Expression of Mosquito Active Toxin Genes by a Colombian Native Strain of the Gram-negative Bacterium *Asticcacaulis excentricus*

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Mosquito control with biological insecticides, such as Bacillus *sp. toxins, has been used widely in many countries. However, rapid sedimentation away from the mosquito larvae feeding zone causes a low residual effect. In order to overcome this problem, it has been proposed to clone the* Bacillus *toxin genes in aquatic bacteria which are able to live in the upper part of the water column. Two strains of* Asticcacaulis excentricus *were chosen to introduce the* B. sphaericus *binary toxin gene and* B. thuringiensis *subsp.* medellin cry11Bb *gene cloned in suitable vectors. In feeding experiments with these aquatic bacteria, it was shown that* Culex quinquefasciatus, Aedes aegypti, *and* Anopheles albimanus *larvae were able to survive on a diet based on this wild bacterium.* A. excentricus *recombinant strains were able to express both genes, but the recombinant strain expressing the* B. sphaericus *binary toxin was toxic to mosquito larvae. Crude protease* A. excentricus *extracts did not degrade the Cry11Bb toxin. The flotability studies indicated that the recombinant* A. excentricus *strains remained in the upper part of the water column longer than the wild type* Bacillus *strains.*

Key words: *Asticcacaulis excentricus* - *Bacillus thuringiensis* - recombinant bacteria - mosquito larvae

Bacillus thuringiensis, a gram-positive bacterium produces toxins active against some insect species belonging to the orders Coleoptera, Diptera, and Lepidoptera. Several highly toxic strains of *B. thuringiensis* have been reported for mosquito control (Goldberg & Margalit 1977, Padua et al. 1984, Orduz et al. 1992, Seleena et al. 1995, Ragni et al. 1996). The classification of the mosquito active strains in three groups, proposed by Delécluse et al. (1995), positions strains potentially important for mosquito control in group 2. These include two strains of *B. thuringiensis* subspecies *medellin*, strain 367 of the subsp. *jegathesan*, and *Clostridium bifermentans* serovar. *malaysia*. These four strains display a crystal protein pattern different from that found in *B. thuringiensis* subsp. *israelensis*, while are nearly as active. Polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified parasporal crystalline inclusions from *B. thuringiensis* subsp. *medellin* strain CIB 163-131 shows a polypeptide of approximately 94 kDa, multiple bands between 80 and 65 kDa, and two doublets at 40-41 and 28-30 kDa. The sequence of the 94 kDa toxin gene of *B. thuringiensis* subsp. *medellin* encoding the Cry11Bb1 protein and its genetic organization has been reported (Orduz et al. 1998).

Parasporal inclusions containing the mosquitocidal toxins rapidly settle on the bottom of the ponds, away from mosquito larvae feeding zone (Murphy & Stevens 1992, Thanabalu et al. 1992, Xudong et al. 1993, Orduz et al. 1995). In order to overcome this problem, it has been proposed to clone mosquito active toxin encoding genes in aquatic bacteria, such as gram negative and cyanobacteria. Several attempts have been done to clone *B. thuringiensis* subsp. *israelensis* toxin genes in cyanobacteria, obtaining variable results (Angsuthanasombat & Panyim 1989, Chungjatupornchai 1990, Murphy & Stevens 1992). At the same time, gram negative bacteria such as *Asticcacalis excentricus*, *Caulobacter crescentus* and *Ancylobacter aquaticus* have been used to clone and to express *B. thuringiensis* subsp. *israelensis* toxin genes, providing better expression of gene products, and extended control by keeping toxins in larval environments at levels of 10^5 to 10^6 cells/ml (Thanabalu et al. 1992, Yap et al. 1994a,b).

