), 240 µL was added to the cell pellet and the solution was gently pipetted to ensure mixing. The purpose of this pre-treatment was to weaken the cell wall so that efficient cell lysis can take place. The sample was then incubated at 37°C for 30–60 min and centrifuged for 2 min at 13,000–16,000 × g. The supernatant was discarded and 600 µL of nuclei lysis solution was added to the cell pellet. The above was gently pipetted until all cells were uniformly resuspended. The samples were incubated at 80°C for 5 min for lysing the cells and then cooled to room temperature. Of an RNAase solution (10 mg/mL), 3 µL was added to above and the tube was inverted 2-5 times to ensure adequate mixing. The sample was incubated at 37°C for 30 min and then allowed to cool to room temperature. Of protein precipitation solution, 200 µL was added to the RNAase-treated cell lysate. Above was vortexed vigorously at a high speed for 20 s so as to ensure proper mixing of the protein precipitation solution with the cell lysate. The sample was then incubated on ice for 5 min and centrifuged at 13,000 × g for 3 min. The supernatant was transferred to a clean 1.5 mL micro centrifuge tube containing 600 µL of isopropanol (at room temperature), gently mixed by inversion until the thread-like strands of DNA could be seen to form a visible mass. DNA was pelleted by centrifugation at 13,000-16,000 × g for 2 min. The supernatant was carefully decanted and discarded; the tube containing the genomic DNA was dried on clean absorbent paper. Subsequently, 600 µL 70% ethanol (at room temperature) was added and the tube gently inverted several times to wash the DNA pellet. This was then centrifuged at 13,000-16,000 × g for 2 min. Ethanol was carefully aspirated away and the tube was drained on clean absorbent paper. The pellet was allowed to air–dry for 10–15 min after which the genomic DNA was dissolved in 100 µL of DNA rehydration solution. The DNA thus obtained was stored at –20°C till further use [25].

Preparation of plasmid DNA from bacterial isolates

Plasmid DNA was prepared as per the protocol described by Green and Sambrook [26]. From each bacterial isolate, a single bacterial colony was transferred into 2 mL of LB medium contained in a loosely capped 10 mL tube. The culture was incubated for 14-16 hr at 37°C with vigorous shaking. Of the liquid culture, 1.5 mL was transferred into a micro centrifuge tube and centrifuged at 12,000 × g for 30 s at 4°C. The bacterial cell pellet was then resuspended in 100 µL of ice-cold solution I (150 mM glucose, 25 mM Tris-HCl, pH 8.0 and 10 mM EDTA, pH 8.0) by vigorous vortexing. Of a freshly prepared solution II (0.2 N NaOH, 1% SDS), 200 µL was then added and the tube was inverted gently three times. This was followed by the addition of 150 µL of ice–cold solution III (5 M potassium acetate glacial acetic acid). The sample was centrifuged at 12,000 × g for 5 min at 4°C after which the aqueous phase was transferred to a new tube. An equal volume of Phenol:Chloroform was mixed into above and the sample was centrifuged at 12,000 × g for 2 min. The aqueous phase was again transferred to a new tube and DNA was precipitated by the addition of one-tenth volume of 3 M sodium acetate and 2 volumes of ethanol. Following 30 min incubation at –20°C, the pellet was washed with 1 mL of 70% ethanol and vacuum dried. The nucleic acid was dissolved in 50 µL of TE buffer pH 8.0 or H_2O and stored at –20°C till further use [27].

