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A Bacterial Strain that Affects Lifecycle of Whiteflies (*Bemisia tabaci* G, *Hemiptera: Aleyrodidae*) Isolated from the Charco Azul, Xichú, Guanajuato (Mexico)

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Soil microorganisms, such as bacteria and fungi, are important biotic factor for the conservation of soil fertility, promotion of plant development and establishment of healthy ecological relation in ecosystems. The download region of Central Mexico, known as Bajío, is an important region placed in the confluence of Nearctic and Subtropical biogeographic regions.The growth of population, agriculture and industrialization seriously impact plants, animal and environmental conservation. We started a global ecological study of those areas, including bioprospection of microbial biodiversity. Hundreds of bacterial and fungi isolates were collected and characterized. Many of them exhibited biological activities for biotechnological purposes. Herein we showed isolated spore forming Gram positive bacteria active against whiteflies (*Bemisia tabaci* G., *Hemiptera: Aleyrodidae*) mediated by 100 kDa S-Layer protein, a protein belonged to *slp2* gene group.

Key words: *Bacillus thuringiensis*, S-layer protein, *Bemisia tabaci*.

Guanajuato state has a 30.491 km territory and it's placed within three great physic geographical provinces: the Mesa del Centro at north, the Sierra Madre Oriental at northeast and neovolcanic at south. Geographically we can distinguish different climatic zones: semidry in the high plains, temperate at the high lands and semitropical in the low plains. The government declared different areas under protection and established Natural Protected Areas, for ecological restoration and sustainable development. These areas are important for a bioprospection program we started, including the study of microbial diversity. Microorganisms play important roles in different biogeochemical cycles and in the mobilization, cycling and transformation of inorganic and organic chemical compounds[1]. Soil microorganisms influence in soil characteristics such as structure[2, 3], fertility[4, 5], plant health[6] and nutrition[7]. Microbial biodiversity play an important role in conservation and restoration of ecosystems and it's also a source of microorganisms useful for further biotechnology developments. One of most important target of our study is the isolation of novel *Bacillus sp*. strains. These Gram positive bacteria are frequent in soils and produce heat-

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resistant endospores, and are extensively uses in industry[8]and agriculture[7]. *Bacillus thuringiensis* has been used as microbial insecticide since the beginning of last century to control insect pest important in agriculture, forestry, animal and human health (9, 10). The biopesticide activity besides in the proteins known as δ-endotoxin or Cry proteins, produced during sporulation. *Bacillus thuringiensis* produces various groups of active proteins, such as Cyt, Vip and Parasporin, in addition to the Cry protein[11, 12], and also produce other extracellular compounds, such as phospholipases, proteases, chitinases and other toxins such as β-exotoxin and vegetative insecticidal proteins, that may contribute to insecticidal activity Isolates of *B. thuringiensis* are mostly active against larvae of lepidopteran, dipteran and coleopteran larvae and constitute safe and clean alternatives to the use of chemical biopesticides[12, 13].

In this study we included S-layer proteins (SLP) as component of parasporal inclusions of *B. thuringiensis* related to the biocide activity. The structure of SLP consists of a two-dimensional lattice structure and is the outermost component of many archaeobacteria and eubacteria. The parasporal inclusion of *B. thuringiensis* strain ITCC80 was found to be not a typical crystal Cry protein, but a proteinaceous inclusion encoded by the S-layer genes *slg1* and/or *slp2*. It has been reported that some *B.thuringiensis* strains produce inclusion bodies composed by 100 kDa S-layer protein (SLP) involved in toxicity against Mexican bean beetlelarvae (*Epilachna varivestis* M., *Coleoptera: Coccinellidae*)[14]. This result encouraged us to carry out prospective works to isolate strains with similar characteristics. SLP is a group of proteins usually covered the surfaces of many archaea and bacteria[15, 16, 17]. It was proposed that these proteins acts in functions related will shape maintenance and conservation of cell integrity and also in macromolecular exchange with the environment because of their location as the outermost cell component[15]. In certain Gram negative pathogens these proteins are involved in virulence and/or resistance to mechanism of complement-mediated killing[18, 19]. The molecular weight of SLP is widely variable since 65 kDa to 255 kDa. It was described that in case of *Bacillus anthracis* exist two different SLP, named Sap and EA1, appear in a growth phase-dependent manner, and the synthesis of Sap preceding the synthesis of EA1[20]. In case of *B. thuringiensis* subs galleria an SLP called SlpA is similar to SAP of *B. anthracis* whereas in case of *B. thuringiensis* subsp. *finitimus* shows high identity with *B. anthracis* EA1[21]. The activity of a SLP protein against insect pest was described previously, when protein termed GP1 was purified and assayed against larvae of Mexican bean beetle[14] an important pest in legume and other crops in world agriculture[22].

