ISOLATION AND CHARACTERIZATION OF THIRTEEN NEW STRAINS OF BACILLUS SPHAERICUS FROM COLOMBIA TOXIC TO CULEX QUINQUEFASCIATUS LARVAE

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Bacillus sphaericus a mosquito pathogenic endospore-former bacterium, has several advantages over *B. thuringiensis* subsp. *israelensis*. The persistence of the *B. sphaericus* spores and toxins in the environment during a considerable period of time, the ability to recycle under certain environmental conditions and to multiply in dead larvae, its specific toxicity to mosquito larvae and innocuity to other aquatic macroinvertebrate fauna and to mammals, account for the importance of this bacterium as a microbial insecticide (J. A. Hornby et al., 1981, *WHO/VBC/81*.830; F. S. Mulligan III et al., 1980, *J. Econ. Entomol., 73:* 684-688; S. Silapanuntakul et al., 1983, *J. Invertebr. Pathol., 42:* 387-392; B. C. Hertlein et al., 1979, *J. Invertebr. Pathol., 33:* 217-221; E. W. Davidson et al., 1984, *Appl. Environ. Microbiol., 47:* 125-129; B. Des Roches & R. Garcia, 1984, *Mosq. News, 44:* 160-165; M. S. Mulla et al., 1984, *Mosq. News, 44:* 336-342; J. P. Siegel & J. A. Shadduck, 1990, *J. Econ. Entomol., 83:* 347-355).

From the 340 strains of *B. sphaericus* belonging to 49 serotypes registered in the International Entomopathogenic *Bacillus* Centre (Institut Pasteur, Paris), 254 have shown pathogenicity for mosquito larvae. Most of them have been reported in the last five years, and have been isolated from 31 countries (H. de Barjac, 1991, *International Entomopathogenic Bacillus Centre.* Institut Pasteur). These mosquito-active strains come from mosquito and non-mosquito origin (W. A. Ramoska, et al., 1977, *J. Invertebr. Pathol., 30:* 151-154; S. Singer, 1973, *Nature [London], 224:* 110; J.

Weiser, 1984, *Zbl. Mikrobiol., 139:* 57-60; R. S. B. Wickremsinghe & C. L. Mendis, 1980, *Mosq. News, 44:* 26-32; O. Lysenko et al., 1985, *J. Am. Mosq. Control Assoc., 1:* 369-371; R. Gai-Xin et al., 1987, *Acta Entomol. Sin., 30:* 20-24; M. Brownbridge & J. Margalit, 1987, *J. Invertebr. Pathol., 50:* 106-112; H. de Barjac et al., 1988, *I Ann Microbiol. [Inst. Pasteur], 139:* 363-377; H. L. Lee, 1988, *Mosq. Borne Dis. Bull., 5:* 39-47; A. H. Gharib et al., 1989, *J. Invertebr. Pathol., 54:* 57-62; A. M. Manonmani et al., 1991, *Indian J. Med. Res. [A], 93:* 111-114; S. Orduz et al., 1992, *J. Invertebr. Pathol., 60:* 107-108; R. G. M. Schenkel et al., 1992, *J. Invertebr. Pathol., 60:* 10-14; I. Thiery et al., 1992, *Appl. Microbiol. Biotechnol., 37:* 718-722).

During a systematic survey conducted between 1988 and 1990 in Colombia to detect mosquito larvae active bacteria, we sampled 134 mosquito breeding places. In this study we report 13 strains of *B. sphaericus* from three localities (Guajira, El Valle and Montelibano) that showed pathogenicity against *Culex quinquefasciatus* Say larvae.

The main sampling area covered the north and west parts of Colombia. The ecological habitats of the sampled areas ranged from the semidesertic areas in the north with an annual rainfall not higher than 400 mm to the tropical rain forest in the west with an annual rainfall of 7000 mm.

Mud or soil samples from the mosquito breeding sites were taken as the possible source of bacteria. Samples were collected in sterile tubes and kept sealed at 4 °C until the screening procedure was initiated. All the isolation procedures were performed under strict aseptic conditions. Strains 2362 and 1593 of *B. sphaericus* were obtained from Dr A. A. Yousten (Virginia Polytechnic Institute and State University, Blacksburg, VA).

The *B. sphaericus* Colombian strains were cultured in an environmental shaker in tubes containing 5 ml of MBS (A. Kalfon et al., 1983, *Europ. J. Appl. Microbiol. Biotechnol., 18:* 168-173), and after 72 hr of incubation at 30 °C and 150 rpm, the screening procedure was initiated. Isolates showing 100% mortality after 48 hr as well as standard strains were grown for 72 hr under continuos shaking (250 rpm, 30 °C), and centrifugated at 9000 rpm at 4 °C, and pellets of the final whole cultures were lyophilized and stored at 4 °C. *Culex quinquefasciatus* was collected in the vicinity of Medellin, Colombia, and are maintained under a 12:12 (light:dark) photoperiod. Bioassays were performed with third instar *C. quinquefasciatus* larvae (S. Orduz et al., *loc. cit.*). The half letal concentration (LC_{50}) was determinated with a computer program designed by E. Frachon (Institut Pasteur, Paris). Spore count was also performed by plating two dilutions with two replicate plates for each dilution.