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In the present study we cloned the *cry11Bb* gene of *B. thuringiensis* subsp. *medellin* and the binary toxin gene of *B. sphaericus* in an *A. excentricus* strain isolated from mosquito larvae breeding ponds in Colombia, and present data on expression and toxicity of recombinant cells to *A. albimanus, Ae. aegypti*, and *Cx. quinquefasciatus* first instar larvae, as well as information regarding flotability properties of the recombinant strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions - Strains JM109, and XL1 Blue MRF´ of *Escherichia coli* were obtained from Stratagene (La Jolla, CA, USA) and were grown at 200 rpm, 37ºC in Luria-Bertani (LB) medium (Sambrook et al. 1989). Recombinant *E. coli* cells were grown at 200 rpm 37ºC in LB medium supplemented with tetracycline 12 µg/ml. *A. excentricus* strain 4274 was obtained from the German Collection of Microorganism and Cell Cultures. Local *A. excentricus* strains were isolated from water samples taken from a lake near Medellin. *A. excentricus* cells were grown in PYE medium (2 g Bacto-peptone, 1 g yeast extract per liter), and their recombinants were grown in PYE supplemented with 6 µg of tetracycline per ml. *B. sphaericus* strain 2362 was grown in NYSM (g/l: nutrient broth, 28; yeast extract, 0.5 and supplemented with salt solution as follows: 0.7 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 µM $MnCl_2$) (Myers & Yousten 1978). Recombinant *B. thuringiensis* expressing the Cry11Bb protein was developed by Restrepo et al. (1997) and cultured by using M1 liquid medium. The Cry11Bb crystals were purified from a final whole culture (FWC) as described by Pendleton and Morrison (1966) with modifications introduced by Otieno-Ayayo et al. (1993). The FWC (250 ml) was washed twice in distilled water, the pellet resuspended in 0.5 M NaCl and gently stirred for 1 h at 30°C; after this, it was washed twice in distilled water supplemented with 0.1 M phenyl-methy-lsulfonyl-fluoride (PMSF) and centrifuged for 20 min at 12,000 rpm. The pellet was then added to a mixture of equal volumes of 1% Na_2SO_4 and CCl_4. This mixture was vigorously shaken in a separation funnel for 10 min and allowed to sit until the formation of two layers. The aqueous phase containing crystals was washed three times in distilled water and stored at -20°C. Crystals were solubilized in 100 mM Caps, 0.05% β-mercaptoethanol, pH 10.6 for 1 h at 30°C, and insoluble material was removed by centrifugation at 12,000 rpm for 10 min. Protein concentration was determined by Bradford method (Bradford 1976) following the recommendations of the Protein Assay Kit (Bio-Rad).

Plasmid pBTM4 containing the *cry11Bb1* gene was constructed by Restrepo et al. (1997). Plasmid pEA1 containing *B. sphaericus* binary toxin gene was a gift from Dr Alan Porter (Institute of Molecular and Cellular Biology, National University of Singapore). The *B. sphaericus* binary toxin gene cloned in plasmid pEA1 is under the control of the $ptac_1$ promoter, a synthetic sequence with the –35 and –10 regions of the *tac* promoter and a concensus ribosome binging site for *C. crescentus*, and downstream of the *B. sphaericus* binary toxin gene, a transcriptional terminator sequence from *B. thuringiensis* subsp. *kurstaki cry* gene (Yap et al. 1994a). Plasmid pSOD2 containing the *cry11Bb* gene of *B. thuringiensis* subsp. *medellin* was constructed as follows: the *cry11Bb* gene from plasmid pBTM4 was cloned into the *Eco*RI site of the plasmid pBlueScript SK(+) to give plasmid pDEMOND (6.3 kb). Plasmid pEA1 (12.4 kb) was digested with *Kpn*I-*Pst*I in order to release a 2.5 kb fragment containing the *B. sphaericus* binary toxin gene. An adapter with the sequence 5´-CTCGCGAAGCTTAGGCCTGCA-3´ was cloned in the *Kpn*I-*Pst*I sites of pEA1 adding the *Nru*I-*Hind*III-*Stu*I sites to give plasmid pSO1A. DNA sequence in both directions was determined in order to confirm the adapter sequence. pDEMOND was digested with *Hind*III, and fragment containing the *cry11Bb* gene (3.3 kb) was cloned into the *Hind*III site of plasmid pSO1A, producing plasmid pSOD2 (13.2 kb). Plasmids pEA1 and pSOD2 containing the same promoter and transcriptional terminator sequences were used to transform *E. coli* and *A. excentricus* cells.