PCR screening of parasporin gene

All isolated nucleic acids either genomic or plasmid DNA were analyzed by horizontal agarose gel electrophoresis using a 1% agarose gel in combination with a 1X Tris Acetate EDTA (TAE) buffer system. The GAF and GAR primers were used to analyze local *Bacillus thuringiensis* strains for the presence of parasporin as well as to isolate the 2.3 kbp parasporin gene from the strains that were positive for parasporin (Table 1). Primers were synthesized at Bioneer, South Korea in accordance with the protocol described [16]. Internal nested primers (GAF350, GAF982, GAR1238, GAF1343, GAF1680, and GAR2021) along with M13F and M13R were only used in sequencing reactions aimed at recovering the complete nucleotide sequence of the nucleotide sequence of the parasporin gene (Table 1). Both genomic, as well as the plasmid DNA extracted from the isolates, were used as templates. The composition of the PCR reaction mixture (50 µL total volumes) was as follows: 200 mM of each dNTP, 0.5 mM primers, 10 mM Tris-

Primer	Primer Sequence
GAF	5'-ATGTCGGTTGTTTACTATGT-3'
GAR	5'-TTTATGAAACAGGACTAA-3'
GAF350	5'-TGAGCATTTATGATAATTTACGATCT-3'
GAF982	5'-GGACAACAAATAGACTCGCAAC-3'
GAR1238	5'-GCACTCATAGCCATTCCAC-3'
GAF1343	5'-CAGGCGGTATAACAAGTCAAG-3'
GAF1680	5'-GGCACCCCCAAATACTAATG-3'
FAR2021	5'-ATTCCATGTGTAAGTTCCCAAG-3'
M13F	5'-GTAAAACGACGGCCAG-3'
M13R	5'-CAGGAAACAGCTATGAC-3'

Table 1: The primers specific for parasporin–1 gene amplification and sequencing.

HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 2.5 U Taq polymerase (AB gene, Surry, UK) and 100 ng of template DNA. The PCR thermocycling parameters were as follows: initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2.5 min. Final extension was carried out for 7 min at 72°C. Table 1 lists the sequences of the gene-specific primers used for parasporin gene isolation as well as the internal nested primers used for DNA sequencing. PCR amplification of the full-length parasporin gene was investigated through DNA gel electrophoresis using a 1% agarose gel.

Purification of PCR products

The Wizard PCR DNA purification system for rapid purification of DNA was used to purify the PCR products from contaminants such as primer-dimmers and amplification primers. The procedure was performed according to the manufacturers' specifications (Promega, USA). Of the completed PCR reaction, 100 µL was transferred to a clean microcentrifuge tube and 100 µL of direct purification buffer was added to it. The two solutions were then mixed by vortexing. Of resin, 1 mL was then added to the sample and vortexed three times over a one–min period. For each PCR product, one Wizard Minicolumn was used. The plunger from a 3 mL disposable syringe was removed and set aside following which the resin/DNA mix was pipetted into the syringe barrel. The syringe plunger was inserted slowly and gently such that the slurry was pushed into the Minicolumn along with the plunger. The syringe was then detached from the Minicolumn and the plunger was removed. The syringe was reattached to the Minicolumn and 2 mL of 80% isopropanol was pipetted into the syringe for washing the column. The Minicolumn was then removed from the syringe and transferred to a new 1.5 mL microcentrifuge tube. To dry the resin, centrifugation was performed for 20 s at 13,000 × g after which the Minicolumn was transferred to a new microcentrifuge tube. Water (50 µL) was added to the new microcentrifuge tube and left undisturbed for 5 min. The tube was then centrifuged for 20 s at 13,000 × g to elute the bound DNA product. The Minicolumn was removed and discarded and the purified DNA stored at –20°C till use.

Cloning of the parasporin gene

The PCR amplified fragment 2.3 kbp was purified using the DNA Purification Kit (Fermentas) and ligated into the pGEM-T cloning vector using the T4 ligase enzyme (Promega). The above mixture was then transformed into *Escherichia coli* DH5α competent cells, and selected on nutrient agar plates containing ampicillin (100 mg/ ml), X-Gal (20 mg/mL), and IPTG (200 mg/mL). White recombinant colonies obtained were screened and verified for the presence of the gene of interest by PCR and restriction enzyme analysis. All DNA manipulations including ligation, transformation, and restriction digestion were performed as described by Sambrook and Russell [28]. The parasporin gene was analyzed and compared with the updated GenBank data using the BLAST program (http://www.ncbi.nlm.nih.gov/blast).