We started an extensive program of microbial prospection from soil, waters and insect corpses, in zones declared as Natural Preserved Areas, in Guanajuato, Mexico. Some spore forming bacteria were isolated and studied exhibiting characteristic interesting for agricultural purposes such as growth stimulating factors synthesis, chitinase and cellulase activities, and siderophores production. The goal of our work was to evaluate if *Bacillus thuringiensis* strains isolated from corpses of *B. tabaci* G. produce SLP active against important insects. Many strains were classified as *Bacillus thuringiensis* by 16S sequence analysis, but only two produce inclusion bodies majorly composed by 100 kDaSLP proteins. Both strains were tested positive for the presence of full length version of *gp1slp* gene[14]. We amplified by PCR two different regions of the SLP gene: the promoter plus the first half of coding region, and the second half of coding region plus the terminator. The isolates were designed as ITCC80 and ITCC81. Purified of crystal inclusions present in selected strain were isolated and tested against whiteflies (*Bemisia tabaci* G.) using green leaves of poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzsch). The results showed remarkable biocide activity of isolate ITCC80 against target insect, making both strains in suitable candidates for further studies and biotechnological applications.

MATERIALS AND METHODS

Isolation and characterization of *Bacillus thuringiensis* strains whose parasporalinclusions are composed by 100 kDa SLP

Soil samples and insect corpseswere collected from Charco Azul, a Natural Protected Area at 2,191 meters upper the level of sea (Xichú, Guanajuato, Mexico). Samples were transferred to

laboratory, in case of soil samples, 2 gr. were taken and transferred to 15 mL sterile Falcon tubes supplied with 5 mL of LifeGuard™ soil preservation solution (MO BIO Laboratories, San Diego, USA). One gram of each sample was used for isolation of microorganism by 48 hours agitation in sterile 1X Phosphate Buffered Saline (PBS buffer, pH 7.4). Isolation from dead insect samples was done according to acetate selection method[23]. Serial dilutions were prepared and plated on LB medium (10 g/Ltryptone, 5 g/L yeast extract and 10 g/LNaCl) and grown at 28°C for one-two days when bacterial colonies grown and were picked and cultured in 5 mL LB medium for two days at room temperature and conserved in glycerol. Isolates were classified into the Gram-positive or the Gram-negative groups[24, 25, 26.]. Gram staining allowed us to differentiate bacterial species into those two large groups: Gram-positive and Gram-negative[27].We also selected visual analysisas well as sporulation tests to identify the *Bacillus* colonies. All bacterial strains were selected and conserved in our culture bank ITCC for further studies.

PCR from total DNA

Total DNA from *Bacillus* isolates were done to carry PCR analysis and the integrity of obtained total DNA was tested by electrophoresis in 0.8 TAE-agarose gels. Two types of PCR analysis were carried out. The first one was to know which of the isolates indeed B. thuringiensis wasby using amplification and sequence comparison of 16S gene[28]. The second one allowed us to determine the presences of S-layer gene similar to those described previously[14]. PCR was performed according standard procedures [14, 28]. The used primers and expected length of amplified fragment are shown in Table 1.

Purification of inclusion bodies from selected strain

Basically we follow a washed pellet method for purification of protein inclusion bodies. Selected strains were grown in solid LB at 28°C until the presence of crystal-spore mixture in the culture was observed. The mixture was harvested in 5 mL of sterile water and centrifuged for 10 min at 10000 rpm and room temperature. The supernatant, containing inclusion bodies plus spores, was transferred to a new tube whereas the pellet containing mainly the spores was discarded. Spores elimination from mixture continues by repeating this washed pellet procedure for five times. The enriched fraction of inclusion crystal protein was analyzed by 12% SDS-PAGE electrophoresis.

Bioassays against whitefly (*Bemisia tabaci* G.)

