

POTENTIAL OF THE MOSQUITO PATHOGEN *Bacillus sphaericus*  
FOR RECYCLING AND GENE TRANSFER IN LARVAL CADAVERS

by

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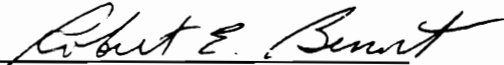
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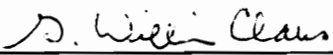
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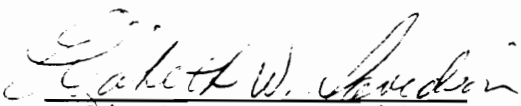
Microbiology


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MICROBIOLOGY

(Abstract)

The ability of spores to germinate, vegetatively multiply and produce new spores and toxin in the larval cadaver is known as recycling. The ability to recycle is an important characteristic since it may enhance effectiveness and persistence of the microbial insecticides in the larval habitat.

The ability of *Bacillus sphaericus* to recycle has only been examined in the low toxicity strain SSII-1 and in the two highly toxic strains 2362 and 1593, both belonging to serotype 5a5ab. This study was expanded and the ability to germinate and recycle of several *B. sphaericus* strains was investigated. Strains tested represented different serological and DNA homology groups, and expressed either or both toxins (the binary toxin, proteins of 51 and 42 kDa or the 100-kDa toxin). Nontoxic strains were also tested as recombinants expressing the toxin genes or with soluble binary toxin.

Results of this study showed that only spores of the highly toxic *B. sphaericus* strains, which normally produce

both, the binary toxin and the 100-Kda toxins, were able to germinate in high percentage and to recycle.

The ability of conjugal transfer of plasmids from *B. sphaericus* to other bacteria was also investigated. The most likely place for conjugation to occur is in the larval cadaver. where spores of *B. sphaericus* germinate and grow vegetatively and interact with bacteria present in the larval cadaver. *Bacillus sphaericus* 2362 carrying the broad host range plasmid pAMB1, was used as donor in filter mating experiments with other *B. sphaericus* strains, a restrictionless *B. subtilis* strain and bacteria isolated from field collected larvae. Conjugal transfer of pAMB1 was observed with strains of the same serotype as the donor and two other serotypes.

The possibility that the large cryptic plasmid present in *B. sphaericus* 2362 (180 kb), could promote its own transfer and mobilize the small nonconjugative plasmids pUB110 to other *B. sphaericus* strains was also tested. No transfer was detected.

Conjugation experiments in vivo (the larval cadaver) were done by feeding *Culex quinquefasciatus* larvae the spores of donor 2362 (pAMB1) along with spores of the recipient strains, 2362a or 1593-P51. No transconjugants were detected in cadavers 72 hours after feeding the spores.

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## TABLE OF CONTENTS

	Page
ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
LIST OF FIGURES	vii
LIST OF TABLES	x
INTRODUCTION	1
LITERATURE REVIEW	4
Bacterial insecticides	4
<i>Bacillus sphaericus</i>	4
Taxonomy	5
The toxins	7
Persistence and recycling	10
Surface layer	13
Spore germination	14
Conjugation	15
Plasmid and gene transfer in <i>B. sphaericus</i>	20
The pAMB1 plasmid	24
Gene transfer by conjugation in <i>B. thuringiensis</i>	25
MATERIALS AND METHODS	32
Bacterial strains	32
Isolation of rifampicin resistant strains	32
Spore suspension stocks	35
Treatment of larvae and recycling determinations	35
Surface contamination of the larvae	36
Alkaline extraction of soluble binary toxin	
from sporulated cultures of <i>B. sphaericus</i>	36
<i>In vitro</i> spore germination experiments	37
Conjugation experiments in broth	38
Filter matings	39
<i>In vivo</i> conjugation in larvae	40
Phage typing	40
Isolation of <i>B. sphaericus</i> auxotrophs	40
Preparation of cell walls and S-layer protein	42
Sodium dodecyl sulfate polyacrylamide	
gel electrophoresis (SDS-PAGE)	43
Plasmid isolation	43
Media formulations	45