Biochemical characterization and antibiotic susceptibility of the Colombian and reference strains (1593 and 2362) was performed as described by S. Orduz et al., (*loc. cit.*). All tests were run using strains 1593 and 2362 of

B. sphaericus as standards. All strains reported here had the typical *B. sphaericus* appearance, with terminal and round to ellipsoidal spores, and swollen sporangia; diameter in all the isolates was less than 1 μm. All strains reported here belong to serotype H5 (H. de Barjac, pers. commun., Institut Pasteur, Paris) and to phage group 3, but strains 143-1110 and 143-1115 of El Valle group that did not respond to any of the bacteriophages and thus could not be tyed (A. A. Younsten, pers. commun. Virginia Polythecnic Institute and State University).

B. sphaericus strains from Colombia as well as the control strains were unable to use any of the carbohydrates tested as it is characteristic to *B. sphaericus* (H. de Barjac et al., (*loc. cit.; B. L. Russel et al., 1989, Appl. Environm. Microbiol., 55:* 294-297). Utilization of citrate, lysine, ornithine, were also negative in all the strains as well as production of H_2S, lecithinase, and amylase. Production of urease, catalase, oxidase, DNase, and alfa hemolysis on sheep blood agar were positive in all the strains tested (data not shown).

The Guajira group of strains and the Montelibano strain had a few differences with the El Valle group of strains. Production of gelatinase

TABLE I

Main biochemical differences between *Bacillus sphaericus* strains 1593, 2362, and thirteen isolates from the Guajira, Montelibano, and El Valle areas in Colombia

CIB[a] number	Gelatinase	Casein hydrolisis	Growth at pH 5.7	Growth in NaCl 7%
Guajira group				
21-0106	+	–	+	+
10-0413	+	w	–	+
14-0420	+	+	–	–
15-0237	+	w	–	+
El Valle group				
143-1108	–	–	+	+
143-1110	–	–	+	+
143-1115	–	+	+	+
143-1120	–	+	+	+
143-1122	–	–	+	+
143-1123	–	–	+	+
143-1310	–	–	+	+
143-1313	–	–	+	+
Montelibano				
82-0608	–	–	+	+
Control strains				
2362	+	–	+	–
1593	+	–	+	+

a: Corporacion para Investigaciones Biologicas, Colombia.
w: weak reaction.

TABLE II

Forty eight four larvicidal activity of *Bacillus sphaericus* strains 2362, 1593 and 13 Colombian isolates on third instar *Cules quinquefasciatus* larvae[a]

CIB isolate	Spores/mg of powder $(x10^6)$	LC_{50} $(x10^{-4}$ mg/100 ml)	95% Confidence interval $(x10^{-4}$ mg/100 ml	Regression equation	Slope
Guajira group					
21-0106	2.27	9.04	7.88-10.46	Y = 3.38 + 0.03x	1.25
10-0413	1.77	9.06	7.10-10.59	Y = 3.52 + 0.03x	1.16
14-0420	1.96	9.89	8.45-11.71	Y = 3.15 + 0.03x	1.12
15-0237	1.35	11.11	9.45-13.24	Y = 2.69 + 0.03x	1.11
El Valle group					
143-1108	3.98	4.92	4.17-5.77	Y = 4.69 + 0.06x	1.10
143-1110	0.56	62.56	57.77-67.39	Y = -3.6 + 0.23x	1.47
143-1115	0.47	58.15	49.42-67.52	Y = 6.22 + 0.05x	1.11
143-1120	0.83	34.50	29.11-40.70	Y = 2.76 + 0.16x	1.07
143-1122	8.86	18.01	15.32-21.26	Y = 3.13 + 0.02x	1.08
143-1123	4.90	56.52	46.49-68.89	Y = 4.28 + 0.05x	1.05
143-1310	13.68	3.09	2.58-3.68	Y = 6.82 + 0.05x	1.06
143-1313	7.15	39.09	34.10-44.69	Y = 0.13 + 0.22x	1.11
Montelibano					
82-0608	4.97	24.12	20.17-28.96	Y = 2.26 + 0.02x	1.06
Control strains					
2362	4.25	8.62	7.19-10.39	Y = 2.01 + 0.001x	1.07
1593	5.30	10.28	8.81-12.12	Y = 3.04 + 0.06x	1.12

a: six to eight concentrations each replicated three times (20 larvae/replicate) and performed in two different days. LC_{50} calculated from lyophilized powder preparations produced from 72-hr whole culture grown in MBS.

was observed in the strains belonging to the Guajira group and in the strains 2362, and 1593, while it was not detected in any of El Valle and Montelibano. Growth in NaCl 7% was positive in all the isolates of the El Valle and Montelibano groups and strain 1593, as well as all but the isolate 14-0420 of the Guajira group and strain 2362. Within the Guajira group, growth under this condition was not observed in the isolate 10-0413 as was the case with strain 2362. Casein hydrolysis was variable in the *B. sphaericus* Colombian groups. Strains 14-0420, 143-1115, and 143-1120 were positive, while isolates 21-0106, 82-0608, and the rest from the El Valle were negative, and isolates 10-0413, and 15-0237 were weakly positive (Table I).