Bioassay against whitefly was performed by dip leaf technique using surfactant to cover uniformly the leaves surface of experimental plants. Mixtures of enriched crystal inclusion body proteins from selected strains were quantified by Bradford method previously to be tested against whitefly on green leaves of poinsettia (*Euphorbia pulcherrima* Will ex Klotzsch). Plants were grown in greenhouse conditions inside isolators on sterile vermiculite substrate and without exposition to pesticide or other chemicals. The isolators consist in wood framed box shade-net covered with side doors (dimensions 80 cm x80 cm x80 cm). The isolators allow the healthy growth of plants in condition of collective experimental greenhouse until the moment of experiment. Plants in isolators were inoculated with adult whiteflies and maintained during fourth weeks at 28 °C (± 3°C) and 60% humidity (± 10%). An intensive light period was maintained to enhance the sexual attraction between male and female insects. The oviposition was confirmed visually and leaves dishes containing insect eggs were carefully cut using sterile punch. Two days before the assay plants infected with whiteflies were irrigated with sterile water solution supplemented with SLP spore mixture from *B. thuringiensis* ITCC80 at 0, 20, 40, 60, 100 µg/mL. The leaves from treated plants were collected and placed on Petridishes and accurately covered by dilution series at concentrations of 0, 20, 40, 60, 100 µg/mL of SLP spore-crystal mixture. Per each assayed concentration30 first instar neonate larvae were used and three independent experiments were carried out. The experiment was carried under the same temperature and humidity conditions during the live cycle of the target insect, including larval (nymph), pupa and adultstages. During the first teen days mortality was measured among the whitefly larvae and LD_{50} was calculated.We also observed the affectation of SLP mixture over the entire life cycle of insect, during the pupation period. The numbers of individuals emerged as adult, completing the life cycle, were counted.

Computational analysis

The evolutionary history was inferred using the Neighbor-Joining method[29]. The optimal tree with the sum of branch length = 1.36131289 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [30]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method[31] and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 2883 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [32].

Statistic approaches

Three independent experiments were carried out. With the obtained data a parametric test was done. We used statistical tests available on-line (http://www.vassarstats.net/anova1u.html). For our experimental conditions LD_{50} was determined using Log Probit analysis[33]. The data were statistically analyzed using ANOVA of each test block to establish if the differences between the means of different experimental lots are significant from statistical point of view. The descriptive statistics, the ANOVA and Tukey´stests were performed according to standard procedures.

RESULTS AND DISCUSSION.

Isolation of *Bacillus thuringiensis* strain active against whitefly (*Bemisia tabaci* G.)

Soil samples, randomly collected from Charco Azul Natural Protected Area (Xichú, Guanajuato, Mexico) and insect corpses[34]were used to extract and isolate bacterial strains. Serial dilutions were prepared and plated on LB medium an incubated. After 48 bacterial colonies were grown, picked and cultured in 5 mL LB medium during two days at room temperature. Because of a large number of isolated strains we previously characterized the isolates by their morphological appearance: white, opaque of translucent and rough, granular or wispy colonies. Additionally we identifiedgram-positive or gram-negative bacteria. Sporulation was evidenced also by staining bacteria with Coomasie Brilliant Blue and observed under Brightfield Microscopy[35]. Spore forming gram-positive strains were incubated in a medium containing 0.25M sodium acetate and after 4 hours of incubation the treated stains were plated and incubated for 48 h at 28°C. This method is reported to select *B. thuringiensis* strains from other spore-forming bacteria. About 60 % of spore-forming isolates didn't grow in sodium acetate containing media while the other 40% grew and were considered *B. thuringiensis.* Total DNA were extracted to characterize it by PCR using primers, specific to *slpgp1* gene [14]. The expected lengths of amplified fragments were 1093 pb and 2042 pb according to the *slpgp1* gene (GenBank accession