Transformation of the competent cells

The competent cells were thawed on ice and 50 µL aliquots were transferred to prechilled Eppendorf tubes. Of the ligation DNA mixture, 7 µL was added to the cells. The tubes were swirled gently and incubated on ice for 30 min. They were then heat pulsed in a 42°C water bath for 30 s followed by incubation on ice for 2 min. Subsequent to this 500 µL of preheated (42°C) LB growth medium was added to the above and the sample was incubated at 37°C for 1 h with shaking at 250 rpm. A sterile spreader was used to plate 100 µL of the transformation mixture onto nutrient agar containing 100 µg/mL ampicillin, 40 µg/mL x-gal, and 0.5 mM IPTG. The plates were incubated overnight at 37°C.

Screening of transformed cells

The blue-white colony screening method is the method of choice for screening recombinants transformed with the pGEM T-easy vector containing a ligated fragment at its MCS (Multiple Cloning Site). The MCS of vector lies within the LacZ gene, which when expressed in response to the presence of the synthetic inducer IPTG, produces the enzyme β–galactosidase. This releases an indigo dye from the chromomeric substrate (X-gal) resulting in the formation of a blue colony. Insertion of a DNA fragment within the MCS would disrupt the LacZ gene resulting in failure of LacZ expression and the consequent formation of the white colony. Hence, this method was used for the screening and identification of recombinants transformed with pGEMT derivatives and plated on nutrient agar.

PCR screening

White colonies were chosen for examination and screening by PCR amplification. The bacterial colony under scrutiny was picked up with a toothpick and resuspended in 50 µL sterile water. This was then, 10 pmol of forward primer, 200 µM dNTPs, 10 pmol reverse primer, 1 unit Taq polymerase, boiled for 5 min. To a PCR tube, 4 µL of this crude cell extract was added in addition to 25 µL PCR reactions containing the following components: 2.5 µL 10X buffer and water to 25 µL volumes. The PCR thermocycling parameters were as follows: 95°C for 30 s, 50°C for 30 s and 72°C for 2 min; the above cycling protocol was repeated for a total of 30 cycles.

Restriction endonuclease digestion

Plasmids were digested with the required restriction enzymes under optimal reaction conditions as described by the manufacturers' protocol. The restriction digestion mix was 20 µL in volume and contained 1 µg DNA along with 2 µL of the 10X buffer 2 (New England Biolabs, USA) and 10 units of the Not1 restriction endonuclease. The reaction mix was incubated at 37°C for 2 h followed by heat inactivation at 65°C for 10 min. Agarose gel (1.2%) was prepared and run as described by Green and Sambrook [26]. Ethidium bromide was added to the gel solution at a concentration of 0.1 µg/mL for the purpose of staining the DNA for visualization using a long wave UV Transilluminator (λ=375). One-tenth volume of sample loading buffer was added to the samples prior to loading them onto the gel.

Sequencing of the parasporin gene

Nucleotide sequencing of the cloned parasporin gene (2.3 kb) was performed by Bioneer in line with the protocol described by Sanger et al. [29]. DNA sequencing reactions were performed using the ABI PRISM Big Dye terminator cycle sequencing ready reaction Kit (PE Applied Biosystems, USA) in conjunction with the ABI PRISM (310 Genetic Analyzer) and PCR 9700 instruments. The reaction was conducted in a total volume of 20 µL containing 8 µL of terminator ready reaction mix, 250 ng of PCR product and 2 pmol of M13 forward, M13 reverse, and internal nested primers in separate sequencing reactions. The sequences were then assembled into a single non-overlapping contiguous sequence using the Fragment Assembly Program of Genetics Computer.

Results and Discussion

Recently we reported the occurrence of a unique *B. thuringiensis*

isolates that produce protein (s) with cytocidal activity against different cancer cell lines [30].

PCR screening of the parasporin gene

The presence of the parasporin–1 gene in local *Bacillus thuringiensis* isolates was confirmed by PCR analysis using GAF/GAR as the forward and reverse primers. Of the 22 *B. thuringiensis* isolates, only four exhibited an amplicon of the expected 2.3 kb fragment size (Figure 1). This entire amplicon was then used for the purpose of protein expression.

