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Isolation and Characterization of *Bacillus thuringiensis* From Various Habitats in Five Locations in the Sudan

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Abstract: In a study aiming at the isolation and characterization of *Bacillus thuringiensis* (*B.t.*) strains from five localities in the Sudan, biochemical characterization revealed that nine of the 33 isolates exhibited typical *B.t.* biochemical characteristics, eleven showed deviation in only one biochemical trait and 13 showed deviation in two or more characteristics. Serological characterization of the isolates against three imported reference *B.t.* subspecies by agglutination tests showed that eight of the isolates were positively agglutinated by the anti-*kurstaki* serum, three by the anti-*israelensis* serum and two by the anti-*tenebrionis* serum. The remaining 20 isolates were untypable by any of the three antisera that were used. The use of these isolates as biocontrol agents against insect pests is discussed.

Keywords: *Bacillus thuringiensis*; agglutination; serotypes; Sudan

INTRODUCTION

Bacillus thuringiensis (*B.t.*) has aroused increasing interest as a biocontrol agent capable of producing, at sporulation, crystalline parasporal inclusions that contain insecticidal proteins. The spectrum of activity of these proteins includes insects in at least three different orders: Lepidoptera, Coleoptera and Diptera, as well as nematodes (Feitelson *et al*. 1992; Krattiger 1997). Today *B.t.* is considered the most promising biocontrol agent, being widely used as a commercial microbial pesticide (Whitlock *et al*. 1991; Lambert and Peferoen 1992; Schnepf *et al*. 1998).

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Bacillus thuringiensis appears to be quite widespread (Martin and Travers 1989). The bacterium has been isolated from diverse habitats, including soil (Ohba *et al*. 2002; Al-Momani *et al*. 2004), plant phylloplane (Smith and Couche 1991), grain dust (Delucca *et al*. 1982; Jung *et al*. 1998), and from diseased or dead insect larvae and pupae (Vassal *et al*. 1993; Itoua-Apoyolo *et al*. 1995).

Available commercial *B.t.* products contain either one of five *B.t.* subspecies, viz. *B.t.* subsp. *kurstaki* and *B.t.* subsp. *morrisoni* which cause diseases in the caterpillars of moth and butterfly, subsp. *israelensis* which is lethal to mosquito and black fly larvae, *B.t.* subsp. *aizawai* which is toxic to wax moth caterpillars, and *B.t.* subsp. *tenebrionis* (*sandiego*) which is poisonous to beetle larvae (Entwistle *et al*. 1993).

Today, there exists a vast array of *B.t.* isolates the delineation of which by biochemical keys alone has proved to be insufficient (Lecadet *et al*. 1999). However, the introduction of serotyping techniques in the delineation of *B.t.* subspecies (serotypes) has progressively led to the recognition of an increasing number of serotypes. For instance, de Barjac (1981) recognized 20 H-serotypes, a number that rose to 34 by 1990 (de Barjac and Frachon 1990). However, Lecadet *et al*. (1999) identified 69 serotypes and 13 sub-antigenic groups within them, resulting in 82 serovars. Nevertheless, researchers continue to produce an ever-expanding list of serotypes and serovars (Li *et al*. 2002; Budatha *et al*. 2008).

To our knowledge, no research has been reported on the *Bacillus thuringiensis* subspecies in the Sudan, their characteristics, or their insect pathogenicity. Accordingly, the present study was undertaken to isolate and characterize *B.t*. strains from different samples that were obtained from various habitats in five locations in the Sudan.

MATERIALS AND METHODS

Habitats and procedure of isolation

Soil, water and plant samples, as well as dead insect larvae and pupae, were obtained from five localities in the Sudan (Table 1). Samples were

aseptically crushed, suspended in saline, and left to equilibrate for about 20 minutes, and were then subjected to a preliminary pretreatment of heating for ten minutes at 80°C. Aliquots were then streaked onto duplicate Nutrient Agar (N.A.) plates (Merck ltd, Darmstadt, Germany), and were incubated inverted at 30°C for 48-72 hours.