Feeding experiments - *Ae. aegypti* and *Cx. quinquefasciatus* were collected in the vicinity of Medellin. *A. albimanus* colony was established with several egg shipments sent by Dr Victor Olano (Colombian National Institute of Health). All mosquito colonies are maintained under laboratory conditions at 30±2°C under a 12:12 (light:dark) photoperiod. For the feeding experiments with all mosquito species, eggs were collected the same day, washed with double distilled and sterile water, and larvae were allowed to hatch. Ten larvae of each mosquito species were placed in sterile 50 mm diameter Petri dishes with 9 ml of sterile water. One ml of the *Asticaccaulis* strain C2 culture containing $1x10^7$, $1x10^8$ or $1x10^9$ cells/ml was added. *Asticcacaulis* control without mosquito larvae was set to verify the viability of the suspensions. Mosquito larvae positive control treatments were also set with food (trout feed for *Ae. aegypti* and *A. albimanus*), and liver powder for *Cx. quinquefasciatus*. Growth of mosquito larvae and presence of exhuvii were recorded daily for five days. All treatments were set in triplicate and experiments were repeated in every

two days. Every 24 h, and after gentle shaking of Petri dishes, 1 ml of suspension was withdrawn, and replaced with 1 ml of a fresh cell suspension in the *Ae. aegypti* and *A. albimanus* treatments, and every 48 h in *Cx. quinquefasciatus* treatments. Larvae of each mosquito species were set without food as negative control. Data were analyzed by Anova and means compared using Tukey´s test (Statistica, StatSoft, Inc.).

Transformation experiments - *A. excentricus* cells (strains 4724 and C2) were grown in PYE to an optical density of OD_{600} = 1.0, harvested by centrifugation at 7,000 rpm, 4ºC, during 15 min, and washed three times alternatively with ice cold distilled water and ice cold 10% glycerol. Cells were finally resuspended in 200 µl 10% glycerol. Electroporation was used to transform 10 µl of *A. excentricus* cells with 3 µg of plasmids pSOD2 or pEA1. After the pulse, cells were placed in 1 ml of PYE and incubated for 2 h at 30ºC. Recombinant cells were selected by plating in PYE agar containing 6 µg/ml of tetracycline and were grown for 48 h. Since no *A. excentricus* strain C2 recombinants were recovered, two alternative procedures were used to treat these bacterial strains: 20 successive passes on solid media, and treatment of the native strain with the ethyl ester of the methane sulfonic acid (EMS), as described by Tao et al. (1993). After treatments, cell suspensions were plated and colonies were checked to select those maintaining *Asticcacaulis* morphology to perform again the electrotransformation assays.

Electrophoresis and Western blot - Recombinant strains were analyzed by 10% SDS-PAGE and stained with Coomasie brilliant blue. Separated proteins were electrotransferred to nitrocellulose membranes, blocked overnight at room temperature in TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.2) with 3% gelatin, and washed three times for 5 min each in TBS with 0.05% Tween-20 (TTBS). Membranes were incubated for 1 h at room temperature with mouse anti-Cry11Bb or with anti-binary toxin polyclonal antibodies raised in mice, diluted 1:1000 in TTBS with 1% gelatin. After three washes in TTBS, membranes were incubated for 1 h with a goat anti-mouse IgG (H+L)-alkaline phosphatase conjugated (1:1000 in TTBS with 1% gelatin). After three washes, color was developed with a solution containing nitroblue tetrazolium chloride (1 mg/ml), 5-bromo-4-chloro-3-indolylphosphate (1.5 mg/ml), and 10 ml of 100 mM $NaHCO_3$-Na_2CO_3, pH 9.86.

Mosquito larvicidal assays - Native and recombinant *A. excentricus* strains were grown in liquid PYE under shaking to an optical density of A_{600} = 1.0. *B. thuringiensis* subsp. *medellin* was grown in M1 at 30ºC, 200 rpm, for 48 h until sporulation (Restrepo et al. 1997). *Ae. aegypti* and *A. albimanus* used in these bioassays are maintained under laboratory conditions as described by Restrepo et al. (1997). Petri dishes (50 mm diameter) were filled with 9.9 ml of distilled water, and 10 mosquito larvae of second instar were added to each dish. One hundred µl of serial dilutions of bacterial cultures were added to obtain dilutions ranging between 10^{-2} and 10^{-7}. Negative controls were set by adding 100 µl of distilled water. Each dose was performed by duplicate and repeated in three different days. One hundred µl of the bacterial cultures were plated on solid PYE or LB in order to determine bacterial concentration. Half lethal concentration (LC_{50}) was determined by probit analysis.