Localization of parasporin gene

The presence of the parasporin gene in the Bt isolates was confirmed by PCR using GAF/GAR as forward and reverse primers. The plasmid and genomic DNA were used as two different templates. The analysis of the results revealed that the parasporin gene was detected only in the genomic DNA and this is with accordance with [16].

Cloning of the parasporin gene and sequencing analysis

The amplified fragment was cloned into the pGEM-T vector. Cloning was confirmed by PCR, restriction digestion as well as sequencing (Figure 2).

Bioinformatics analysis

Nucleotide sequence analysis revealed a 99% homology to the known parasporin gene sequence (Blast software) while a 98% homology was observed at the amino acid level. The parasporin gene sequence isolated in this study was submitted to GenBank (Accession no.: KJ576792). The 2371 nucleotides long parasporin gene was found to encode a 789-amino acid protein with an estimated molecular weight 84 kDa and a calculated isoelectric point (pI) of 6.7. Restriction mapping of the parasporin gene revealed that it has both EcoR1 as well as Xba1 restriction sites (Figure 3) and this is in agreement with Mizuki et al. [13]. Phylogenetic analysis with other parasporin–1 members also revealed high degree of relatedness between this gene and parasporin 1 genes been isolated previously (Figure 4).

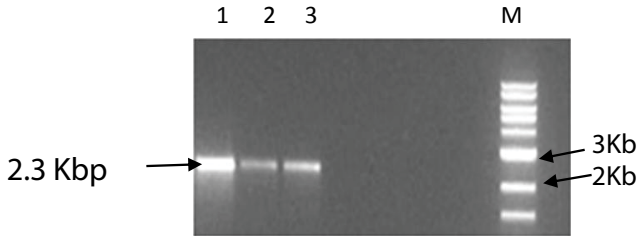

Figure 1: Ethidium bromide stained agarose gel resolving the PCR amplification fragments of parasporin–1 lanes: 1 to 3 (~2.3 Kbp) from Bt using specific primers, M: 1 Kb ladder.

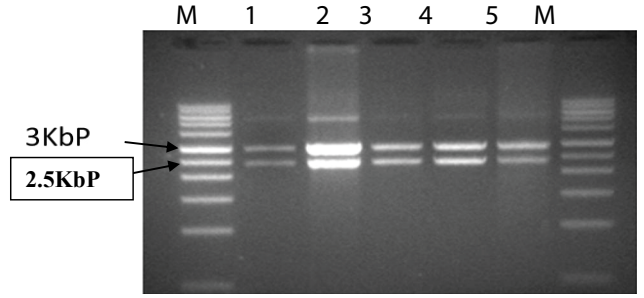

Figure 2: Screening for the presence of cloned parasporin–1 fragment at 2.3 Kbp, using *Not* 1 cut in pGEM-T vector at 3 kbp, M: 1 Kb marker, the higher 3rd band at 5.3 Kbps incomplete digestion.

Parasporin–1 protein analysis and 3D structure prediction

Careful analysis of the nucleotide sequence indicated that the 2371 nucleotides of the parasporin gene could be sorted as follows: %A=36, %G=16.5, %T=31.48 and % C=16.5. This shows that the gene has a combined %A-T=67 and %G-C=33. From this analysis, it can be concluded that the parasporin gene is a typical bacterial gene with a low %G-C and high %A-T content (Figures 5 and 6). The protein sequence of the gene showed that the amino acid composition can be classified as follows: aliphatic amino acids=171, aromatic amino acids=82, positive amino acids=83, negative amino acids=73 and small size amino acids=169. Further analysis revealed the presence of two domains; details of these domains have been discussed below:

Domain 1: Endotoxin_N (Interval 210–426 aa), Pssm-ID: 252266; Cd Length: 224; Bit Score: 94.27; E-value: 1.78e–21. Superfamily cl04339, e-value=1.78e–21, Delta endotoxin, N-terminal domain (pfam 03945). This family contains insecticidal toxins produced by the Bacillus species. During spore formation, the bacteria are known to produce crystals of this protein. Ingestion by an insect causes proteolytic cleavage of these proteins resulting in their activation. The N-terminus is cleaved in all of the proteins while the C-terminal extension is cleaved only in some selected members. Once activated the endotoxin binds to the gut epithelium and causes cell lysis which leads to death. This activated region of the delta endotoxin is composed of three structural domains. The N-terminal helical domain is involved in membrane insertion and pore formation while the second and third domains are involved in receptor binding.