At the end of the incubation period, plates were microscopically examined for spore-forming colonies. These were purified by further streaking on fresh N.A. plates. The cultural characteristics of the resulting isolates were recorded; the presumptive isolates were given identification codes and were kept on N. A. slopes at ≈ 4°C.

Microscopic examination

Smears were prepared from the presumptive isolates and were examined by phase-contrast microscopy. Cell shape, presence and position of spores and presence of parasporal crystals were noted Parasporal crystals and spores were visualized using differential stains on post-stationary phase cultures of the isolates.. Cell dimensions were recorded using ocular and stage micrometers. Smears from the isolates were examined for their Gram reaction following the procedure described by Tortora *et al*. (1998). Motility of the isolates was determined by stab-inoculating with a straight wire into semi-solid N.A. tubes that were examined for swarming for up to four days.

Biochemical tests

Various biochemical tests were conducted on the isolates, including catalase production, anaerobic growth, citrate utilization, production of indole, starch hydrolysis, gelatin liquefaction, lecithinase production, glucose utilization, production of acid from mannitol and growth at 5°C and 40°C.

The catalase test was conducted following the procedure described by Collee *et al*. (1990). An anaerobic jar system was used for the anaerobic culturing of the test strains. Water was added to the contents of a disposable packet to activate its contents of chemicals to evolve sufficient hydrogen for the catalytic removal of oxygen present in the jar as well as CO_2 to a final concentration of 10% (v/v) in the jar atmosphere.

Table 1. Localities and habitats of isolation of *Bacillus thuringiensis* isolates*

Serial No.	Isolate code	Locality of isolation	Habitat
1	ED2	El Obeid	Sandy soil
2	EP1	El Obeid	Dead insect pupa
3	EP3	El Obeid	Dead insect pupa
4	EW1	El Obeid	Stagnant water
5	EW2	El Obeid	Stagnant water
6	EW3	El Obeid	Stagnant water
7	FC1	El Fao	Clay soil
8	FL1	El Fao	Dead insect larva
9	FL2	El Fao	Dead insect larva
10	KC1	Kosti	Clay soil
11	KC2	Kosti	Clay soil
12	KC3	Kosti	Clay soil
13	NC1	Kenana	Clay soil
14	NC2	Kenana	Clay soil
15	NC3	Kenana	Clay soil
16	SA1	Shambat	Okra leaves
17	SA2	Shambat	Okra leaves
18	SA3	Shambat	Okra leaves
19	SA4	Shambat	Okra leaves
20	SC1	Shambat	Clay soil
21	SC2	Shambat	Clay soil
22	SC3	Shambat	Clay soil
23	SC4	Shambat	Clay soil
24	SC5	Shambat	Clay soil
25	SC6	Shambat	Clay soil
26	SC7	Shambat	Clay soil
27	SG1	Shambat	Cotton leaves
28	SG2	Shambat	Cotton leaves
29	SL2	Shambat	Dead insect larva
30	SL3	Shambat	Dead insect larva
31	SL5	Shambat	Dead insect larva
32	SL7	Shambat	Dead insect larva
33	SW1	Shambat	Stagnant water

*All isolates were Gram-positive, contained central spores, and showed no cell swelling. All were motile except EW2 and SC5. Cell dimensions were in the range 3.0-4.5 x 1.0-1.2 μm.

The ability to use citrate as a sole carbon and energy source was tested on Simmon´s Citrate Agar medium to which 40 ml of 0.2% bromothymol blue solution were added as indicator. Appearance of a blue colour and a streak of growth were taken as a positive result.

The ability to decompose tryptophan to produce indole was tested for in a medium containing 20.0 g peptone, 5.0 g NaCl in a litre of distilled water. Kovac´s reagent was used for indication of indole production.