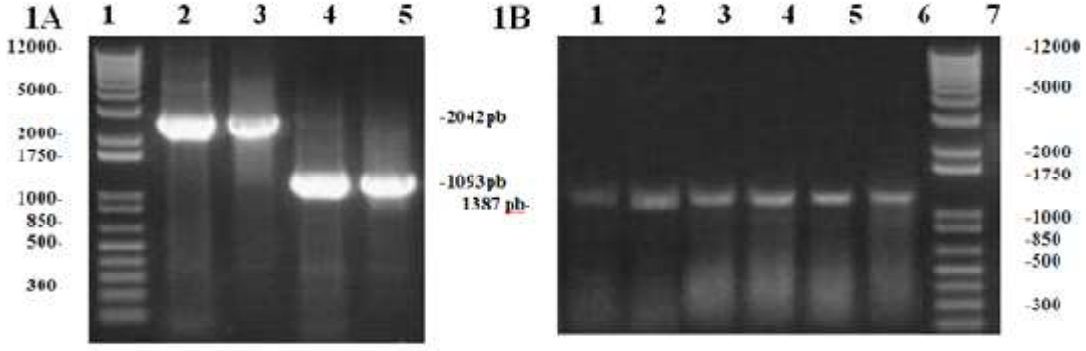

Fig. 1. Electrophoresis in 1% TAE of PCR products amplified using specific to S-Layer GP1 primers (1A) and 16 primers (1B). **1A:** 1) 1 kb Plus DNA Ladder, Lanes 2 and 3) Amplified 2042 pb fragments from *Bacillus thuringiensis* strains ITCC80 and ITCC81; 4 and 5. Amplified 1093 pb fragments from *Bacillus thuringiensis* strains ITCC80 and ITCC81. **1B:** Amplified 1100 pb fragments of 16S gene from *Bacillus thuringiensis* strains ITCC80 and ITCC81 (Lanes 5 and 6)

number AY956311). PCR and sequencing of 16S rRNA using specific primers was used to confirm the identity the *Bacillus thuringiensis* strains. Additionally we tried to amplify genes encoding for the most common *cry* genes described: *cry1A, cry3A, cry2A* using specific primers but amplification did not achieved, so we considered that the corresponding genes are not present in the bacteria. We confirmed the identification of *Bacillus* ssp. by PCR amplification and sequencing of 16S rRNA[35], using primers 63f and 1387r (Table 1, Fig. 1). About 20 isolates harboring *slpgp1* gene was identified as *Bacillus thuringiensis*[36]and the amplified fragments, obtained when primers pairs SLProt1-SLProt2; and SLProt3-SLProt4 were used, have 1093 pb and 2042 pb respectively, as it was expected from the data of the *slpgp1* gene (GenBank accession number AY956311, Table 1, Fig. 1).

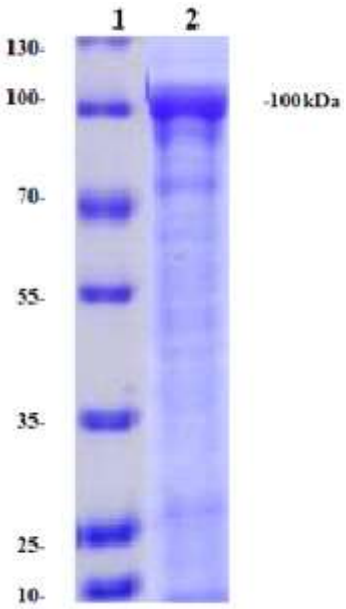

Fig. 2. SDS-PAGE inclusion bodies SL- spore mixture. Lane 1: Molecular weight makers. Lane 2: preparative SL protein spore-crystal mixture obtained from inclusion bodies from sporulated *B. thuringiensis* ITCC80

Purification of inclusion body composed by 100 KDaproteins

Bacillus thuringiensis isolates selected positive by PCR were grown until sporulation and thecolonies producing predominately 100 KDa proteins were selected by 12.5% SDS-PAGE electrophoresis. Two strains, ITCC80 and ITCC81 were selected. With this we additionally tried to amplify genes encoding for the most common cry genes described: cry1A, cry3A, cry2A using specific primers but amplification did not achieved, so we considered that the corresponding genes are not present in the Bacteria. We found both strains suitable to be included in insecticidal activity experiment against *Bemisia tabaci* G. Up to this moment the only difference found between both strains is that ITCC80 was isolated from corpses of *B. tabaci* while ITCC81 was isolated from a soil sample. In other series of experiments strain ITCC80 didn't show other biological activities, such as chitinase and syderopho reproduction were measured with negative results. Batch culture with total volume of 10 L **(**20 3-liters flask with 0.5 L medium) was grown until sporulation and bacterial cells were harvested by centrifugation, the inclusion bodies were collected and its proteins were obtained and analyzed by 12% SDS-PAGE electrophoresis. We observed an enriched 100 KDa protein fraction of inclusion body (Fig. 2).The molar relation of SL protein in the final mixture was determined by densitometry

Table 1. Primers for PCR amplification of *slpgp1* gene[14]and 16S rRNA gene[28]