Protease activity of A. excentricus extracts - Protease activity of *A. excentricus* extracts was tested in order to determine if Cry11Bb protein could be degraded in the cytoplasm of the recombinant bacteria. Cry11Bb protein was produced and purified as mentioned above. Five µg of toxin were incubated with 13 µg of crude protease extract prepared by sonication as described by Liu et al. (1996). Samples were ajusted to 30 µl with PBS buffer and incubated for 16 h at 30ºC, separated by SDS-PAGE, and proteins were transferred to nitrocellulose membranes. Membrane was developed as mentioned before.

Flotation experiments - Cells of *A. excentricus* reference strain transformed and untransformed with plasmid pEA1, *Asticcacaulis* native strain C2, transformed with plasmid pSOD2 and untransformed, and final whole cultures of *B. sphaericus*, *B. thuringiensis* subsp. *medellin*, *B. thuringiensis* subsps. *israelensis* and *jegathesan* were grown in appropriate media as indicated above, collected by centrifugation and resuspended to an A_{600} = 1 in water. Two ml were placed in sterile plastic cuvettes, sealed and kept at room temperature during six days. Cuvettes were photographed every two days in order to register their sedimentation.

RESULTS

Feeding experiments - Development of mosquito larvae was significatively different between controls (with and without normal food) and treatments containing *A. excentricus* cells as larval food in the three mosquito species tested; however, at the *A. excentricus* lower concentrations (10^7 and 10^8 cells/ml) larvae reached only second instar after five days, while in treatments containing 10^9 *A. excentricus* cells/ml, 7.5, 8.2, and 4.5 third instar larvae were observed for *Cx. quinquefasciatus*, *Ae. aegypti*, and *A. albimanus*, respectively (Table I), and in the negative control (no food added) no mosquito larvae reached second instar in any of the mosquito species tested.

Transformation efficiency and expression of toxin genes by A. excentricus cells - Transformation of native *A. excentricus* C2 cells with plasmid pEA1 or pSOD2 was only obtained in cells treated with EMS. Transformation efficiency of *A. excentricus* C2 with plasmid pEA1 after treatment with EMS was $5.8x10^2$ to $2.8x10^3$ transformants/µg of DNA, 2 to 9 times lower than results obtained with *A. excentricus* strain 4724. However, transformation efficiency of *A. excentricus* cells with plasmid pSOD2 was $2.2x10^2$ and $2.9x10^2$ transformants/µg of DNA in *A. excentricus* strains 4724 and C2, respectively.

A. excentricus cells harboring plasmids pEA1, and pSOD2, and *B. sphaericus*, *B. thuringiensis* subsp. *medellin*, and recombinant *B. thuringiensis* grown as mentioned in materials and methods were boiled in Laemmli sample buffer and subjected to SDS-PAGE and Western blot. Proteins detected in the nitrocellulose membrane indicate that both *A. excentricus* clones produce proteins corresponding to the *B. sphaericus* binary toxin (Fig. 1, lanes 2, 4) as well as in *B. sphaericus* FWC culture (Fig. 1, lane 1). These two bands were absent in *Asticcacaulis* strains untransformed (Fig. 1, lanes 3 and 5). Expression of Cry11Bb toxin in *A. excentricus* recombinant strains was also observed (Fig. 2, lanes 3, 5). Although the band seen in these recombinant strains were not as strong as the one shown by recombinant *B. thuringiensis* strain (Fig. 2, lane 2), the molecular weight of the band corresponds to the original Cry11Bb toxin.

Bioassays - Toxicity (LC_{50}) of recombinant *A. excentricus* strain C2 transformed with plasmid pEA1, expressing the *B. sphaericus* binary toxin was $1.0x10^5$ and $1.08x10^6$ cells/ml in *Cx. quinquefasciatus* and *A. albimanus* second instar