RESULTS	47
Spore germination and recycling	47
<i>In vitro</i> spore germination	68
Conjugation	72
<i>In vitro</i> conjugation experiments using plasmid pAMB1	72
Effect of incubation time on conjugation frequencies	77
Effect of incubation temperature on conjugation frequencies	78
Broth matings vs. mating on filter membrane	78
Effect of donor/recipient ratio on conjugation frequency	82
Range of recipient strains	82
Interspecies conjugation: Bti, <i>B. subtilis</i> and <i>B. mycoides</i> as recipients of pAMB1	92
<i>In vitro</i> conjugation experiments to detect mobilization of pUB110	95
Mating experiments with Bti carrying the pBC16 plasmid	102
<i>In vivo</i> conjugation in larval cadavers	102
DISCUSSION	107
Spore germination and recycling	107
Effects of rifampicin resistance on sporulation	117
<i>In vitro</i> spore germination	119
Conjugation	121
CONCLUSIONS	141
Spore germination and recycling	141
Conjugation	142
LITERATURE CITED	143
CURRICULUM VITA	157

## LIST OF FIGURES

Figure		Page
1.	Recycling of <i>B. sphaericus</i> 2362-7 in larvae fed in two different concentrations of spores	49
2.	Recycling of <i>B. sphaericus</i> 2362-7 in larval cadavers	51
3.	Recycling of <i>B. sphaericus</i> IAB 59 in larval cadavers	53
4.	Recycling of <i>B. sphaericus</i> 2297 in larval cadavers	54
5.	Recycling of <i>B. sphaericus</i> LP12AS in larval cadavers	56
6	Recycling of <i>B. sphaericus</i> 1883 in larval cadavers	57
7	Recycling of <i>B. sphaericus</i> 1883 in larval cadavers. Spores were fed along with strain 2362 soluble binary toxin	58
8	Recycling of <i>B. sphaericus</i> Q in larval cadavers	60
9	Recycling of <i>B. sphaericus</i> ATCC 14577 in larval cadavers. Spores were fed along with strain 2362 soluble binary toxin	61
10	Recycling of <i>B. sphaericus</i> NRS 718 in larvae	62
11	Recycling of <i>B. sphaericus</i> NRS 718 in larval cadavers. Spores were fed along with strain 2362 soluble binary toxin	63
12	Recycling of <i>B. sphaericus</i> NRS 718 (pUE382) in larval cadavers	65
13	Recycling of <i>B. subtilis</i> DB104 (pUE382) in larval cadavers	66
14	Recycling of <i>B. subtilis</i> DB104 in larvae	67
15	Recycling of <i>B. sphaericus</i> 1693 (pC35) in larval cadavers	69

Figure	Page
16 Recycling of <i>B. sphaericus</i> 1693 (pC35) in larval cadavers. Spores were fed along with strain 2362 soluble binary toxin	70
17 Recycling of <i>B. thuringiensis</i> serovar. israelensis (Bti) in larval cadavers	71
18 Comparison of in vivo and in vitro spore germination by <i>B. sphaericus</i> 2362-7	73
19 Comparison of in vivo and in vitro spore germination by <i>B. sphaericus</i> 1883	74
20 Comparison of in vivo and in vitro spore germination by <i>B. sphaericus</i> LP12AS	75
21 Agarose gel electrophoresis of plasmid DNA from the relevant strains used to demonstrate transfer of pAMB1 to <i>B. sphaericus</i> 2362a recipients	88
22 SDS-PAGE patterns of surface layer proteins of <i>B. sphaericus</i> 1593 and bacteriophage resistant mutants	89
23 Bacteriophage sensitivity of <i>B. sphaericus</i> 2362 (pAMB1) donor	90
24 Agarose gel electrophoresis of plasmid DNA from the relevant strains used to demonstrate transfer of pAMB1 to <i>B. sphaericus</i> 1593 recipients	91
25 Agarose gel electrophoresis of plasmid DNA from the relevant strains used to demonstrate transfer of pAMB1 to <i>B. sphaericus</i> 2297 recipients	93
26 Agarose gel electrophoresis of plasmid DNA from the relevant strains used to demonstrate transfer of pAMB1 to <i>B. sphaericus</i> 31-2 recipients	94
27 Growth of <i>B. sphaericus</i> 1691 histidine auxotroph and 2362 (pUB110) on the defined medium BAT+	98

Figure		Page
28	Growth of <i>B. sphaericus</i> 1691 and 1691 histidine auxotroph on the defined medium BAT+	99
29	Growth of <i>B. sphaericus</i> 1691 and 1691 histidine auxotroph on the defined medium BAT+ supplemented with histidine	100
30	Agarose gel electrophoresis of plasmid DNA from some of the relevant strains used to attempt transfer of pUB110 to other <i>B. sphaericus</i> recipients	101