Primer	Oligonucleotide Sequence	5' Position on S-layer GP1 gene	Length of PCR products
SLProt1	5'-GCTCTAGATGAGAGAGTGCTTTATAGGAAAAT-3'	-21	1093 pb
SLProt2	5'-AAAACTGCAGAAGTACCGTCAGCACTTGCTTC-3'	1114	
SLProt3	5'-AACGCTGCAGTTGTAACACTTGGTGGTAAAG-3'	1108	2042 pb
SLProt4	5'-CGGGATCCTCCTCGACCTGCGTCACTATCA-3'	3150	
Primer	Oligonucleotide Sequence	5' Position on 16S rRNA gene	Length of PCR products
63f	5'-GTGCCTAATACATGCAAGTC-3'	63	1324pb
1387r	5′-GCCTTGTACACACCGCCCGT-3'	1387	

and total protein concentration in final preparation established previous to the activity assay.

Characterization of whitefly colony

Previous to the bioassay we established whitefly colony and characterized it during two lifecycles. Whiteflies are major pest of both greenhouse and field horticultural crops and there are about 1200 different species affecting more than 500 different crops and other plants [37, 38]. *Bemisia tabaci* causes severe damage directly through phloem feeding and indirectly by the infection of 100 different plant viruses to a wide range of plant in tropical and subtropical zones. The insect development rates on green leaves of poinsettia (*Euphorbia pulcherrima* Will ex Klotzsch) were similar to those reported on this host plants recorded in the literature. Adult whiteflies are small, 0.8 to 1.2 mm long, sap sucking, flying insects. Very high populations can develop within three to four weeks at described plant host, temperature and humidity conditions. This pest provokes several damages to vegetable crops through indirect effect of feeding on plants, injecting into plants a toxin which causes physiological damage, producing honeydew which encourages sooty mould that contaminates the product and because of its ability to transmit Gemini viruses such as tomato leaf curl viruses (TLCV and TYLCV). Each female randomly lays 50-400 eggs (average 160), usually on the underside of a leaf. The new eggs are whitish yellow then turn brown and hatch after seven to 10 days.

The dynamic development of insect colony in our conditions was also studied because of our intention to study the effect of treatment, applied in the early stage of development over the whole lifecycle.There are four nymph stages before the adults emerge: 1) Crawlers (first instar): When the eggs hatch greenish-yellow, flattened, oval first instar nymphs about 0.3 mm long emerge. They crawl a short distance until they tap into a sap source in the phloem tissue. They remain in that position until they emerge as adults. This is the only nymph stage where the insect can move and at this stage we can translocate the larvae, from one piece of host leaf to other, according to the experimental design. Second and third instar nymphs: When the nymphs look like soft scale insects, oval but slightly pointed towards the tail. These stages are stationary and have no legs or

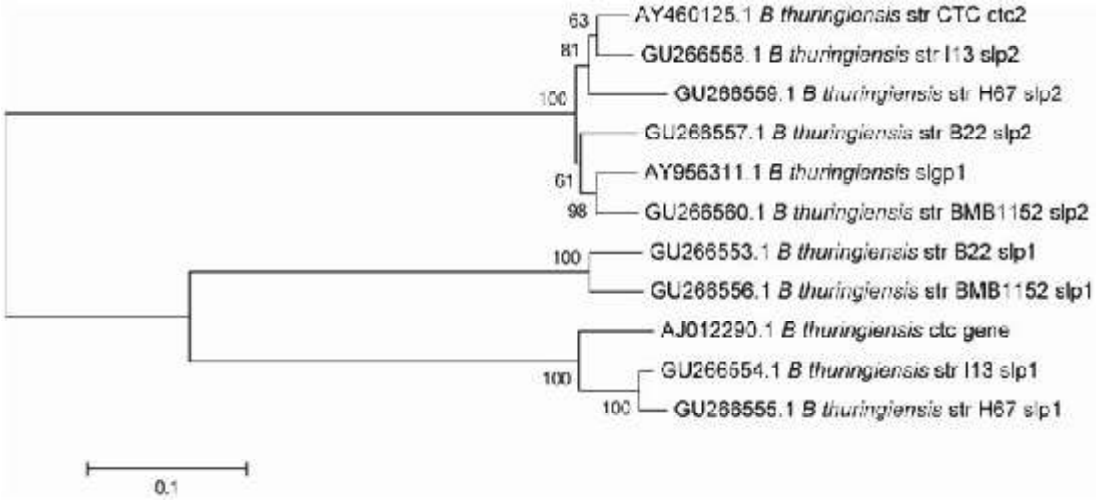

Fig. 3. Evolutionary relationships of *slp* genes among *Bacillus thuringiensis* ssp. The evolutionary history was inferred using the Neighbor-Joining method[29]. The optimal tree with the sum of branch length = 1.36131289 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [30]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method[31]and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 2883 positions in the final dataset. Evolutionary analyses were conducted in MEGA5[32]