Domain 2: CBM6_cellulase-like (Interval 695–786 aa), Pssm-ID: 271146; Cd Length: 144; Bit Score: 35.67; E-value: 0.01, Superfamily cl14880, e-value=0.01, Carbohydrate Binding Module 6 (CBM6) and CBM35_like superfamily. Carbohydrate-binding module family 6 (CBM6, family 6 CBM), also known as cellulose binding domain family VI (CBD VI), and related CBMs (CBM35 and CBM36). These are non-catalytic carbohydrate-binding domains found in multiple enzymes that display activity against a diverse array of carbohydrate targets including mannans, xylans, beta-glucans, cellulose, agarose, and arabinans. These domains facilitate the binding between the appended catalytic modules and their dedicated, insoluble substrates. Many of these CBMs are associated with Glycoside Hydrolase (GH) domains. CBM6 is an unusual CBM as it represents a chimera of two distinct binding sites with different modes of binding: the binding site I within the loop regions and binding site II on the concave face of the beta-sandwich fold. CBM36s are calcium-dependent xylan binding domains. CBM35s display conserved specificity through extensive sequence similarity but display divergent function through their appended catalytic modules. This alignment model also contains the C-terminal domains of bacterial insecticidal toxins which may play a vital role in determining insect specificity via carbohydrate binding functionality.

Conserved features/sites (Domain 2) feature 1: Ca binding site [ion binding site], 8 residue positions. Conserved feature residue pattern: [EQ] [EQ] × x × x [DE] [DNQ]. Evidence: Comment 2, Ca^{2+} ions are bound at these sites. Structure: 1UZ0: Cellvibrio mixtus CBM6cm–2 binds 2 Ca^{2+}, contacts at 4A. Feature 2: ligand binding site I [chemical binding site] Evidence: Comment: CBM6 represents a

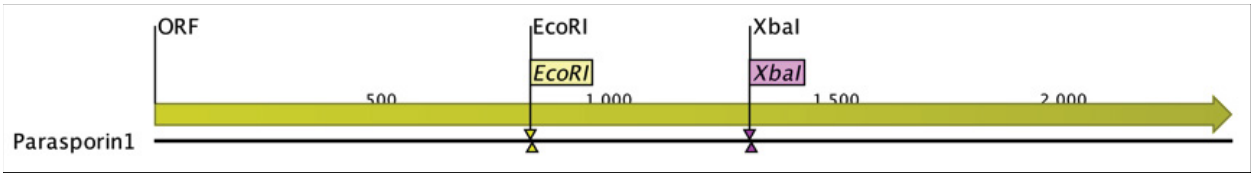

Figure 3: DNA restriction map of parasporin–1.