Starch Agar medium (10.0 g peptone, 10.0 g yeast extract, 5.0 g K_2HPO_4, 3.0 g Starch, 15.0 g agar in a litre of distilled water) was used for testing the ability to hydrolyze starch as indicated by appearance of clear zones after flooding with iodine solution.

Tests for gelatin liquefaction and lecithinase production were carried out as described by Collee *et al*. (1990). Production of acid, or acid and gas, from glucose was tested in test tubes containing peptone water to which 1% separately-sterilized glucose was added. Durham tubes were included to trap any gas produced, while acid production was tested for by the addition of a few drops of methyl red indicator. Likewise, the production of acid from mannitol was tested in peptone water containing 1% mannitol, and methyl red indicator was used to test for acid production.

Serological typing of the local isolates

Preparation of antisera against the reference strains

The following three *Bacillus thuringiensis* subspecies were imported from the United States of America to be used in the serological grouping of the isolates:

1/ *B.t.* subsp. *kurstaki* (B.t.*k*, serotype H 3a 3b) [Bonide Products Inc., Yorkville, N.Y.],

2/ *B.t*. subsp. *tenebrionis* (B.t.*te*, serotype H 18) [Bonide Products Inc., Yorkville, N.Y.], and

3/ *B.t.* subsp. *israelensis* (B.t.*i*, serotype H 14) [Summit Chemical Co., Baltimore, MD, USA].

Cultures of reference subspecies were grown in Luria-Bertani (LB) broth (Maniatis *et al*., 1982) with continuous shaking. When cell counts of these cultures were in the range of 10^7-10^9 CFU/ml, drops of formalin were added and cultures were stored at 4°C.

Six healthy rabbits were reared in the laboratory, two for each of the reference subspecies. For rabbit immunization a modification of the procedure of Somasegaran and Hoben (1985) was adopted. Each rabbit was first injected subcutaneously with 1.0 ml of a 1:1 mixture of the broth suspension of cells and Freund´s complete adjuvant (Difco). A second injection was carried out one week later. After another week, rabbits were bled and the blood was allowed to clot for two hours at room temperature, followed by 12 hours at 4°C at aseptic conditions. Antisera were carefully decanted, centrifuged, diluted 25-fold with saline, heated at 56 °C for 30 minutes and were then frozen till needed.

Conducting the agglutination tests

The tube agglutination test (de Barjac 1981) was used as a serological technique for characterization of the local isolates. Motility was encouraged through growing the test strains in LB broth without shaking to allow for production of flagella. Aliquots of 0.5 ml of the broth culture of each of the 33 local isolates (and the three reference subspecies) were suspended in saline, and were pipetted into agglutination tubes to serve as whole-cell antigens.

The stored antisera were further diluted to give concentrations of 1/50, 1/100 and 1/200. Twelve tubes were used for each isolate, four for each antiserum. One of the tubes received 0.5 ml of the 1/50 concentration, another received the 1/100 concentration, while the third received the 1/200 concentration. The fourth tube served as a control, receiving saline alone. Tubes were covered with cellophane sheets and were incubated at 37 °C for 24 hours. At the end of the incubation period, tubes were examined for the presence of visible clumps of agglutinated cells, and reactions of the test strains were compared to reaction in the saline controls, and to those of the reference subspecies. The results were recorded as positive or negative.

RESULTS AND DISCUSSION

The present study revealed that *Bacillus thuringiensis* strains are ubiquitous in Sudan's environment, as 33 *B.t.* isolates could be obtained in a relatively short survey of soil and water samples, and phylloplanes of okra (*Abelomoschus esculentus*) and cotton (*Gossypium* spp.) plants from five localities. Isolates were presumptively identified as *B. thuringiensis* strains based on cultural and staining characteristics. Colonies were white to cream-coloured, flat, with a ground-glass appearance; some were mucoid while others were brittle. All isolates were Gram positive and produced central spores with no swelling of the cell. All were motile except NC2 and SG2.