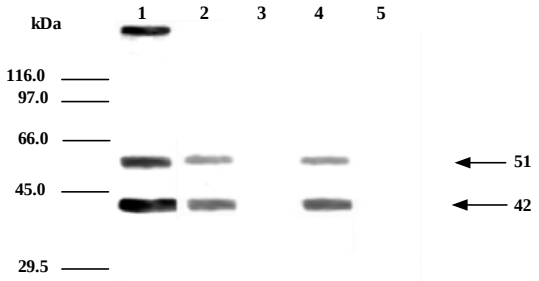

Fig. 1: expression of the binary toxin of *Bacillus sphaericus* by *Asticcacaulis excentricus* recombinant cells detected by Western blot with polyclonal antibodies developed against the *B. sphaericus* binary toxin. Lane 1: final whole culture of *B. sphaericus* strain 2362; lane 2: *A. excentricus* strain 4724 transformed with plasmid pEA1; lane 3: *A. excentricus* strain 4724; lane 4: native strain C2 of *A. excentricus* transformed with plasmid pEA1; lane 5: native strain C2 of *A. excentricus*. Arrows indicate the two bands of the binary toxin. Molecular weight is indicated to the left of the figure.

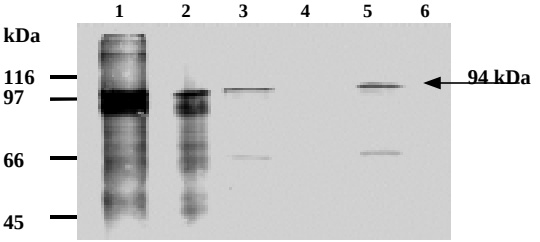

Fig. 2: expression of the Cry11Bb toxin of *Bacillus thuringiensis* subsp. *medellin* by *Asticcacaulis excentricus* recombinant cells detected by Western blot with polyclonal antibodies developed against the Cry11Bb toxin. Lane 1: final whole culture of *B. thuringiensis* subsp. *medellin*; lane 2: recombinant *B. thuringiensis* strain expressing the Cry11Bb toxin; lane 3: *A. excentricus* strain 4724 transformed with plasmid pSOD2; lane 4: *A. excentricus* strain 4724; lane 5: native strain C2 of *A. excentricus* transformed with plasmid pSOD2; lane 6: strain C2 of *A. excentricus*. Arrows indicate the 94 kDa band of the Cry11Bb toxin. Molecular weight is indicated to the left of the figure.

TABLE I

Percent of *Aedes aegypti*, *Anopheles albimanus* and *Culex quinquefasciatus* larvae reaching third instar after five days of feeding on different concentrations of fresh cultures of the gram negative bacterium *Asticcacaulis excentricus* C2, and mosquito larvae laboratory food

	Mean number of mosquito larvae reaching third instar[a]		
Treatments	*Cx. quinquefasciatus*[b]	*Ae. aegypti*[c]	*A. albimanus*[d]
Without food	0.0c[e]	0.0c[f]	0.0c[f]
With food	9.1a	10.0a	10.0a
A. excentricus C2 10^7 cells/ml	0.0c	0.0c	0.0c
A. excentricus C2 10^8 cells/ml	0.0c	0.0c	1.0c
A. excentricus C2 10^9 cells/ml	7.5b	8.2b	4.5b

a: means within each column followed by the same letter are not significantly different (*b*: F=194.5, df=25; *c*: F=165.4, df=25; *d*: F=238.7, df=25; alpha=0.05, Tukey´s HSD); mean values are the result of two tests, each one with three replicates, and 10 larvae per dish; *e*: mosquito larvae fed with liver powder; *f*: mosquito larvae fed with trout feed.

larvae respectively (Table II). This strain was 11 and 7 times less toxic to *Cx. quinquefasciatus* and *A. albimanus* larvae, respectively, than final whole culture of wild type *B. sphaericus*. On the other hand, *A. excentricus* strain 4724 transformed with the same plasmid was 1.5 times less toxic to both mosquito larvae species than *B. sphaericus*. The *A. excentricus* recombinant strains (C2 and 4724) harboring plasmid pSOD2 did not show toxicity to first instar *Cx. quinquefasciatus* larvae in any of the concentrations tested (data not shown).

Since no toxicity was found in treatments containing *A. excentricus* cells transformed with plasmid pSOD2, we investigated whether *A. excentricus* protease extract could have degraded the Cry11Bb recombinant protein once it was exported to the cytoplasm. The Western blot shown in Fig. 3 indicates that the Cry11Bb protein is degraded to a fragment of ca. 39.5 kDa in all treatments including the Cry11Bb protein without *A. excentricus* strain 4724 protease extract (Fig. 3, lane 7). This same pattern was observed when different concentrations of Cry11Bb protein were incubated with cell extracts of *A. excentricus* strain C2 (Fig. 3, lanes 5 and 6).