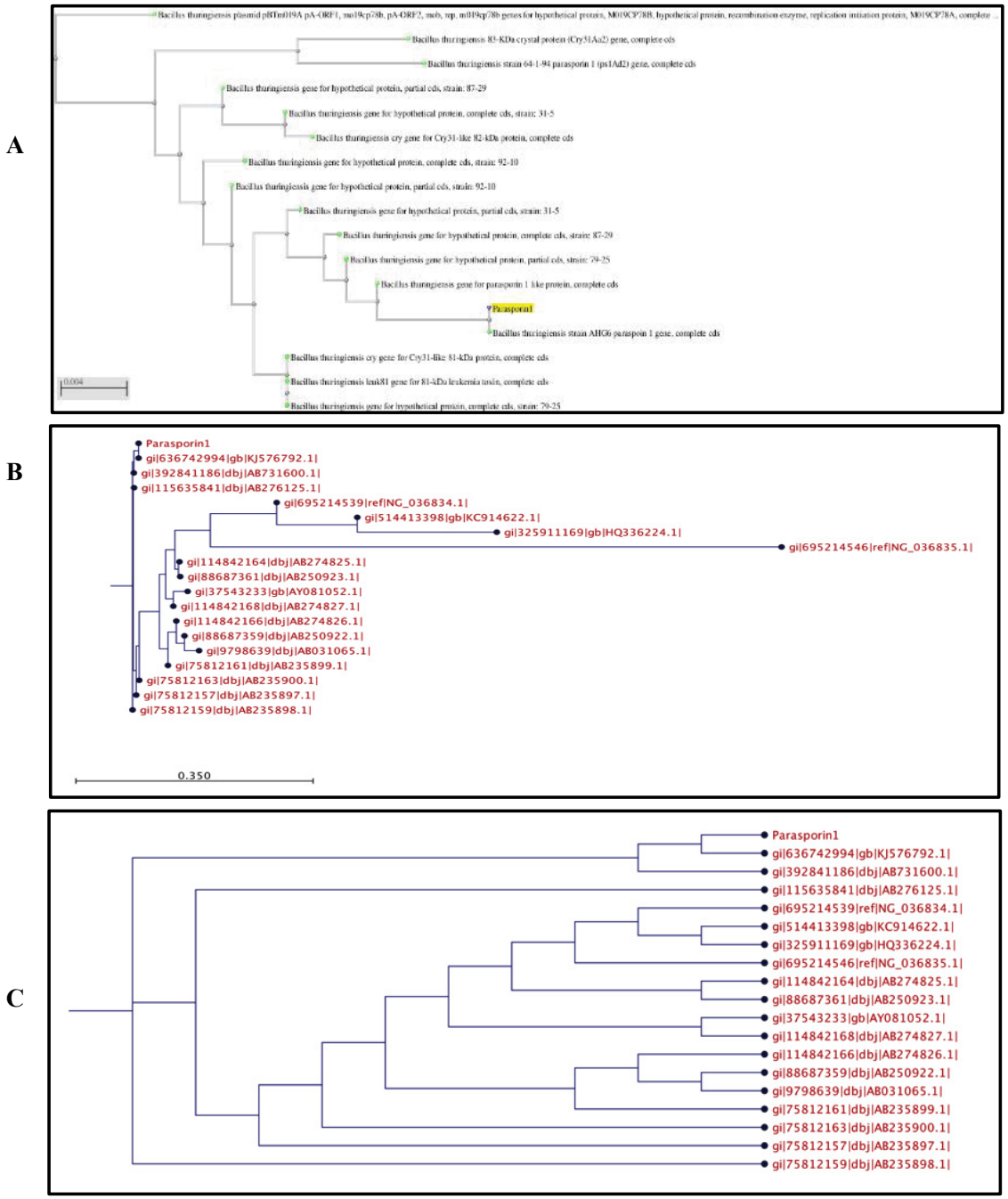

Figure 4: A: Distance tree of BLAST results; B: Phylogenetic analysis of parasporin–1; and C: Cladogram analysis of parasporin–1.

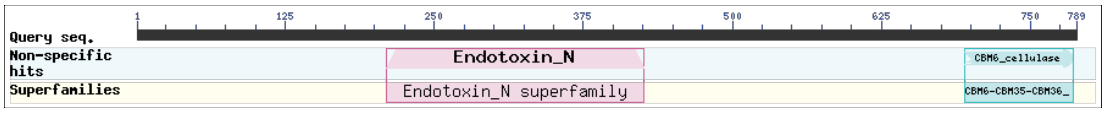

Figure 5: Domain analysis of parasporin–1.