## LIST OF TABLES

Table		Page
1	Bacteria used in recycling studies	33
2	Bacteria used in the conjugation experiments	34
3	Effect of mating/expression time on frequency of conjugation	79
4	Effect of temperature on frequency of conjugation	80
5	<i>B. sphaericus</i> 2362 (pAM $\beta$ 1) conjugation frequencies on membranes and in broth	81
6	Effect of donor:recipient ratio on frequency of conjugation between <i>B. sphaericus</i> 2362 (pAM $\beta$ 1) and <i>B. sphaericus</i> 2362a	83
7	Conjugation frequencies between <i>B. sphaericus</i> 2362 (pAM $\beta$ 1) and other bacteria	84
8	Conjugation frequencies between <i>B. sphaericus</i> 2362 (pUB110) and other bacteria	97
9	Conjugation frequencies between <i>B. thuringiensis</i> serovar. <i>israelensis</i> (pBC16) and <i>B. sphaericus</i>	103
10	Conjugation in mosquito larval cadavers	104
11	Conjugation in mosquito larval cadavers	105

## INTRODUCTION

*Bacillus sphaericus* is a heterogeneous species that is divided into at least 5 DNA homology groups. The pathogenic strains belong to group IIA and produce either one or two toxins. The first is a binary toxin consisting of two distinctive proteins (51.4 and 41.9 kDa), and it is found in a parasporal body and produced during sporulation. The second is a 100-kDa toxin is produced by vegetative cells, and its exact location in the cell is unclear. Strains producing the binary toxin (alone or in combination with the 100-kDa toxin) are considered highly toxic strains, and strains producing only the 100-kDa toxin are among the low toxicity strains.

In the field, *B. sphaericus* has been reported to persist and provide control of mosquito larvae for extended periods of time. Although many factors may influence persistence and effectiveness of this bacterium in the mosquito habitat, the ability to recycle in the larval cadaver is considered an important characteristic of this mosquito pathogen.

Recycling is the ability of spores to germinate, multiply vegetatively and resporulate in the larval cadaver. Recycling allows production of new spores and toxin that will be available to control new generations of mosquito

larvae.

The recycling ability of *B. sphaericus* has only been studied with the low toxicity strain SSII-1 (of serotype 2) and with the highly toxic strains 2362 and 1593 (of serotype 5a5b). However, no information was available about the recycling ability of strains belonging to other serotypes or DNA homology groups.

Despite the availability of highly toxic *B. sphaericus* strains, efforts are being made to expand the spectrum of larval toxicity or growth efficiency for commercial production. Expression of new traits are commonly achieved by introducing a gene on a plasmid which generally carries an antibiotic resistance marker.

It is possible that gene exchange in the environment is relatively common. It has led to the spread of antibiotic resistance among bacteria and probably leads to extensive reorganization of the genotypic structure of bacterial populations. Conjugation is suggested to be the most prevailing mechanism for dissemination of plasmids in the natural environment.

Bacterial conjugation is the transmission of genetic material between bacteria, and it requires cell to cell contact. DNA transfer is generally mediated by plasmids which may encode all functions specific for transfer. Large self-transmissible plasmids can mobilize small



nonconjugative plasmids.

If recombinant strains of mosquito pathogens are able to transfer genetic information, the most likely place for this exchange to occur is in the larval cadaver where these recombinants are in close proximity with larval flora. Here, germinated spores of the invading pathogen interact with vegetative bacteria present in the larval cadaver increasing the probability of DNA transfer.

The specific objectives of this research were:

1) To determine the ability to recycle of *B. sphaericus* strains expressing different toxins, presenting different range of larvicidal activity and representing different serological and DNA homology groups.

2) To investigate the possibility of conjugal transfer of plasmids from *B. sphaericus* to other bacteria.

3) To determine if *B. sphaericus* exchange genetic information by conjugation mechanism, in vivo, in the larval cadaver.

## LITERATURE REVIEW

### **Bacterial insecticides**

Bacterial insecticides are important as alternatives to chemical pesticides. Among their advantages are that they are effective in controlling insect pests and are safe to non-target organisms. Furthermore, they do not produce the adverse effects on the environment that chemical insecticides like DDT produce (99, 35, 98). Among the spore forming bacteria, *Bacillus sphaericus* and *B. thuringiensis* have already been shown to be effective microbial insecticides (108) and the latter is widely used in many countries (116).