Flotation experiments - Flotability is a desirable character in recombinant toxin-producing bacteria, since anopheline mosquito larvae feed in the top of the water column. A comparison of flotability of *B. sphaericus* and gram negative aquatic *A. excentricus* is shown in Fig. 4. Spores of *B. sphaericus* started to sediment after two days, and were completely sedimented six days later; however, cells of *A. excentricus* transformed or untransformed remained in suspension, even six days after initiated the experiment.

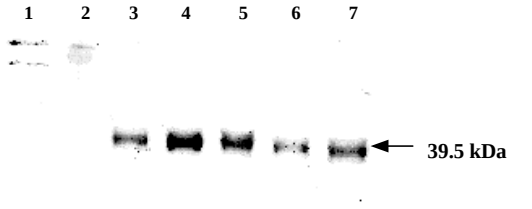

Fig. 3: Western blot of Cry11Bb protein incubated with *Asticaccaulis excentricus* cell protease extract developed with mouse anti-Cry11Bb antibody. Lane 1: *Bacillus thuringiensis* supbsp. *medellin*; lane 2: pure Cry11Bb protein; lanes 3 and 4: 5 µg of Cry11Bb protein incubated with 13 and 1.3 µg of *A. excentricus* strain 4724 cell protease extract; lanes 5 and 6: 5 µg of Cry11Bb protein incubated with 13 and 1.3 µg of *A. excentricus* strain C2 cell protease extract; lane 7: 13 µg of the Cry11Bb used as control.

DISCUSSION

An important point to be considered in developing recombinant bacteria for mosquito control is the ability of mosquito larvae to feed on a given bacterium host candidate. Pure cultures of *A. excentricus* strains C2 and 4724 were able to support mosquito larvae development in the three species tested. However, the degree of development reached in the mosquito species evaluated was significantly lower when compared to treatment with normal larval food (trout feed or liver powder), but higher than negative control, in which all larvae failed to reach second instar. Differences in larval growth between larvae in *A. excentricus* and normal larval food could be due to the kind of nutrients contained by each treatment. Merrit et al. (1992) have reported that mosquito larvae also ingest green algae, cyanobacteria, protozoa, and suspended organic material. Wotton et al. (1997) reported that development of *A. albimanus* larvae is directly related to the presence of suspended organic material as a diet complement. Differences in larval growth between the mosquito species could be due to specific nutrient requirements and/or to the specific ingestion rates for each mosquito species as indicated by Merrit et al. (1992) and

TABLE II

Toxicity of recombinant *Asticcacaulis excentricus* strain C2 and *A. excentricus* strain 4274 expressing the binary toxin of *Bacillus sphaericus* and *B. sphaericus* strain 2362 against *Culex quinquefasciatus* and *Anopheles albimanus* second instar larvae

	LC_{50} (cells/ml, 48 h)[a]	
Bacterial strain	*Cx. quinquefasciatus*	*A. albimanus*
A. excentricus (native)	0.0	0.0
A. excentricus C2 (pEA1)	$1.0x10^5$ ($7.6x10^4$ – $1.2x10^5$)[b]	$1.08x10^6$ ($7.01x10^5$ – $1.46x10^6$)
A. excentricus 4724 (pEA1)	$2.5x10^4$ ($1.93x10^4$ - $3.3x10^4$)	$2.47x10^5$ ($1.4x10^5$ - $3.4x10^5$)
B. sphaericus 2362	$9.0x10^3$ ($6x10^3$ – $1.11x10^4$)	$1.6x10^5$ ($1.22x10^5$ – $1.96x10^5$)

a: LC_{50} calculated by probit analysis; *b*: numbers in parenthesis indicate the confidence interval.