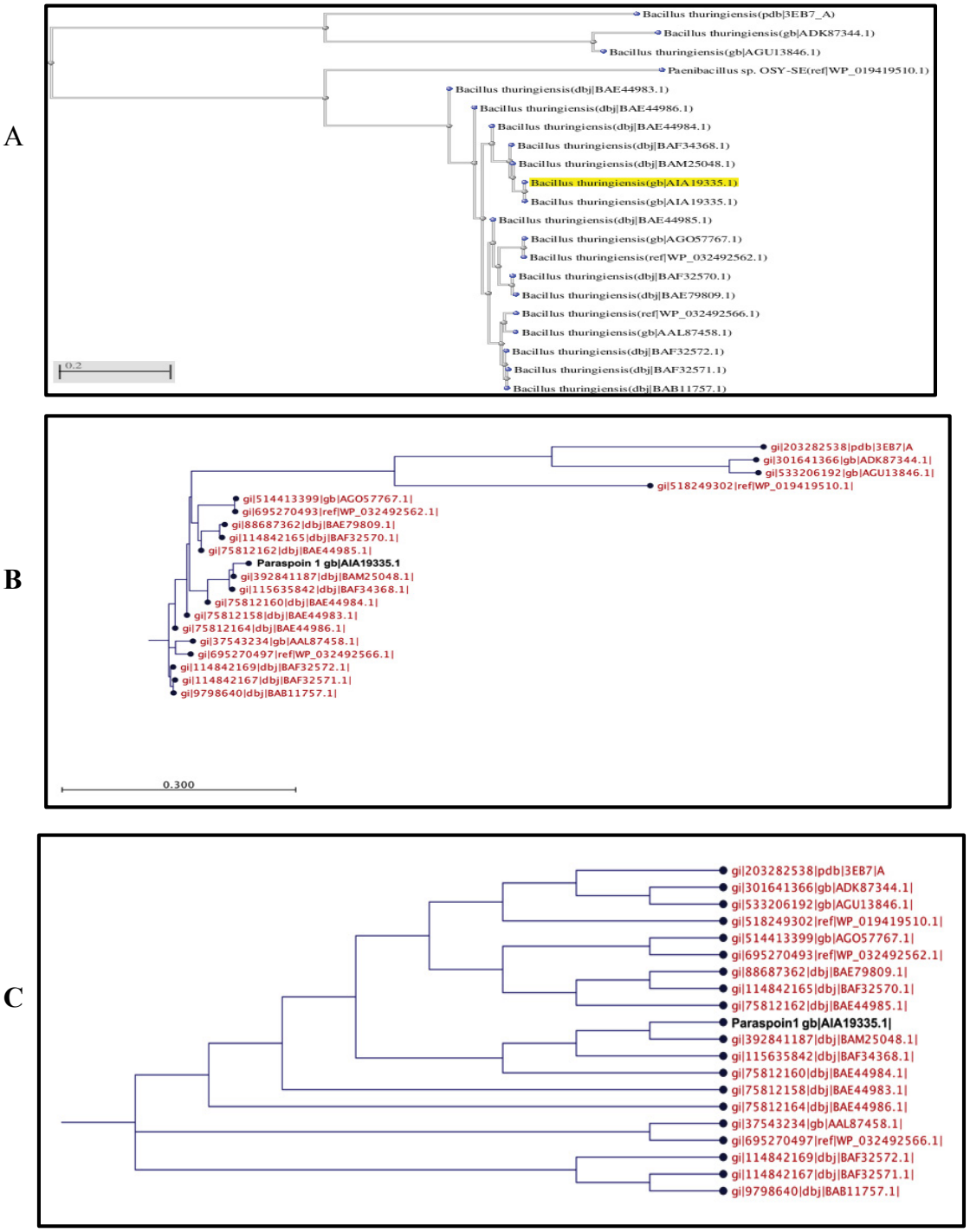

Figure 6: A: Distance tree constructed from the BLAST results; B: Phylogenetic analysis and phylogram of parasporin; and C: Cladogram of parasporin.