Soil was the major source of isolates of this bacterium as 45.5% of the isolates were obtained from soil samples. *Bacillus thuringiensis* is primarily a soil-inhabiting bacterium, and soil has been one of the principal sources of novel *B. thuringiensis* isolates (Dulmage and Aizawa 1982; Smith and Couche 1991). However, the rest of the isolates have been obtained from other sources, including plant phylloplanes (18.2%), stagnant water (12.1%) and dead insect larvae and pupae (24.2%). Isolation from the latter source indicated that invertebrates can be an important source of *B. thuringiensis* isolates. Itoua-Apoyolo *et al*. (1995) isolated five different *B. thuringiensis* subspecies from a single laboratory colony of the European sunflower moth (*Homoeosoma nebulella*) which indicates the ubiquity of this bacterium and the complexity of its ecology. The present results indicate that this bacterium is widespread in Sudan's environment, probably playing a hitherto not fully explored role in the habitats where it is found.

No clear relationship between serotypes and habitats or locations was evident. Insects in the orders Lepidoptera (bollworms in the genera *Earias* and *Heliothis*, and leaf worms such as *Spodoptera* spp.), Diptera (*Liriomyza* leaf miners), and Coleoptera (the African ladybird beetle *Epilachna elaterii*) are known to be among the most prevalent pests of cotton and okra in the Sudan (Schmutterer 1969; Hassan and El Khidir 2005). Nevertheless, all the isolates that were obtained from cotton (SG1

and SG2) or okra phylloplanes (SA2, SA3 and SA4, but not SA1) were not agglutinated by any of the antisera to the three serotypes tested (Table 3), although it is known that insects in the above orders are vulnerable to attack by the insecticidal proteins embodied in the three serotypes. However, it could be that the bacterium was merely attracted by the more hospitable environment created by the crop's phylloplane.

The results of the biochemical tests, that were carried out on the 33 *B.t.* isolates to ascertain their identity, are shown in Table 2. Nine of the isolates (EW2, EW3, KC1, KC3, NC3, SA1, SC6, SL2 and SL5) exhibited biochemical traits completely conforming with those typical of *B. thuringiensis* as has been documented by Claus and Berkeley (1984), eleven (ED2, FL2, KC2, NC1, SA2, SA3, SC4, SC5, SC7, SG1 and SG2) showed deviation in one biochemical attribute, while the remaining 13 isolates differed in two or more traits.

Of the present isolates, eight (20.5%) belonged to serotype 3a, 3b, 3c (*B.t.* subsp. *kurstaki*), three to serotype 14 (*B.t.* subsp. *israelensis*), and two to serotypes 8a, 8b (*B.t.* subsp. *tenebrionis*); while 20 isolates (51.3%) were untypable by any of the three antisera that were available.

As mentioned above, twenty of the present isolates (51.3%) could not be typed by the three antisera that were used. These obviously belong to other serotypes. However, the rest of the isolates could be assigned to the present three serotypes (3, 8 and 14) which are active against the insect orders Lepidoptera, Coleoptera and Diptera which comprise the most prevalent insect pests in the Sudan (Schmutterer 1969; Hassan and El Khidir 2005).

It is noteworthy that one of the untypable isolates was SC5, which was non-motile. Non-motile *B. thuringiensis* usually do not lend themselves to typing by agglutination (de Barjac and Frachon 1990) due to absence of flagella. However, the other non-motile isolate (EW2) was agglutinated by the anti-*israelensis* serum, a result that could not be explained except on the possibility of apparently false motility or apparent agglutination results.