Early attempts to develop *B. sphaericus* as a microbial pesticide included the search for strains with increased pathogenicity. This was followed by their characterization and study of their physiology and their nutritional requirements. More recently, molecular and cloning techniques have been used to understand the mechanism of toxin action and toxin specificity.

### ***Bacillus sphaericus***

*Bacillus sphaericus* is aerobic, rod-shaped and forms round, terminal spores. This bacterium has a worldwide distribution and is commonly found in soil and aquatic habitats (34). Some strains are pathogenic to mosquito

larvae (99). *Bacillus sphaericus* is a heterogeneous species (5, 6, 119), typically producing negative reactions in the traditional phenotypic tests (1, 121). Strains of *B. sphaericus* were found to be naturally resistant to streptomycin and chloramphenicol (1, 24, 60). The susceptibility of mosquito larvae to the insecticidal strains varies. Larvae of *Culex* and *Anopheles* species are more susceptible to the action of *B. sphaericus* than those of *Aedes* (3). Larvae of *Psorophora* and *Mansonia* have also been shown to be highly sensitive. It is not toxic to black fly larvae (34, 35). Its safety to nontarget animals is well documented (35, 99).

#### **Taxonomy**

Using the classical biochemical methods, it has not been possible to distinguish between the insecticidal and non-insecticidal strains of *B. sphaericus* (39, 100).

The *B. sphaericus* strains are divided into six DNA homology groups (65). All toxic strains are grouped into the DNA homology subgroup IIA, and homology group I contains the type strain of the species, *B. sphaericus* ATCC 14577 (65). Studies on numerical classification of phenotypic features (1, 25), ribosomal RNA restriction patterns (5, 6), multilocus enzyme electrophoretic patterns (100, 115) and random amplified polymorphic DNA (RAPD) fingerprinting (119) are in agreement with the DNA homology grouping.

According to phage sensitivity, *B. sphaericus* pathogenic strains are placed in bacteriophage groups 1, 2, 3, 4 and 8 (121).

*B. sphaericus* strains are also classified according to their H (flagellar) antigens. The mosquito pathogenic strains were distributed in flagellar serotypes H1, H2a2b, H3, H5a5b, H6, H25 and H48 (39, 40, 110).

Woodburn et al. (119) were able to identify individual serotypes among the pathogenic strains by random amplified polymorphic DNA (RAPD) fingerprinting. The RAPD data also correlated with bacteriophage type, flagellar serotypes, and the type of toxin produced (binary or 100 kDa).

Singer (100), using multilocus enzyme electrophoresis and vertical polyacrylamide gel electrophoresis (PAGE), found no relationship with the bacteriophage groups of Yousten (121) nor the serogroups of De Barjac et al. (39) and the multilocus enzyme groups. However, the electrophoretic data agreed with the DNA homology groups of Krych et al. (65).

Recently, Frachon et al. (44) and Nicolas et al. (79) used gas-liquid chromatography of bacterial fatty acid methyl esters (FAME) to classify *B. sphaericus* strains and predict mosquitocidal activity. They found that the mosquito pathogens were grouped into two clusters.

Liu et al. (71) isolated five new high toxicity strains

of *B. sphaericus* in Singapore. The interesting feature about these strains is that, although in many aspects they resemble the high toxicity strains e.g they belong to DNA homology group IIA and have the binary toxin genes, they differ from them in that they lack the 100-kDa toxin gene. Also, they were classified as members of phage group 8, where only low toxicity strains have been reported.

### **The toxins**

Among the pathogenic *B. sphaericus* strains, great variation in toxicity is observed. However, investigators often speak of the strains in two groups, the high toxicity strains and the low toxicity strains. Among the high toxicity strains, two types of toxin have been characterized. A binary toxin containing proteins of 51.4 and 41.9 kDa (14, 20, 21, 36) is found in a parasporal body sometimes called a "crystal". It is toxic following ingestion by mosquito larvae (36, 53, 59, 124). A second toxin of 100 kDa is cell-associated but its exact location is not known (109).

The parasporal crystal (binary toxin) appears during stage III of sporulation and is partially enclosed by the exosporium. The interior of the crystal shows a crystalline lattice structure. It is surrounded by an envelope that is retained after dissolution of the matrix in the larval gut or treatment with alkali (124). Yousten and Davidson (124)

first suggested that the parasporal crystal could be the source of toxin. Davidson (33) and Tinelli and Bourgoïn, (111) extracted the toxin from spore and crystal mixtures.