Spore content of the *B. sphaericus* lyophilized powder preparations ranged from 0.47 x 10^6 to 13.68 x 10^6 spores/mg. Half lethal concentration was very similar within the isolates of the Guajira group, ranging from 9.04 x 10^{-4} to 11.11 x 10^{-4} mg/100 ml, whereas the Montelibano isolate had a LC_{50} of 24.12 x 10^{-4} mg/100 ml. In the El Valle group, toxicity within the isolates showed greater variation than in the Guajira group. The most active isolates

from El Valle (143-1108 and 143-1310) had LC_{50},s of 4.92 x 10^{-4} and 3.09 x 10^{-4} mg/100 ml respectively. Other isolates had LC_{50},s ranging between 18.01 x 10^{-4} and 62.56 x 10^{-4} mg/100 ml. Toxicity of standard strains 2362 and 1593 is also shown in Table II.

Spore-crystal complex was collected by centrifugation of the final whole cultures and toxins solubilized as described by H. de Barjac et al. (*loc. cit.*) protein analysis of the alkali-solubilized toxins was performed by electyrophoresis on a 10% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) according to U.K. Laemmli, 1970, *Nature (London) 227:* 680-685. The results of this analysis revealed similar protein patterns between the Colombian strains and the 2362 and 1593 (data not shown). The major components of these alkali extracts are proteins of 53 and 42 kDa. Other proteins with higher and lower bands were also present in all the strains.

Although the 13 strains of *B. sphaericus* presented in this study came from very different ecological environments, they seem to be a homogeneous group regarding their phenotypic characteristics and posses a wide degree

of variation in the insecticidal properties. These findings and other recently reported, confirm the cosmopolitan distribution of this pathogenic species and indicate that strains of *B. sphaericus* can be recovered from biotic and abiotic sources in many geographical and ecological locations (O. Lysenko et al.; R. Gai-Xin et al.; M. Brown-bridge & J. Margalit; H. de Barjac et al.; H. L. Lee; A. H. Gharib et al.; A. M. Manonmani et al.; S. Orduz et al.; R. G. M. Schenkel et al., all authors).

In the Colombian strains, susceptibility to trimethoprim sulfamethoxazole, cephalotin, cephoxitin, cephoperazone, and piperaciline was observed. In all the CIB isolated strains from the mud samples and strains 2362 and 1593 natural resistance to nalidixic acid was observed. Natural resistance of *B. sphaericus* to streptomycin, chloramphenicol and tetracycline has been previously reported (W. Burke & K. McDonald, 1983, *Curr. Microbiol., 9:* 69-72; J. Polack & R. P. Novick, 1982, *Plasmid, 7:* 152-162). Further studies of this finding could permit the possibility of using the resistance to nalidixic acid as genetic marker or as a mean to retrieve the bacterium from the environment as streptomycin resistance was used by A. A. Yousten et al., (1985, *Appl. Environ. Microbiol., 49:* 1532-1533).

LC_{50} determined from the lyophilized powder preparations of the CIB isolates indicate that strain 143-1310 has equivalent toxicity to the strains M2-1 and M7-1 reported by H. L. Lee (*loc. cit.*), when tested against the same instar *Cx. quinquefasciatus* larvae.

This Colombian strain produced higher toxicity in *Cx. quinquefasciatus* larvae when compared to the acetone powders of the strains TS-1 (8.9 x 10^{-4} mg/100 ml), Ghar. 2&20 (0.162 mg/100 ml), and 2615 (4.32 x 10^{-4} mg/100 ml), (R. Gai-Xin et al.; A. H. Gharib et al.; M. Brown-bridge & J. Margalit all, *loc. cit.*) for third and fourth instar *Cx. pipiens,* and strain 143-1310 from El Valle group has higher potency than the Nigerian isolates recently reported by Orduz et al. (*loc. cit.*) and I. Thiery et al., (*loc. cit.*).

Parasporal crystal production is related to the sporulation (A. Kalfon et al., 1983, *Europ. J. Appl. Microbiol. Biotechnol., 18:* 168-173; A. A. Yousten A. A. & E. W. Davidson, 1982, *Appl. Environ. Microbiol., 44:* 1449-1455); however, we were not able to correlate spore production with the degree of toxicity displayed against *Cx. quinquefasciatus* larvae. The most potent strain, 143-1310 had the higher spore yield; strain 143-1123 with spore yields similar to those of the reference strains, had five times lower toxicity, and strains with half the spore yields of the reference strains had similar toxicity; this indicates that differences in toxicity could be due to the amount of proteins deposited in the parasporal crystals or to a variation in the amino acid composition of their active fragments.