Journal of Industrial Microbiology, 1 (1986) 245–250
Elsevier

SIM 00033



# Isolation and description of antimicrobic-resistant (marker) strains of select insecticidal and noninsecticidal varieties of *Bacillus sphaericus*

Marcia A. Yurks and Samuel Singer*

*Department of Biological Sciences, Western Illinois University, Macomb, IL 61455, U.S.A.*

Received 23 April 1986
Revised 27 June 1986
Accepted 14 July 1986

*Key words:* Antibiotic-resistance; *Bacillus sphaericus*; Insecticidal strain

## SUMMARY

Single and multiple antibiotic-resistant strains were isolated from four insecticidal and two noninsecticidal parent strains of *Bacillus sphaericus*. Several of the single resistant strains isolated were also cross-resistant to several antibiotics of different modes of action. All of the parent strains were naturally susceptible to 11 and naturally resistant to two out of 22 antimicrobics examined. The majority, 17 out of 23, of antibiotic-resistant strains, that were isolated from the insecticidal parent strains, retained their insecticidal activity while the noninsecticidal strains remained noninsecticidal. Among the isolates were several strains that would be potentially useful as marker strains in the genetic manipulation of the insecticidal strains.

## INTRODUCTION

*Bacillus sphaericus* strains 1593, 2297 and 2362, with insecticidal activity against mosquito larvae, have been useful both as field candidates [17,22,26,27] and material for analysis of toxin production [5,7–9]. To date very little information is available about the genetics of *B. sphaericus* [4]. None of the classic modes of genetic transfer (transduction, conjugation and transformation) has been demonstrated in *B. sphaericus* [27], although protoplast plasmid transformation has been reported [19]. Some recent work has been in the direction of using recombinant DNA technology to exploit further the potential of these strains [1,11,19]. Availability of marker strains particularly in terms of antimicrobic-resistant strains, still maintaining larvicidal activity, would be most useful. The purpose of this study was to isolate, from natural populations of *B. sphaericus*, strains resistant to a series of antibiotics for use as marker strains for future genetic manipulation and physiological studies. Although some information on the antimicrobic susceptibility is available [6,14], a more complete examination of these candidates would be of value not only to obtain marker strains but for systematics and environmental considerations.

* To whom correspondence should be addressed.

## MATERIALS AND METHODS

*Bacteria*

The following noninsecticidal (14577, derived from ATCC No. 14577; 7054, derived from ATCC No. 7054) and insecticidal (SSII-1, WHO/CCBC No. 1321; 2297, WHO/CCBC No. 2297; 2362, WHO/CCBC No. 2362; 1593, WHO/CCBC No. 1593) strains of *B. sphaericus* were used [21].

*Media and growth conditions*

The primary medium used was nutrient agar (Difco) supplemented with salts ($5 \cdot 10^{-5}$ M $MnCl_2$; $7 \cdot 10^{-4}$ M $CaCl_2$; $10^{-3}$ M $MgCl_2$) and vitamins: calcium pantothenate, thiamine hydrochloride, nicotinic acid at 10 g/l and biotin at 1 g/l. Brain heart infusion (Difco) supplemented with vitamins was also used in preliminary experiments. Modified nutrient agar was chosen for experiments because the greatest sensitivity was shown in this medium. For example, using brain heart infusion medium there was a lack of sensitivity not only to streptomycin, nalidixic acid and lincomycin, but also to colistin, bacitracin, trimethoprim and sulfadiazine. All of the strains were maintained on the modified nutrient agar. The appropriately filter-sterilized antibiotic was added aseptically to the medium prior to use. Cultures were grown in accordance with a standardized inoculum buildup procedure described by Singer [22]. Material from the inoculum buildup production flask was used for larval bioassays. For the disc-diffusion and MIC tests, inoculum was taken from a 3 h old seed flask.

*Mosquito bioassay*

The standard mosquito bioassay [22] was performed. The higher the negative number of the $LC_{50}$ value, the greater the insecticidal activity. As discussed elsewhere [23] when dealing with broth cultures, larvicidal activity measured in terms of colony forming units is unsatisfactory since it has been shown that nonviable cells may be as insecticidal as viable cells [23]. As a result, the log (dose) of the final whole culture of a carefully standardized inoculum buildup and fermentation is the more appropriate approach to be used. When testing antibiotic-resistant strains grown in the presence of an antibiotic, a control test using decimal dilutions of the antibiotic solution was performed to ensure that the antibiotic did not kill the larvae.