The toxin crystal from *B. sphaericus* 2362 was studied by Broadwell and Baumann (22). It contained four proteins having a mass of 125, 110, 63 and 43 kDa, as determined by SDS-PAGE. The two higher molecular weight proteins appeared to be precursors of the 63 and 43 kDa proteins. However, Hindley and Berry, (53) analyzed the nucleotide sequence representing the 43-kDa protein and concluded that it does not arise from a higher-molecular-weight precursor. Bowditch et al. (19) found later that the 122 and 110 kDa proteins were contaminants originating from the S-layer protein that covers the vegetative cell.

Cloning studies also helped elucidate the nature of the genetic determinants involved in *B. sphaericus* toxicity. The genes for the binary toxin and the 100-kDa toxin have been characterized (7, 12, 14, 15, 17, 53, 109) and cloned into *Escherichia coli* (14, 16, 21, 36, 45, 103, 109), *Bacillus subtilis* (13, 20, 26, 103), and both non-pathogenic and low toxicity *B. sphaericus* (20, 21, 26), *Bacillus thuringiensis* (18, 82), *Caulobacter crescentus* (108) and the blue green bacterium *Anacystis nidulans* (41). It is now known that each protein plays a different role in toxicity. Berry et al. (16) demonstrated that the 41.9 kDa protein was responsible

for host range, however both the 42 and 51 kDa proteins are required for toxicity to *Culex* larvae (14, 20, 36).

Plasmid hybridization experiments with the cloned toxin genes suggested that the genes for the binary toxin are located in the chromosome rather than on plasmids (5).

A typical representative of the low toxicity strains is *B. sphaericus* SSII-1. The 100-kDa toxin in this strain is not present as a parasporal crystal but associated with the vegetative cell (32). The genes encoding the binary toxin are not present in the low toxicity strains (12). The gene for the 100-kDa toxin has been isolated and characterized. It is widely distributed among both high and low toxicity strains (109). All highly toxic strains tested by Thanabalu et al. (109), contained the 100-kDa gene in addition to the genes for the 51.4 and 41.9 kDa toxins. This gene has been cloned and its sequence showed that the N terminus had significant homology to bacterial toxins which act by ADP-ribosylation of G proteins (106, 107 and 109). A domain in the C-terminal region was sufficient for toxicity to cultured *Culex* cells, however both regions were necessary for toxicity to mosquito larvae (106).

The mechanism of action of the binary toxin is not completely known (124). Several studies have been carried out to understand the mechanism of action and specificity of the *B. sphaericus* toxins. Susceptibility of different

species of mosquito larvae to the 43-kDa toxin is not due to differential activation by gut proteases (3, 23, 80).

Studies of the histopathology caused by *B. sphaericus* toxins in mosquito larvae showed that the primary site of action is gastric caecum cells and posterior midgut (32, 36). After ingestion of toxin, the epithelium was distended, the gut was paralysed, cytolisosomes were increased in number and size producing epithelial cell lysis, epithelial cells were separated from one another, followed by larval death (32, 34). Recently, the presence of specific receptors for purified binary toxin on brush border membrane fractions from *C. pipiens* larvae was demonstrated by in vitro binding assays (81).

#### **Persistence and Recycling**

Persistence is defined as the presence in the environment of spores and/or vegetative bacteria. Recycling is spore germination, followed by replication of resulting vegetative cells and production of new spores and toxin in larval cadavers (120). Therefore, recycling of *B. sphaericus* in cadavers may extend larvicidal activity, because more toxin is produced in larval cadavers to control subsequent populations of larvae.

*B. sphaericus* is able to recycle and persist in the aquatic environment for longer periods of time than *Bacillus thuringiensis* serovar. *israelensis* (Bti). Hertlein et al.



(52) recovered infective spores from a roadside ditch up to 9 months after application. Singer (99) reisolated *B. sphaericus* from water and larval cadavers from treeholes 7 months after treatment. Hornby et al. (54) reported persistence of spores for several months in sewage and for 30 to 50 days in fresh water. DesRoches and Garcia (42) demonstrated that *B. sphaericus* persisted and was toxic in sewer and spring water for up to 30 days, and the presence of larvae increased persistence even longer.

Several studies demonstrated the ability of *B. sphaericus* to recycle in larval cadavers (27, 37, 38, 90, 95). Recycling was also demonstrated under field conditions (38). These studies showed that, during *B. sphaericus* recycling in larvae, there was a decrease in the number of spores during the first 12 to 24 h. During the next 48 h, the spore count increased to  $10^5$  or  $10^6$  per larva. The final number of spores, after the 72 h, was at least 100 times the lowest concentration observed, an indication of new spore formation.

