FIVE NEW MOSQUITO LARVICIDAL STRAINS OF *BACILLUS SPHAERICUS* FROM NON-MOSQUITO ORIGINS

OLEG LYSENKO[1], ELIZABETH W. DAVIDSON[2], LAWRENCE A. LACEY[3] AND ALLEN A. YOUSTEN[4]

ABSTRACT. Five new strains of *Bacillus sphaericus* having larvicidal activity similar to that of strains 1593 and 2362 are described. These strains were isolated from caterpillars or grasshoppers, but have no insecticidal activity toward these insects.

INTRODUCTION

Approximately 40 strains of *Bacillus sphaericus* are currently known to have insecticidal activity for mosquito larvae (Yousten 1984a, 1984b). With one exception, these strains have all been isolated from mosquitoes or mosquito habitats; the exceptional strain 2362 was isolated from a black fly (Weiser 1984). None of the *B. sphaericus* strains obtained from culture collections and originating from non-insect sources were found to be insecticidal (Krych et al. 1980). The insecticidal and noninsecticidal *B. sphaericus* strains have been classified by flagellar (H-) antigen serotyping and by lytic bacteriophage typing (Yousten et al. 1980, de Barjac et al. 1980). These two methods of classification produce the same groups of strains, and strains classified together within a phage- or serovar appear to be similar in insecticidal activity as well (op cit, Yousten 1984b). The highly insecticidal strains of *B. sphaericus* including those in phage group 3 (serovar H5a5b) and phage group 4 (serovar H25) all produce parasporal crystals accompanying the spore (Davidson and Myers 1981). The crystals of strains 1593 and 2297 have recently been shown to be highly insecticidal to mosquito larvae (Yousten and Davidson 1982, Kalfon et al. 1984, Payne and Davidson 1984).

During an examination of insect specimens and isolated strains sent for routine diagnostic service in the Culture Collection of Entomogenous Bacteria (CCEB, Prague, Czechoslovakia), one of the authors (OL) identified five strains of *B. sphaericus*. These strains were found to produce microscopically-visible inclusions accompanying the spore, and to have insecticidal activity for *Culex quinquefasciatus* Say larvae in preliminary assays. These strains originated from insects other than mosquitoes (Table 1). We report assays of these strains against mosquito larvae, lepidopteran larvae and grasshoppers.

METHODS AND MATERIALS

Strains are designated according to accession numbers assigned in the World Health Organization Collaborating Center for Biological Control, The Ohio State University, USA.

The sensitivity of each strain to a series of lytic bacteriophages was determined as previously described (Yousten et al. 1980).

Two 125-ml Erlenmeyer flasks containing 25 ml nutrient broth-yeast extract-salts medium (NYSM) (Myers and Yousten 1978) were each inoculated with a loopful of growth from a slant culture. Cultures were incubated at 28°C and 150 rpm for 48 hr on an orbital water bath shaker. Cells were harvested by centrifugation, washed once in 0.9% saline, and frozen in 5 ml of saline. For bioassays, spores were thawed and sonicated on ice with one or two brief pulsed bursts to disrupt clumps of spores. Spore suspensions were diluted in saline and plated on NYSM agar to provide an estimate of viable colony forming units. Spore suspensions were bioassayed against 48-hr old second instar *Cx. quinquefasciatus* and *Aedes aegypti* (Linn.) larvae using procedures prescribed in Lacey (1984). Briefly, these procedures consisted of introducing 20 larvae into each 125 ml plastic container containing 100 ml bacterial suspension diluted in deionized water. Three containers/bacterial concentration and 5–7 concentrations of each isolate were used in tests conducted against *Cx. quinquefasciatus*. Four to six replicate tests were run on each isolate on separate dates. Only one test of two high concentrations of each isolate was run against *Ae. aegypti* larvae. Fifty mg of debittered brewer's yeast was added to each cup at initiation of assay. Survivors were counted 48 hr later.

Spore suspensions were assayed using larvae of the cabbage looper, *Trichoplusia ni* (Hbn). Cubes of diet ca. 1 mm^3 (prepared without preservatives) were dipped into the spore suspensions, placed into jelly cups, and one first-instar *T. ni* larva was placed on the surface of

[1] Deceased. Department of Insect Pathology, Institute of Entomology, Czechoslovak Academy of Sciences, 16609 Prague 6, Czechoslovakia.

[2] Department of Zoology, Arizona State University, Tempe, AZ 85287; address for correspondence.