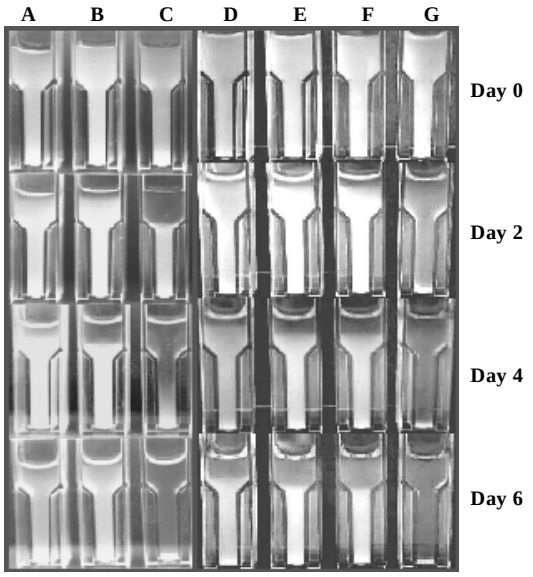

Fig. 4: flotation characteristics of *Asticaccaulis excentricus, Bacillus sphaericus* and *B. thuringiensis* subsp. *medellin*. A: cells of *A. excentricus* strain C2 untransformed; B: cells of *A. excentricus* strain C2 transformed with plasmid pEA1; C: final whole culture of *B. sphaericus*; D: *A. excentricus* trasnformed with plasmid pSOD2; E: *A. excentricus* strain C2 transformed with plasmid pSOD2; F: *A. excentricus* strain C2 untransformed; G: final whole culture of *B. thuringiensis* subsp. *medellin* were placed in plastic cuvettes as described in Materials and Methods. Pictures were taken at the interval of days indicated in the figure.

Thiery et al. (1993). The number of cells/ml of the *A. excentricus* cultures used to perform the feeding experiments resembles those found in mosquito larvae breeding ponds as reported by Grant and Long (1989). Once it was determined that *A. excentricus* strain C2 could support mosquito larvae growth, the transformation experiments were performed.

Transformation of native *A. excentricus* C2 cells with plasmid pEA1 or pSOD2 was only obtained in cells treated with EMS. Level of transformation efficiency of *A. excentricus* C2 with plasmid pEA1 after treatment with EMS is in correspondence to those reported by Tao et al. (1993), who only obtained transformants of *Streptococcus mutans* after treatment with EMS.

Expression of *B. sphaericus* binary toxin by recombinant strains of *A. excentricus* was confirmed by Western blot, as it was previously shown by Liu et al. (1996). Expression of Cry11Bb protein was also seen by Western blot, but the level of expression was low as compared to the expression of the *B. sphaericus* binary toxin.

The level of toxicity reached by native *A. excentricus* strains transformed with plasmid pEA1 is significantly greater than the toxicity obtained by unicelular cyanobacteria as reported by de Marsac et al. (1987), Xudong et al. (1993), and Sangthongpitag et al. (1997). In the same way, the LC_{50} of *A. excentricus* strain C2 transformed with plasmid pEA1 is two and four times higher than the toxicity obtained in previous reports by *A. aquaticus* and *C. crescentus*, respectively (Thanabalu et al. 1992, Yap et al. 1994b). *A. excentricus* recombinant strains (C2 and 4724) harboring plasmid pSOD2 did not show toxicity to first instar *Cx. quinquefasciatus* larvae in any of the concentrations tested (data not shown). Although the same vector was used to clone the *B. sphaericus* binary toxin gene and *B. thuringiensis* subsp *medellin* Cry11Bb toxin gene, and both contain the identical promoter and transcriptional terminator sequences, negative results shown in the bioassays could be due to modifications caused by protease activity in the cytoplasm of recombinant *A. excentricus* strains, post-translational modifications, different codon usage of the host bacteria, or a combination of the above mentioned factors. We investigated if protease activity of *A. excentricus* cytoplasm extracts could be responsible for the lack of toxicity, and found that in the protease extract prepared by sonication of *A. excentricus* cells, as well as in the Cry11Bb control treatment, the 94 kDa protein was degraded to a 39.5 kDa fragment (Fig. 3), and this protein fragment was probably produced by activity of contaminant crystal proteases left during the sample preparation. Further experiments under way aimed to design new expression vectors for these gram negative aquatic bacteria will bring light on gene expression in these bacteria.

Results of flotability experiments of *A. excentricus* recombinant cells indicated that this characteristic could be possibly due to cell division that had occurred during the time of the experiment, and/or by movement mediated by flagella in those cells that reach the movile stage. Transformation of *A. excentricus* strains, and expression of the *B. sphaericus* binary toxin by these recombinants did not affect the flotability properties of this bacterium.

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