*Antibiotics*

Bacto-sensitivity discs (6 mm diameter, Dispens-O-Discs, DIFCO) were used initially to test for antibiotic response. These discs included: ampicillin (AM), 10 μg; bacitracin (B), 10 U; cephalothin (CR), 30 μg; chloramphenical (C), 30 μg; chlortetracycline (A), 30 μg; colistin (CL), 10 μg; erythromycin (E), 15 μg; kanamycin (K), 30 μg; lincomycin (L), 2 μg; nalidixic acid, 30 μg; neomycin (N), 30 μg; nitrofurantoin (FD), 300 μg; novobiocin (NB), 30 μg; oxytetracycline (T), 30 μg; penicillin G(P), 10 U; polymyxin B (PB), 300 U; rifampin (RA), 5 μg; streptomycin (S), 10 μg; sulfadiazine (SD), 300 μg; tetracycline (TE), 30 μg; trimethoprim (TMP), 5 μg; and vancomycin (VA), 30 μg. Ampicillin, chloramphenicol, erythromycin, kanamycin and vancomycin powders were obtained from Sigma. Rifampin powder was obtained from Calbiochem. These powders were used for gradient plates, fermentation procedures and bioassays. Antibiotic solutions used for incorporation into media were prepared according to Barry [3], filter-sterilized and used immediately or frozen at −20°C until used.

*Determination of antimicrobic susceptibility*

Strains of *B. sphaericus* were tested for antibiotic susceptibility according to the standard method currently recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [20] with the following variations. Modified nutrient agar was used (instead of Mueller-Hinton agar) due to the growth needs of the bacilli. Also, instead of transferring 4 – 5 colonies from a plate into Tryptic Soy Broth, inoculum for a test was taken from a 3 h seed flask and adjusted to the NCCLS turbidity standard with a spectrophotometer (Spec. 20).

The minimum inhibitory concentration tests were done according to Finefold et al. [10].

*Gradient plates*

Gradient plates were made according to Szybalski and Bryson [25].

## RESULTS

Insecticidal strains of *B. sphaericus* are naturally resistant to streptomycin, nalidixic acid and lincomycin when grown on modified nutrient agar. Non-insecticidal strains of *B. sphaericus* were resistant to these three antibiotics, plus novobiocin. When grown on modified nutrient agar, all strains tested were naturally susceptible to penicillin G, tetracycline (including oxytetracycline and chlortetracycline), erythromycin, neomycin, cephalothin, vancomycin, ampicillin, kanamycin and rifampin, as well as showing intermediate susceptibility to chloramphenicol (with strains 2297, SSII-1 and 14577 being fully susceptible), bacitracin, colistan (with strain 7054 being resistant) and polymyxin B. The strains were all naturally susceptible to nitrofurantoin, as well as showing partial zones of inhibition to trimethoprin, and sulfadiazine.

Minimum inhibitory concentrations of the antibiotics used in the selection of the resistant isolates for the parents were determined (Table 1). All of the MIC values for the specific antibiotic for the stable resistant isolates were 100 or greater.

Sixty resistant strains were initially isolated; of these 36 were isolated in terms of single resistance (RA, C, E, K. AM, or VA for each of the six *B. sphaericus* parents), 12 strains isolated in terms of double resistance (RA + E (RA/E), C + K (C/K) for each of the six *B. sphaericus* parents) and 12 isolated in terms of triple resistance (RA + E + VA (/RA/E/VA), C + K + AM (/C/K/AM) of each of the six *B. sphaericus* parents). Thirty-four of these 60 strains were found to be stable; there was no loss of antibiotic resistance after being transferrred at least eight times slant-to-slant in the presence of at least 50 μg/ml of the antibiotic followed by being transferred at least three times in the absence of the antibiotics. Twenty-five of these isolates, 24 isolates of the six parents singly resistant to RA, C, E, or K, as well as strain 2297/AM, were stable; nine isolates, six isolates of the six parents resistant to RA + E, plus three isolates strains 2362, SSII-1, and 7054 resistant to C + K, were stable. None of the triple-resistant isolates was stable. In addition to these double-resistant strains several of the strains isolated in terms of a single antibiotic were also found to be multiresistant (Table 2). With the exception of strain SSII-1/K, all of the strains resistant to kanamycin were also resistant to neomycin or showed a marked reduction in susceptibility to neomycin.

Mosquito larval bioassays were conducted on parent strains and antibiotic-resistant strains. The higher the negative number of the $LC_{50}$ value, the greater the insecticidal activity (Table 3). Seventeen out of 23 antibiotic-resistant strains that were isolated from the insecticidal parent strains retained their insecticidal activity, while the 11 noninsecticidal strains remained noninsecticidal (Table 3).