[3] USDA-ARS, P.O. Box 14565, Gainesville, FL 32604.

[4] Microbiology Section, Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

Table 1. Origin of *Bacillus sphaericus* strains.

WHO/CCBC No.	OL[1] accession number	Geographic origin	Original host	Contributor
2532-2	OL324-62	Guyana	*Diatrea saccharalis* (Lepidoptera)	A. J. Rajindera
2533-1 (K1)	OL324-K1	Guyana	*Castria daedalus* (Lepidoptera)	A. J. Rajindera
2533-1 (K2)	OL324-K2	Oligosporogenous mutant of K1		
2601	OL325-1	Budapest, Hungary	grasshopper	L. Szalay-Marso
2602	OL326-21-1	Prague, Czechoslovakia	*Mammestra brassicae* (Lepidoptera)	J. Weiser

[1] Oleg Lysenko Culture Collection, Czechoslovakia Academy of Sciences.

each diet cube. Ten days later, surviving larvae and pupae were counted.

Spore suspensions were also assayed against the grasshopper, *Melanoplus differentialis* (Thos.). Third instar nymphs were individually caged in shell vials, starved for 24 hr, and then fed 5 μl of spore suspension on a 7 mm lettuce disc. Each suspension was fed to 20 nymphs. The nymphs were reared in groups of 5 per rearing tube for 15 days after inoculation (Henry et al. 1979).

RESULTS AND DISCUSSION

All strains were found to express a pattern of sensitivity to lytic phages identical to strains in phage group 3. This phage group corresponds to serovar H5a5b, which also includes strains 1593 and 2362. Several strains in phage group 3 are highly insecticidal for *Cx. quinquefasciatus* larvae (Yousten 1984a, 1984b).

Cultures of all strains except 2533-1(K2) were at least 90% sporulated after 48 hr incubation, according to microscopic observation. Strain 2533-1(K2) is an oligosporogenous mutant of 2533-1(K1). The former strain produced few spores; however, these spores were accompanied by small crystals. Strains 2532-2 and 2601 produced small crystals although not every spore was accompanied by a crystal. All spores produced by the remaining strains were accompanied by medium to large crystals.

Insecticidal activity of broth cultures of the 5 strains is given in Table 2. Strain 2532-2 was less insecticidal than the other strains; the remaining strains were about equally insecticidal, and their activity is similar to that observed for strains 1593 and 2362, i.e. LC_{50} = ca. 100–1000 spores/ml. In bioassays against *Ae. aegypti* larvae, no strain produced 100% mortality even at 10^{-2} dilution of the original spore suspension.

No mortality was observed among *T. ni* caterpillars fed high dosages of spores of these *B. sphaericus* strains. Likewise no mortality was observed among the grasshoppers, *M. differentialis* after *per os* inoculation with the bacterial suspensions.

The five strains of *B. sphaericus* described here are the first highly mosquito-larvicidal strains to be isolated from sources other than mosquitoes, mosquito habitats or aquatic insects. Although these *B. sphaericus* strains were originally isolated from lepidopteran larvae or from grasshoppers, they apparently are not significantly pathogenic for these insects, assuming the species chosen for our assays are representative. *Trichoplusia ni,* for example, is highly sensitive to *B. thuringiensis* (Dulmage and Cooperators 1981). *Bacillus sphaericus* may have only an opportunistic relationship with these insects, or may act as a secondary pathogen (Lysenko 1981). Similarly, strain 2362 is not pathogenic for black flies, even though it was isolated from these insects (Weiser 1984). However, all larvicidal strains of *B. sphaericus* identified to date have been associated with insects. Phage typing provided an accurate prediction of insecticidal activity of these strains. The sensitivity of *Cx. quinquefasciatus* and insensitivity of *Ae. aegypti* to these strains was similar to the

Table 2. Activity of liquid cultures of *Bacillus sphaericus* to *Culex quinquefasciatus* larvae.

Culture	Cfu/ml[1]	Mortality (%) at: 10^{-5}	10^{-6}	10^{-7}
2532-2	4.6×10^9	78–100[d]	3–42[e]	0[a]
2533-1 (K1)	2.6×10^9	100[a]	48–72[d]	1.7[a]
2533-1 (K2)	3.3×10^9	100[a]	78–93[e]	2–18[c]
2601	3.6×10^9	100[a]	22–92[c]	0–5[b]
2602	1.7×10^9	100[a]	75–95[e]	7–15[e]

[1] Colony forming units/ml of final whole culture.
[a] 1 replicate.
[b] 3 replicates.
[c] 4 replicates.
[d] 5 replicates.
[e] 6 replicates.

activity of other strains of *B. sphaericus* in the same phage group. Each insecticidal strain bore a crystal; the least insecticidal strain (2532-2) produced the smallest crystals in the lowest numbers.