Aly et al. (2) demonstrated that the entomocidal bacterium *B. thuringiensis israelensis* recycled in mosquito larvae of *A. aegypti* and *Anopheles albimanus*. However Bti exhibits shorter persistence in the field. This indicates that other factors in the environment may also play a role in the persistence of entomocidal bacteria in the field.

Karch et al. (61) observed a four-year persistence of *B. sphaericus* in a natural pond and suggested that associated nontarget species could influence the maintenance of *B. sphaericus*. The role of nontarget arthropod species found in association with *Culex* sp. in natural breeding sites, was studied. Nontarget species contributed to the action of the bacterium by their predator activity or by redistributing the bacterium in the water column.

Yousten et al. (122) tried to determine the fate of the spore-toxin complex after ingestion by nontarget invertebrates, i.e. midge larvae, snails and oysters. They found differences in the elimination rate of spores by the three invertebrate organisms studied that could be related to the complexity of their guts. There was no indication of spore recycling in the animals, and complete or partial detoxification of the toxin could occur after gut passage, depending on the animal. Results indicated that unaffected but exposed nontarget organisms might transport microbial control agents to untreated sites. The information available about the fate of *B. sphaericus* following its delivery into the aquatic environment is still very limited. To gather more of such information, Yousten et al. (123) studied spore interactions with three larvae of aquatic insects. The predatory stonefly, *Paragnetina media*, the leaf shredding stonefly, *Pteronarcys proteus* and the

cranefly, *Tipula abdominalis*. They found that both stonefly larvae eliminated the spores after spore feeding was stopped. The crane fly larvae retained one fifth of the spores for up to five weeks. Although spores recovered in crane fly faecal material were detoxified by gut passage.

Other factors may influence the greater persistence of *B. sphaericus* in the field compared to Bti. Yousten et al. (126) reported on the greater tendency of Bti spores and paraspores to adhere to and settle with suspended sediment and fine particulates compared to paraspores of *B. sphaericus*. Once resuspended by turbulence in water, toxin adhering to particles will sink more rapidly and will be less accessible to larvae.

Ohana et al. (82) followed the fate of an antibiotic resistant Bti under laboratory-simulated field conditions. They also found loss of larvicidal activity due to the masking of the toxin caused by the adsorption of the spores or/and toxin on the mud.

### **Surface Layer**

Many gram positive and gram negative bacteria possess a surface layer (S layer) composed of protein or glycoprotein. The function of the S layer is not well understood, but because of its location at the cell surface, several functions have been proposed. These include maintenance of the cell shape, a barrier against internal or external

factors or even as a channel for conjugal gene transfer between bacteria which lack pili. Wiwat et al. (117, 118) cloned the Bti S-layer protein gene and found that antibody against the S-layer protein inhibited plasmid transfer by conjugation in this bacterium (117). In the case of *B. sphaericus*, the S layer consists of a linear array of glycoproteins (70), and it functions as a site of bacteriophage attachment (69). Bowditch et al. (19) cloned and sequenced the gene that encodes a 125-kDa precursor of the 122-kDa S-layer protein of strain 2362.

### **Spore Germination**

Most gram-positive, endospore-forming bacteria are soil organisms which predominate as spores under poor nutrient conditions or as vegetative forms when enough nutrients are present (76). Spores are important for maintenance and dispersal of the organisms. The spore can resist considerable environmental extremes and is still able to respond to low concentrations of germinant substances in the medium. The conversion of a spore into a vegetative cell occurs in two stages. First, the **germination** process involves the breakage of dormancy, loss of spore resistance properties and resumption of metabolic activity. During the **outgrowth** stage, the spore protoplast resumes growth and emerges from the coat remnants. During this second stage, there is onset of RNA, protein and DNA synthesis. The cell

mass increases, causing the germinated spore protoplast to swell, following DNA replication and cell division.

Several events take place during germination in *Bacillus*. **Activation** or pretreatment of spores before exposure to germinants, generally increases the subsequent germination response. Sublethal heating at 70 or 80°C or prolonged storage at 4°C are activation factors. The mechanism of activation is not understood, but it probably involves an alteration in the permeability properties of the spore. **Commitment** is the time that a spore suspension must be exposed to germinant to allow the progress of germination events. The germination process is essentially hydrolytic with approximately 30% of the dry weight of the spore being excreted and this precedes significant macromolecular synthesis. Other events in germination include loss of heat resistance, resumption of metabolic activity and enzyme reactivation as the water activity in the core increases. The small, acid-soluble proteins (SASPs), which are located in the spore core and which bind DNA, are hydrolyzed and supply amino acids to support protein synthesis. The earliest defined germinant identified was L-alanine. The D isomer acts as a competitive inhibitor, but many other compounds have been identified for particular species (Reviewed in references 76, 87, 88).