Table 2. The evaluation of LD_{50} of *Bacillus thuringiensis* ITCC80 for *B. tabaci* are the result of the data from three independent experiment and it was established in 62.03 µg/mL of SLP-spore mixture using Log Probit™analysis

Exp 1	Estimate	Standard	t-value	p-level	Lo. Conf	Up. Conf
n	6.92769	0.651056	10.64070	0.000442	5.12007	8.73531
CI50	63.2	0.893538	70.69657	0.000000	60.68919	65.65090
R2	0.99846051					
Exp 2	Estimate	Standard	t-value	p-level	Lo. Conf	Up. Conf
n	7.68934	0.539405	14.2552	0.000141	6.19171	9.18697
CI50	60.7	0.510096	118.9543	0.000000	59.26183	62.09433
R2	0.99943687					
Exp 3	Estimate	Standard	t-value	p-level	Lo. Conf	Up. Conf
n	7.96909	0.421996	18.8843	0.000046	6.79744	9.14074
CI50	62.2	0.393612	158.0533	0.000000	61.11874	63.30442
R2	0.99964988					
LD_{50}	62.03 µg/mLSL-spore mixture from *B. thuringiensis* ITCC80					

Table 3. Primary data of biological activity experiments of *Bacillus thuringiensis* ITCC80 against whiteflies (*Bemisia tabaci*). **A:** Mortality during the larval stage. **B:** Number of converted to nymphs. **C:**Number of adults emerged from larvae. In each case the total number of larvae assayed per concentration was 30 representing the 100 % for percentage calculation

A	SL proteinspore -crystal			Nymph Mortality			
N°	C µg/mL	Exp. 1	%	Exp. 2	%	Exp. 3	%
1	0	0	0.0	0	0.0	0	0.0
2	20	0	0.0	0	0.0	0	0.0
3	40	1	1.9	1	2.0	1	2.0
4	60	16	42.5	17	48.6	15	43.2
5	80	21	80.9	24	87.5	24	87.0
6	100	30	100.0	30	100.0	30	100.0
B	SL proteinspore -crystal			Number nymphs emerged			
N°	C µg/mL	Exp. 1	%	Exp. 2	%	Exp. 3	%
1	0	30	100	30	100	30	100
2	20	30	100	30	100	30	100
3	40	28	93.3	29	96.6	29	96.6
4	60	14	46.6	13	43.3	15	50
5	80	9	30	6	20	6	20
6	100	0	0	0	0	0	0
C	SL proteinspore -crystal			Number of Adults emerged from nymphs			
N°	C µg/mL	Exp. 1	%	Exp. 2	%	Exp. 3	%
1	0	28	93.3	26	86.6	28	93.3
2	20	21	70	22	73.3	25	83.3
3	40	16	53.3	15	50	21	70
4	60	6	20	0	0	0	0
5	80	1	3.3	0	0	0	0
6	100	0	0	0	0	0	0

n=Initial number of larvae is 30 representing 100%, in all case, of assayed larvae

distinguishing features, they suck sap from the plant. Late in the third instar and through the fourth instar nymphs develop obvious red eyes and are referred to as red-eyed nymphs. The fourth instar nymphs or are the pupae stage. At this stage the nymphs are yellow and about 0.6-0.8 mm long. Late in the fourth instar they stop feeding and pupate, the yellowish-white body of the adult develops then emerges. The empty white cases the adults emerged from can be seen under the leaf of host plants. Peak emergence of adults occurred between 6 and 9 a.m. The average number of eggs laid per female was 81 at 26.7°C and 72 at 32.2°C. Males lived an average of 7.6 and 11.7 days, and females lived an average of 8.0 and 10.4 days, at 26.7 and 32.2°C, respectively.

Bioassays against whitefly (*Bemisia tabaci* G.)