chimera of two distinct binding sites with different modes of binding: the binding site I (cleft A) within the loop regions and binding site II (cleft B) on the concave face of the beta-sandwich fold. Structure: 2Y8K: Clostridium thermocellum Ctgh5-Cbm6 binds glycerol in cleft B, contacts at 4A. Feature 3: ligand binding site II [chemical binding site]. Evidence: Comment: CBM6 represents a chimera of two distinct binding sites with different modes of binding: the binding site I (cleft A) within the loop regions and binding site II (cleft B) on the concave face of the beta-sandwich fold. Structure: 1UZ0: Cellvibrio mixtus CBM6cm–2 binds glc–4glc–3glc–4glc in cleft A, contacts at 4A. In addition to the 3 D structure of parasporin been studied (Figure 7) and this in accordance with [31]. The first step in this study was to target a parasporin gene. PCR screening detect the parasporin in genomic. To the best of our knowledge, this is the first study of its kind to report the of isolation of parasporin-producing Bt strains in Saudi Arabia. The parasporin–1 gene isolated in our study exhibits 99% nucleotide identity and 98% amino acid identity with previously isolated parasporin found in the database. In PCR-based experiments with parasporin specific primers, the DNA fragments of 2.3 Kb were generated from four of our isolates. These fragments were found to encode for a 789 amino acids protein with a predicted molecular weight of 88 kDa. The protein sequence obtained by translation of DNA sequences exhibited a very low amino acid sequences homology (<25%) to known Cry proteins of *Bacillus thuringiensis*. Phylogenetic analysis with other parasporin–1 members revealed a high degree of relatedness between this gene and the parasporin–1 gene. Future work: Experiments to elucidate the mechanism of discrimination between cancer cells and normal cells by parasporin are under way. Identification of the cell receptor, if any, may provide insights into the specificity of this unique protein.

Conclusion

The parasporin gene obtained from the four isolates identified in our study can be categorized into the parasporin 1. In addition, the results of our study suggest that the parasporin–1 genes may be widely distributed in the western region (Al-Konfodhah City, Makkah City and Jizan City) of Saudi Arabia. The parasporin gene sequence obtained from this study was submitted to GenBank (Accession no. KJ576792). The 84-kDa protein showed very low amino acid sequence homology (25%) to the existing Cry and Cyt proteins of *B. thuringiensis*. Obviously, this protein is not allied to the known classes of insecticidal Cry and Cyt proteins and constitutes a new class of Cry protein, designated Cry31Aa1 by the *Bacillus thuringiensis* Pesticide Crystal Protein Nomenclature Committee (Follow N. Crickmore's nomenclature website at http://epunix.biols.susx.ac.uk./Home/Neil_Crickmore/Bt/index.html). The overall sequencing data, coupled with unusual biological activity of the protein, support the creation of a new category of protein, the parasporin, defined as the bacterial parasporal protein that is capable of discriminately killing cancer cells. In a preceding study, we found that the parasporal protein(s) from our local *B. thuringiensis* isolate, a similar cytocidal spectrum, active on different cancer cell lines but nontoxic to normal cells [30]. Thus, the protein of this isolate is likely related to the parasporin.

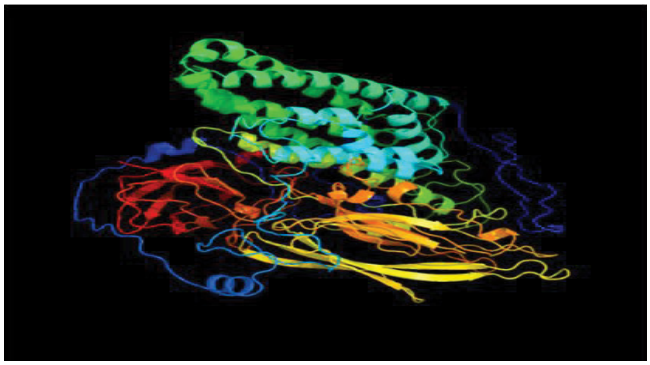

Figure 7: The predicted 3D structure of parasporin1. 3D structure prediction was performed using "Phyre2".

Author's Contributions

Parasporin 1 isolation and cloning was done by Osman GH, Bioinformatics analysis was done by Assaeedi AS and the manuscript was written by Osman GH and Assaeedi AS.

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