Table 1

Minimum inhibitory concentration (MIC) for parent strains of antibiotics used in the selection of isolates

| Antibiotic | Parent strains | | | | | |
|---|---|---|---|---|---|---|
| | 14577 | 7054 | 1593 | SSII-1 | 2297 | 2362 |
| Rifampin (RA) | 0.39* | 12.50 | 25.00 | 0.39 | 25.00 | 0.39 |
| Erythromycin (E) | 1.56 | 12.50 | 0.78 | 0.39 | 0.78 | 0.78 |
| Chloramphenicol (C) | 3.125 | 6.25 | 0.78 | 6.25 | 6.25 | 12.50 |
| Kanamycin (K) | 1.56 | 1.56 | 1.56 | 0.39 | 0.78 | 1.56 |
| Ampicillin (AM) | 1.56 | 3.125 | 0.78 | 0.39 | 3.125 | 0.78 |
| Vancomycin (VA) | 1.56 | 3.125 | 6.25 | 0.78 | 0.78 | 1.56 |

* μg/ml of antibiotic.

Table 2

Summary of available stable multiple-resistant strains of *B. sphaericus*

| Strain | Antibiotic resistance |
|---|---|
| 14577/K | K, N, VA, CL |
| 7054/RA | RA, E |
| 1593/RA | RA, E |
| 2362/E | E, AM, CR, C, K, T, TE |
| 2295/AM | AM, CR, CL, N, SD |
| 14577/RA/E* | RA, E |
| 7054/RA/E | RA, E |
| 7054/C/K | C, K, N |
| 1593/RA/E | RA, E |
| SSII-1/RA/E | RA, E |
| SSII-1/C/K | C, K, N |
| 2362/RA/E | RA, E |
| 2362/C/K | C, K, N |
| 2297/RA/E | RA, E |

* Order of resistance - - RA/E (rifampin/erythromycin) means that isolated strain was initially resistant to RA then to E. Antibiotic code: ampicillin (AM); cephalothin (CR); chloramphenicol (C); colistin (CL); erythromycin (E); kanamycin (K); neomycin (N); oxytetracycline (T); rifampin (RA); sulfadiazine (SD); tetracycline (TE); vancomycin (VA).

## DISCUSSION

*B. sphaericus* strains have been divided into bacteriophage groups [28] as well as into DNA homology groups [16]. No apparent relationship existed between phage or DNA homology grouping (Table 1) and antibiotic susceptibility of the parent strains for the six antibiotics, used for the isolation of the resistant strains. For example, insecticidal strains 1593 and 2362 both belonging to phage group 3, showed a 60-fold difference in rifampin MIC values, while strain 2297 (phage group 4) showed equivalent rifampin MIC values to that of strain 1593. Similarly, strain SSII-1 (phage group 2) had a rifampin MIC value equivalent to that of strain 2362. Susceptibility aside, what is important is that the natural parent populations are not equivalent in antibiotic response and each should therefore be a good source for derived marker strains. This, indeed, was the case, since an assortment of single- and multiple-resistant strains were isolated with interesting combinations of antibiotic susceptibility and larvicidal activity.

Choosing a sufficiently large natural population,

Table 3

$LC_{50}$ values for sensitive and antibiotic-resistant *B. sphaericus* strains

| Strain subisolate | Strain | | | | | |
|---|---|---|---|---|---|---|
| | 1593 | 2362 | SSII-1 | 2297 | 14577 | 7054 |
| Original parent | −3.87* | −4.50 | −5.88 | −5.50 | NI | NI |
| /RA | NI | −6.43 | −3.72 | −3.65 | NI | NI |
| /C | −4.98 | −5.09 | −3.89 | −5.78 | NI | NI |
| /E | NI | NI | NI | −3.87 | NI | NI |
| /K | −4.20 | −3.87 | −6.00 | −4.13 | NI | NI |
| /AM | - - | - - | - - | −4.93 | - - | - - |
| /VA | - - | - - | - - | - - | - - | - - |
| /RA/E | NI | −0.73 | NI | −2.63 | NI | NI |
| /C/K | - - | −4.49 | −3.87 | - - | - - | NI |

* Average of duplicates; - -, isolate unstable; $LC_{50}$ = log (dose) of the dilution of the final whole culture from a standardized inoculum buildup that kills 50% of the larvae, *Culex quinquefasciatus*. NI, noninsecticidal (after 48 h).

one may expect to have present minor populations resistant to a single antibiotic and, as it turned out, multiple resistance to several antibiotics (Table 2). This preliminary report describes the partial characterization of spontaneous antibiotic-resistant mutants. It holds the prospect of providing antibiotic-resistant raw material from strains of *B. sphaericus* itself. Difficulty in constructing any vector for use in genetic manipulation always involves the question of whether the proper DNA control sequences (information) has been inserted [2]. Material from natural *B. sphaericus* populations should have this information readily available. The remaining questions that need to be addressed are what material do we have and in what direction do we need to go to determine the biochemical mechanism(s) of resistance and the location of the gene(s) conferring added resistance.