Bioassays were performed by dip leaf technique using surfactant to cover uniformly the leaves surface of experimental plants on *Bemisia tabaci*larvae. The experiments were performed using green leaves of poinsettia cultivated in green house conditions. The leaves from treated plants were collected and placed on Petri dish at 0, 20, 40, 60, 100 µg/mL of SL protein spore-crystal mixture. We evaluated the activity of the of SL protein

Table 4. Statistical analysis of the biological activity experiments of *Bacillus thuringiensis* ITCC80 against *Bemisia tabaci.*G. The analyzed data are the result of three independent experiments.B. Results of the ANOVA test for correlated blocks the number of samples is k=3. C. Results of the Tukey´s test; where M1: mean of sample 1, M2: mean of sample 2 and so forth. HSD: the absolute [unsigned] difference between any two sample means required for significance at the designated level. HSD[.05] for the .05 level; HSD[.01] for the .01 level.

A	Experiments 1	2	3	Total
n	6	6	6	18
$\sum_{i=1}^{n} X_i$	38	37	24	99
$\overline{X}_i$	6.3333	6.1667	4	5.5
$\sum_{i=1}^{n} X_i^2$	334	329	182	845
σ_X	18.6667	20.1667	17.2	17.6765
σ_X^2	4.3205	4.4907	4.1473	4.2043
Std. Error	1.7638	1.8333	1.6931	0.991

C Tukey HSD Test

HSD[.05]=1.5; HSD[.01]=2.04
M_1 vs M_2 nonsignificant
M_1 vs M_3 P<0.01
M_2 vs M_3 P<0.01
M1 vs M3 P<0.01

M1 Nymphs (1, 2, 3 Instars)
M2 Pupae (4 Instar nymphs)
M3 Emergedadults

ANOVA for correlated samples *k=3*

Source	SS	df	MS	F	P
Treatment [between groups]	20.3333	2	10.1667	11.3	0.002716
Error	9	10	0.9		
SS/BI	271.1667	5			
Total	300.5	17			

spore-crystal mixture among the life cycle of whitefly. Basically we applied the mathematical model proposed to see the effect of chemical insecticides over the entire lifecycle of insects[39]. We introduced some modifications because in our case we intend to evaluate the effect of a protein (SLP mixture) instead chemical products[39, 40]. In addition, other important experimental conditions are different in our case: the characteristics of our target insect and the dynamic of established insect colony and maintenance conditions are different. We established three indicative moments in the insect lifecycle describe the putative effect of SLP-spore mixture from *B.* ITCC80: accumulated mortality, transition to pupae and transition to adults. Accumulated mortality during nymph stages 1, 2, and 3 (approximately 10 days) was quantified, as well as the transitions from to the stage 4 (pupae), and then to adults. With the obtained data of accumulated mortality of three independent experiments the $LD_{50}$was calculated in 62.03 µg/mLSLP-spore mixture from *B. thuringiensis* ITCC80 (Table 2). During the first three nymph stages, we observed highest activity just at highest concentration of SLP-spore mixture (100 µg/ml), when all insects arrested their development at third instar stage and didn't develop to the pupae (Table 3). The statistic treatment of raw data showed that the differences between the transitions to the pupae stage and then are significant in relation with the accumulated mortality during the early stage of lifecycle (nymph 1, 2 and 3 stages) to adults showed differences well correlated with the assayed SLP-spore mixture concentrations (Table 4).

***Bacillus thuringiensis* and S-Layers**

B. thuringiensis is widely used bacteria in agriculture as biopesticide[41, 42]. Typical parasporal inclusions of *B. thuringiensis* consist of Cry proteins and are usually encoded by *cry* genes. In this case the isolated Bt ITCC80 produces only parasporal inclusion bodies composed by SLP encoded by *slp* genes. The nucleotide sequences of theamplified S-layer gene fragments were "*in silico"* assembled as a single gene and it was compare with the previously reported *slp1s* and *slp2s* gene groups[43]. The evolutionary relationships was inferred (Fig. 3) and we found that our sequence grouped with *slp2* genes and it is close related with *slp2* gene from *B. thuringiensis* strain BMB 1152 (Figure 4).