### **Conjugation**

Bacterial conjugation is the transmission of genetic material between bacteria by a process that requires cell to cell contact. DNA transfer is generally mediated by plasmids or transposons which may encode all functions specific for genetic transfer (51).

Conjugation was first reported in 1946 by Lederberg and Tatum (67). Using auxotrophic mutants of *Escherichia coli*, they demonstrated genetic recombination between strains. Reversion to prototrophy resulted from exchange of chromosomal genes between the strains. They predicted that recombination was mediated by a bacterial fertility factor called "F". Now, it is known that the F plasmid is only one representative among a broad spectrum of conjugative plasmids found in bacteria (114).

A self-transmissible or conjugative plasmid is one that carries genes that determine the formation of specific cell contacts necessary for conjugation and whose DNA can be prepared for transfer to a recipient cell. Genes required to transmit a self-transferable plasmid are called *tra*, for transferable. Trans-acting functions encoded by and required for the transmission of mobilizable plasmids are called *mob*, for DNA mobilization. And both groups act at specific DNA sequence called *oriT*, for origin of transfer. *oriT* is the only cis-acting function required for plasmid transmission (51).

The F plasmid encodes the complete set of functions required for DNA mobilization and transfer. Approximately 31 genes in F are involved in conjugation, of which 13 deal with production, assembly and erection of pili and cell surface structures necessary for its transmission (55).

Although F has been the paradigm for conjugation studies in *E. coli*, other transmission systems are now known in the eubacteria. Examples include the colicinogenic factors transmissible among the enterobacteria, the resistance transfer factors responsible for the spread of antibiotic resistance genes in the enterobacteria (114), the broad host range plasmids in gram-positive bacteria (73) and in the gram-negative bacteria (50), the conjugal transfer of the *Agrobacterium* plasmid Ti (43) and the sex-pheromone responding plasmids of *Enterococcus faecalis* (28).

The ability of plasmid DNA to transfer or to be mobilized within species and among different species has been demonstrated in numerous studies. Plasmid mobilization has been studied both under laboratory conditions and in simulated field conditions, or in microcosms representing natural habitats (93), in sterilized wastewater (46), in soil (89) and even in filter sterilized drinking water (94). Indigenous bacteria isolated from wastewater were demonstrated to be capable of acting as plasmid donors to other indigenous wastewater recipients (74).

Certain factors can discourage the exchange of related plasmids between bacteria, e.g. incompatibility and surface exclusion. Incompatible plasmids are considered to be closely related because of their overlapping replication and segregation requirements, similarity in their DNA sequences, and interchangeability of their conjugal functions. Based on these criteria, plasmids are placed in incompatibility (Inc) groups, such as IncF or IncP (62, 114). Donor cells can encode conjugal elements for surface exclusion proteins that discourage conjugation between two cells containing incompatible plasmids. The exchange of related plasmids between donors is discouraged but not prevented by surface exclusion. The actual mechanism for surface exclusion remains unknown (51, 91).

Studying conjugation in bacteria as a mechanism for gene transfer may aid the understanding of important bacterial interactions and their evolutionary implications. For example, conjugation plays an important role in the dissemination of antimicrobial resistance (51). Conjugation may be implicated in the production of new combinations of delta-endotoxins within different populations of *B. thuringiensis* (47, 91).

Conjugation seems to be a likely transfer process occurring in natural environments (74, 89). Engineered organisms can come in contact with indigenous bacteria and



transfer the plasmids. This creates the possibility of survival and spread of this DNA. Most cloning vectors used in genetic engineering are small plasmids that lack *tra* genes and therefore cannot mediate their own transfer. However, if conjugative plasmids are present in a cell, they may be capable of mobilizing small non-conjugative plasmids for transfer to potential recipients during conjugation (92).