The unstable isolates mentioned above had growth or sporulation problems when grown in the presence or the absence of the particular antibiotic. In the case of our efforts to obtain multiple-resistant strains an increased sensitivity to the second or third antibiotic appeared in some cases, to preclude the easy isolation of the desired mutant [12]. Double or triple mutants were isolated but quickly died out on subsequent slant-to-slant transfer. This was particularly the case with vancomycin. According to Lorian [18] rapid and spontaneous back mutation from resistance to vancomycin sensitivity occurred. Hammond and Lambert [13] stated that no substantial degree of resistance to vancomycin has developed, while Jawetz et al. [15] say that vancomycin-resistant strains do not emerge rapidly. Even though these reports referred to a clinical situation, the same may be true for *B. sphaericus*. The only vancomycin-resistant strain isolated was strain 14577/K. Similar difficulties were encountered in attempts to obtain ampicillin-resistant strains. The only strains resistant to AM were strains 2362/E and 2297/AM, both of which exhibited resistance to an array of antibiotics (Table 2). The insecticidal parent population least amenable to the isolation of antibiotic-resistant mutants was strain 1593.

Massive resistance to rifampin may develop as a one-step acquisition. Highly resistant mutants occur in all microbial populations in a frequency of $10^{-7}$ or greater [15]. This seemed to be the case with *B. sphaericus* where we obtained a high degree of resistance quickly. Isolation of resistance to the other antibiotics required a step-wise procedure.

One can easily fall into the trap of comparing differences in larvicidal activity of the antibiotic-resistant strains. It should be noted that modified nutrient medium, used here in order to best express insecticidal activity and response, is not the most ideal fermentation medium for expressing larvicidal activity. Differences in larvicidal activity as great as shown here can be seen when examining the effect of fermentation media on larvicidal activity [21]. What is pertinent is that a majority of the insecticidal resistant strains remained insecticidal. Of the five antibiotic-resistant strains that were originally insecticidal, only one, 2362/E, was fully sporulated. Lack of sporulation per se may not account for lack of insecticidal activity, since both parent strains, 1593 and SSII-1, have been shown to be insecticidal under nonsporulating or low sporulation conditions [21,23]. Loss of larvicidal activity by the five strains therefore may be the result of the selection of resistance to the antibiotic(s) which interfered with the isolate's ability to produce toxin.

Of the 25 single antibiotic-resistant strains isolated, five strains displayed multiresistance to an array of antibiotics in addition to the target antibiotic (Table 2). This would imply the presence of multi-resistant plasmids. The plasmid DNA complement of *B. sphaericus* has been shown to be quite simple compared to the complex arrays observed in *B. thuringiensis* [24]. Work in our laboratory (to be reported elsewhere) has demonstrated through the examination of plasmid profiles that no new plasmids appear when antibiotic-resistant isolates were obtained from the insecticidal populations. Unless the multiresistance somehow relates to the cryptic plasmids present, it would appear that these multiresistant events involved mutational resistance in the chromosomal DNA. It is difficult to conceive of a singular chromosomal event that would account for the sudden appearance of multiple resistance except possibly the alteration in the surface receptor(s) or loss of capacity for active transport

through the cell membrane. Since the antibiotics involved belong to a variety of modes of action, mutational changes in the chromosome would need to be (selectively?) nonspecific. The other possibility, which is more likely, is that we have yet to identify a multiresistant plasmid.

## REFERENCES


1 Abe, K., R.M. Faust and W.C. Bulla. 1983. Plasmid deoxyribonucleic acid in strains of *Bacillus sphaericus* and in *Bacillus moritai*. J. Invertebr. Pathol. 41: 328–335.

2 Aronson, A.I., W. Beckman and P. Dunn. 1986. *Bacillus thuringiensis* and related insect pathogens. Microbiol. Rev. 50: 1–24.

3 Barry, A.L. 1976. The Antimicrobic Susceptibility Test: Principles and Practices, p. 236, Lea and Febiger, Philadelphia.