The nucleotide identity varies between 73 to 79% among the genes of SLP1 group, and 93 to 94% among the genes of *slp2* group. The amplified sequences also show 92% identity at the primary sequence *Bacillus anthracis* EA1, including both coding and regulating regions. This high identity infers us to think that in *B. thuringiensis* the regulation of *slp*genes is a complex process depending by sigma factors as it occurs in *B. anthracis*with *eag* genes[20]. In *B. anthracis* another type of SLP named Sap protein, is necessary for the temporal control of AE1 protein, as it acts as a transcriptional repressor of the *eag* gene. As a result of this regulation, the *sap* gene is expressed during exponential phase whereas the expression of *eag* gene is produced during the exponential phase and for this reason it is associate only with sporulated cells. The regulator protein SAP is found in both growth phases probably because the EA1 protein displaces SAP protein from the cell wall and during sporulation phase is found in the medium. The SLP proteins comprise a family of proteins widely distributed on the surface of very different bacteria living in different environmental conditions and this fact resulted in great diversity of functions and targets. The phylogenic studies, showing evolutionary history of these proteins among different bacterial groups, have been done and demonstrated that diversity[14]. The regulation of *slp* expression in *B. thuringiensis* and elucidation of their function in these bacteria are a matter for further studies but, as it occurs in other bacteria, we suppose that SLP play an important role in the establishment of supramolecular structure of the three major classes of prokaryotic cell. In Gram-negative Archaea, the cell envelope contains crystalline SLP as the only component external to the cytoplasmic membrane. In Gram positive Eubacteria, the rigid cell wall is composed majorly by peptidoglycan and SLP is connected with this polymer, playing and important role in the functional architecture of cell envelope. Concerning the Gram positive Archeae, other polymers as pseudomurein and methanochondroitin are found rather peptidoglycan but they form the same type of supramolecular structure as it was found in Eubacteria[44]. In Gram negative Eubacteria the SLP are found in closed association with

lipopolysaccharide of the outer membrane[45]. Chemically the hydrophobic, crystalline structure of SLP can explain the formation of inclusion bodies composed by this type of proteins. Overproduction of SLP during sporulation of *B. thuringiensis* can induce the formation of inclusion SLP bodies in the bacterial cells. We observed that the *slp1*and *slp2* genes are present in *Bacillus ssp*. strains unable to form SLP inclusion bodies (data not shown)**.** We also found that *B. thuringiensis* produce twogroups of genes *slp1* and *slp2*, coexisting in the S-layer and in parasporal SLP inclusions, but we focused our work to find strains producing SLPG1, a protein belonging to SLP 2 group, encoding by *slpgp1* gene, because it has been previously reported the SLP-based insecticidal activity (Fig. 4).

The majority of bacteria, Archaea and Eubacteria, have a clear defined cell wall external to the cytoplasmic membrane [15, 16, 20]. It's believed that this wall evolved by selection as adaptive response to specific environmental condition. One common feature of this wall is the presence of S-layers, an abundant regularly ordered planar protein. This protein functions in many manners: as protective coats, structure involved in cell adhesions and surface recognition, as molecular sieves, molecular and ion traps, as scaffolding for enzymes and lately as virulence factor. The abundance in the bacteria (about 15% total protein content) and wide distribution among all types of bacteria indicated us the complexity of their role in the cell and how vital for the bacteria is its function. In *Bacillus cereus* the S-layer protein promotes interaction with human leukocytes with the host, enhancing pathogenicity[44]. In *B. anthracis*it is proposed that S-layer protein the capsule cooperate in the interaction with the hosts[20]. In our case, both stains, ITCC80 and ITCC81, the predicted amino acid sequence homology of SLP genes reveals 87% (Bt ITCC80) and 80% (Bt ITCC 81) identities with the surface layer (S-layer) protein Sap (GenBank Z36946) in *B. anthracis.* Many interesting aspects about the role and function of SLP in *B. thuringienis*strains still unclear and requires further studies. These include the range of specific activity, the function of these inclusions in the cell and the mechanism of insecticidal activity. Up to now, we found only one report of SLP with insecticidal activity against *Epilachna varivestis*[14].Because we found not homologies between *slp* and *cry* genes we considered that mechanisms of action greatly differ.

CONCLUSIONS

The expression of SLP layer gene in *B. thuringiensis* strain ITCC80 resulted in the formation of inclusion bodies which exhibit insecticidal activity against *Bemisia tabaci* G. affecting the normal development of the target insect during the life cycle. Screening procedures for *B. thringiensis* strains with specific insecticidal activity from insect corpses is a promising and effective methodology. Bt ITCC80 produces only parasporal inclusion bodies composed by SLP encoded by *slp* genes. Many interesting aspects about these *B. thuringienis* strains still unclear and requires further studies, including the range of specific activity, the function of these inclusions in the cell and the mechanism of insecticidal activity.

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