The results presented confirm that highly insecticidal strains for mosquito larvae may be found associated with insects in habitats other than the aquatic habitat. We urge that any *B. sphaericus* isolates found associated with insects be submitted for phage- or serotyping and determination of mosquito larvicidal activity. Isolates may be submitted to Dr. Yousten (address above) for phage typing, or to Dr. H. deBarjac, Institut Pasteur, Paris for serotyping.

ACKNOWLEDGMENTS

Assays against grasshoppers were performed by Dr. John Henry, USDA-ARS, Rangeland Insects Laboratory, Bozeman, MT. This research was supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

References Cited

Barjac, H. de, M. Vernon and V. Cosmao Dumanoir. 1980. Caracterisation biochimique et serologique de souches de *Bacillus sphaericus* pathogenes ou non pour les moustiques. Ann. Microbiol. (Inst. Pasteur) 131B:191–201.

Davidson, E. W. and P. Myers. 1981. Parasporal inclusions in *Bacillus sphaericus*. FEMS Microbiol. Lett. 10:261–265.

Dulmage, H. T. and Cooperators. 1981. Insecticidal activity of isolates of *Bacillus thuringiensis* and their potential for pest control, pp. 193–222. *In* H.D. Burges (ed.), Microbial control of pests and plant diseases 1970–1980. Academic Press, London.

Henry, J. E., E. A. Oma, J. A. Onsager and S. W. Oldacre. 1979. Infection of the corn earworm, *Heliothis zea*, with *Nosema acridophagus* and *Nosema cuneatum* from grasshoppers: Relative virulence and production of spores. J. Invertebr. Pathol. 34:125–132.

Kalfon, A., J.-F. Charles, C. Bourgouin and H. de Barjac. 1984. Sporulation of *Bacillus sphaericus* 2297: An electron microscope study of crystal-like inclusion biogenesis and toxicity to mosquito larvae. J. Gen. Microbiol. 130:893–900.

Krych, V. K., J. L. Johnson and A. A. Yousten. 1980. Deoxyribonucleic acid homologies among strains of *Bacillus sphaericus*. Int. J. Syst. Bacteriol. 30:476–484.

Lacey, L. A. 1984. Production and formulation of *Bacillus sphaericus*. Mosq. News 44:153–159.

Lysenko, O. 1981. Principles of pathogenesis of insect bacterial diseases as exemplified by the nonsporeforming bacteria, pp. 163–188, *in* E. W. Davidson (ed.), Pathogenesis of invertebrate microbial diseases. Allanheld, Osmun, Totowa, New Jersey.

Myers, P. and A. Yousten. 1978. Toxic activity of *Bacillus sphaericus* SSII-1 for mosquito larvae. Infect. Immun. 19:1047–1053.

Payne, J. M. and E. W. Davidson. 1984. Insecticidal activity of the crystalline parasporal inclusions and other components of the *Bacillus sphaericus* 1593 spore complex. J. Invertebr. Pathol. 43:383–388.

Weiser, J. 1984. A mosquito-virulent *Bacillus sphaericus* in adult *Simulium damnosum* from Northern Nigeria. Zbl. Mikrobiol. 139:57–60.

Yousten, A. A. 1984a. Bacteriophage typing of mosquito pathogenic strains of *Bacillus sphaericus*. J. Invertebr. Pathol. 43:124–125.

Yousten, A. A. 1984b. *Bacillus sphaericus:* Microbiological factors related to its potential as a mosquito larvicide. Adv. Biotechnol. Processes 3:315–343.

Yousten, A. A. and E. W. Davidson. 1982. Ultrastructural analysis of spores and parasporal crystals formed by *Bacillus sphaericus* 2297. Appl. Environ. Microbiol. 44:1449–1455.

Yousten, A. A., H.de Barjac, J. Hedrick, V. Cosmao Dumanoir and P. Myers. 1980. Comparison between bacteriophage typing and serotyping for the differentiation of *Bacillus sphaericus* strains. Ann. Microbiol. (Inst. Pasteur) 131B:297–308.