4 Baumann, P., B.M. Unterman, L. Baumann, A.H. Groadwell, S.J. Abbene and R.D. Bowditch. 1985. Purification of the larvicidal toxin of *Bacillus sphaericus* and evidence for high-molecular-weight precursors. J. Bacteriol. 163: 738–747.

5 Bourgouin, C.M., R. Tinelli, J.P. Bouvet and R. Pires. 1984. *Bacillus sphaericus*: 1593-4, purification of fractions toxic for mosquito larvae. FEMS Symp. 24: 387–388.

6 Burke, F.W., Jr. and K.O. McDonald. 1983. Naturally occurring antibiotic resistance in *Bacillus sphaericus* and *B. licheniformis*. Curr. Microbiol. 9: 69–72.

7 Davidson, E.W. 1981. Bacterial diseases of insects caused by toxin-producing bacilli other than *Bacillus thuringiensis*. In: Pathogenesis of Invertebrate Microbial Diseases (Davidson, E.W., ed.), pp. 269–291, A-lanheld, Osmun, Totowa, New Jersey.

8 Davidson, E.W. 1982. Purification and properties of soluble cytoplasmic toxin from the mosquito pathogen *Bacillus sphaericus* strain 1593. J. Invertebr. Pathol. 39: 6–9.

9 Davidson, E.W. 1984. Microbiology, pathology and genetics of *Bacillus sphaericus*: Biological aspects which are important to field use. Mosq. News 44: 147–152.

10 Finegold, S.M., W.J. Martin and E.G. Scott. 1978. Bailey and Scott's Diagnostic Microbiology, 404 p., The C.V. Mosby Company, St. Louis.

11 Ganesan, S., H. Kamdar, K. Jayaraman and J. Szulmajster. 1983. Cloning and expression in *Escherichia coli* of a DNA fragment from *Bacillus sphaericus* coding for biocidal activity against mosquito larvae. Mol. Gen. Genet. 189: 181–183.

12 Goldberg, H.S. 1959. Antibiotics, 608 p., D. Van Nostrand Company, Inc., New York.

13 Hammond, S.M. and P.A. Lambert. 1978. Antibiotics and Antimicrobial Action, 63 p., The Camelot Press, Ltd., Southampton.

14 Hertlein, B.C., R. Levy and T.W. Miller, Jr. 1979. Recycling potential and selective retrieval of *Bacillus sphaericus* from soil in a mosquito habitat. J. Invertebr. Pathol. 33: 217–221.

15 Jawetz, E., J.L. Melnick and E.A. Adelberg. 1982. Review of Medical Microbiology, 553 p., Lange Medical Publications, Los Altos, California.

16 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.

17 Lacey, L.A. and A.H. Undeen. 1986. Microbial control of black flies and mosquitoes. Annu. Rev. Entomol. 31: 265–296.

18 Lorian, V. 1980. Effects of subminimum inhibitory concentrations of antibiotics on bacteria. In: Antibiotics in Laboratory Medicine (Lorian, V., ed.), pp. 342–408, Williams and Wilkins, Baltimore.

19 McDonald, K.O. and W.F. Burke, Jr. 1984. Plasmid transformation of *Bacillus sphaericus* 1593. J. Gen. Microbiol. 130: 203–208.

20 National Committee on Clinical Laboratory Standards. 1975. Performance standards for antimicrobial disc susceptibility tests. Approved Standard ASM 2, Villanova, Pennsylvania.

21 Singer, S. 1980. *Bacillus sphaericus* for the control of mosquitoes. Biotechnol. Bioeng. 22: 1335–1355.

22 Singer, S. 1982. The biotechnology for strains of *Bacillus sphaericus* with vector control potential. In: Proceedings IIIrd International Colloquium on Invertebrate Pathology, University of Sussex, Brighton, U.K., pp. 485–489, Society for Invertebrate Pathology.

23 Singer, S. 1985. *Bacillus sphaericus* (Bacteria). In: Biological Control of Mosquitoes (Chapman, H.C., ed.), pp. 123–131, American Mosquito Control Association, Fresno.

24 Singer, S. 1986. Current status of the microbial larvicide *Bacillus sphaericus*. In: Biotechnology Advances in Insect Pathology and Cell Culture (Maramorosch, K., ed.), Academic Press, New York, in the press.

25 Szybalski, W. and V. Bryson. 1952. Genetic studies on microbial cross resistance to toxic agents. J. Bacteriol. 64: 489–499.

26 WHO. 1985. Information consultation on the development of *Bacillus sphaericus* as a microbial larvicide, 24 p., TDR/BCV/SPHAERICUS/85.3. (Mimeo, doc.).

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

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