Table 2. Biochemical characteristics of the *Bacillus thuringiensis* isolates obtained from five locations in the Sudan*

Isolate code	Growth at 5 °C	Catalase test	Citrate utilization	Starch hydrolysis	Lecithinase test	Gas from glucose	Acid from mannitol
ED2	-	+	-	+	+	-	-
EP1	-	+	-	+	+	+	-
EP3	-	+	-	+	+	+	-
EW1	+	-	-	+	+	+	+
EW2	-	+	+	+	+	-	-
EW3	-	+	+	+	+	-	-
FC1	-	+	-	+	+	-	+
FL1	-	+	-	+	+	-	+
FL2	-	+	+	+	+	+	-
KC1	-	+	+	+	+	-	-
KC2	-	+	-	+	+	-	-
KC3	-	+	+	+	+	-	-
NC1	-	+	+	+	+	-	+
NC2	-	+	-	+	+	-	-
NC3	-	+	+	+	+	-	-
SA1	-	+	+	+	+	-	-
SA2	-	+	+	+	+	+	-
SA3	-	+	+	+	-	-	-
SA4	-	-	-	+	+	+	-
SC1	-	-	-	-	+	-	-
SC2	-	+	-	+	+	+	+
SC3	-	+	-	+	+	-	+
SC4	-	+	-	+	+	-	-
SC5	-	+	-	+	+	-	-
SC6	-	+	+	+	+	-	-
SC7	-	+	-	+	+	-	-
SG1	-	+	+	+	+	+	+
SG2	-	+	+	+	+	+	-
SL2	-	+	+	+	+	-	-
SL3	-	+	+	+	+	+	+
SL5	-	+	+	+	+	-	-
SL7	-	+	+	+	-	-	-
SW1	+	+	-	+	+	-	-

*All isolates were capable of growth at 40 °C; none could produce indole from tryptophan; all were capable of gelatin liquefaction except isolate SL3; all were capable of anaerobic growth except isolate SL7; and all produced acid from glucose except isolate SC1.

Table 3. Agglutination patterns of the isolates against three reference antisera

Isolate code	Anti-*kurstaki* serum	Anti-*tenebrionis* serum	Anti-*israelensis* serum
ED2	-	-	+
EP1	-	-	-
EP3	-	-	-
EW1	-	-	-
EW2	-	-	+
EW3	-	+	-
FC1	-	-	-
FL1	-	-	-
FL2	-	-	-
KC1	+	-	-
KC2	-	-	-
KC3	+	-	-
NC1	+	-	-
NC2	-	-	-
NC3	-	-	+
SA1	+	-	-
SA2	-	-	-
SA3	-	-	-
SA4	-	-	-
SC1	-	-	-
SC2	-	-	-
SC3	+	-	-
SC4	-	-	-
SC5	-	-	-
SC6	+	-	-
SC7	-	-	-
SG1	-	-	-
SG2	-	-	-
SL2	+	-	-
SL3	-	-	-
SL5	-	+	-
SL7	-	-	-
SW1	+	-	-

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عزل بكتيريا *Bacillus thuringiensis* من عدة بيئات في السودان وتحديد خصائصها

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موجز البحث : هدفت هذه الدراسة لعزل سلالات بكتيريا *Bacillus thuringiensis* وتحديد خصائصها في خمسة مواقع في السودان. أظهرت الاختبارات الكيموحيوية أن تسعاً من ثلاث وثلاثين عزلة كان لها خصائص متطابقة تماماً مع الخصائص النمطية لهذه البكتيريا ، وأظهرت إحدى عشرة عزلة اختلافا في خاصية كيموحيوية واحدة ، بينما أظهرت الثلاث عشرة عزلة الباقية اختلافاً في خاصيتين أو أكثر. وباستعمال اختبارات التلازن المصلية وثلاثة تحت أنواع (أنماط مصلية) مرجعية مستوردة، أظهرت النتائج أن ثمان عزلات أظهرت تلازناً موجباً مع المصل المضاد للنمط المرجعي *kurstaki*، وتلازنت ثلاث عزلات مع المصل المضاد للنمط *israelensis*، وعزلتان مع المصل المضاد للنمط *tenebrionis*، بينما لم تتلازن أي من العشرين عزلة الباقية مع أي من الأنماط المصلية الثلاثة التي تم استخدامها. ناقشت الورقة إمكانية الاستفادة من العزلات التي تم الحصول عليها في المكافحة الحيوية للآفات الحشرية.

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