Three types of gene transmission have been identified in gram positive bacteria. One system represents the "pheromone-responding plasmids" typical of the enterococci and their close relatives (28). Instead of pili, conjugation is facilitated by a proteinaceous aggregation substance produced by the donor. The recipient cells secrete a peptide pheromone which is sensed by a donor pheromone receptor and transmission is initiated. Plasmids of 25 kb or more transfer with high efficiency in liquid media. The recipient cells produce a small peptide sex pheromone inducing in the donor production of surface components and cell clumping. Frequency of transfer can be as high as  $10^{-2}$  per donor in broth matings. This group of plasmids often carries genes associated with pathogenic functions i.e. hemolysins, bacteriocins and antibiotic resistance.

A different type of system is composed of the conjugative transposons (29). These elements encode factors

for their serial transposition and also mediate their own conjugal transfer. They usually carry antibiotic resistance genes and are 15-20 kb, but can be larger than 50 kb. They do not generally transfer in liquid but rather on a solid surface. Transfer frequencies are low compared with those observed with the pheromone-responding plasmids,  $10^{-6}$  versus  $10^{-2}$  or higher.

A third type of transfer encompasses the so-called broad host range conjugative plasmids (73). These plasmids carry drug resistance markers, have a size of 15-20 kb and do not respond to pheromone signals. Frequency of transfer varies in the  $10^{-3}$  to  $10^{-6}$  range. Transfer works better on solid surface than in liquid.

#### **Plasmids and gene transfer in *B. sphaericus***

Reports of plasmid profiles in *B. sphaericus* are incomplete and inconsistent. This is probably due to differences in techniques. Seyler et al. (95) and Singer (100) reported the presence of a large cryptic plasmid (approximately 180 kb), in most of the highly toxic strains and in many of the noninsecticidal strains. No plasmids were detected in the highly toxic strain 1691. Aquino et al. (5) analyzed the plasmid content of strains used in their ribosomal restriction pattern study and reported a similar pattern of sizes and distribution of plasmids as those found by Singer (100).

The first report of genetic transfer in *B. sphaericus* was by McDonald and Burke (83). They obtained transformation of polyethylene glycol (PEG)-treated *B. sphaericus* 1593 protoplasts with plasmids pUB110 and pBC16. Lower transformation frequencies were obtained when the DNA was isolated from *B. subtilis*. This was due to the presence of a restriction modification system in strain 1593 which inactivated pUB110 isolated from *B. subtilis*.

Although the optimized protoplast transformation provided some transformants, there are more effective and easier ways of obtaining genetic transfer. Using the method of filter mating, the promiscuous plasmid pAMB1 (26.5 kb) was conjugally transferred from *Streptococcus (Enterococcus) faecalis* to *B. sphaericus* and between derivatives of *B. sphaericus* strain 1593 (84).

Taylor and Burke (104) improved the transformation process for *B. sphaericus* by using electroporation (105). This technique involves application of brief, high intensity electric fields to reversibly permeabilize the plasma membrane to allow entry of the genetic material. Transformation frequencies obtained were 100-fold greater than efficiencies obtained by McDonald and Burke (83) using protoplast transformation.

More recent reports on genetic manipulation of *B. sphaericus* involved the introduction of new genetic

determinants to improve its characteristics as a microbial control agent. Genes encoding the Bti delta-endotoxin have been cloned and expressed in *B. sphaericus* to increase its spectrum of activity to include *Aedes* and *Simulium* species (10, 112). Poncet et al. (86) transferred Bti toxin genes cryIVB and cryIVD in *B. sphaericus* 2297 to obtain transformed cells with a broader spectrum of activity to mosquito larvae and good persistence in the environment. Broadwell et al. (20, 21) introduced plasmids carrying the binary toxin genes into non-toxic strains of *B. sphaericus* to make them pathogenic. Plasmids carrying the *P. putida* xylE gene coding for catechol 2,3-dioxygenase were introduced into strain 1593 to provide a simple colorimetric system for detecting the bacteria in the environment. The amyE gene, encoding amylase in *B. subtilis*, was introduced in *B. sphaericus* using pUB110 as the vector. This was intended as a first step to alter metabolism and obtain strains with increased manufacturing efficiency.

Sedimentation of the bacterial spores limits the duration of effective mosquito control following the application of *B. sphaericus* and *B. thuringiensis* (126). To prolong control by maintaining mosquitocidal toxins in larval feeding zones, toxin genes from these bacilli have been transferred to bacterial species that are found in almost every aquatic habitat, located at or near the water

surface. *Bacillus sphaericus* binary toxin genes were inserted into and expressed by the cyanobacterium *Anacystis nidulans* (41). Thanabalu et al. (108) expressed the binary toxin genes from *B. sphaericus* 2297, the 100-kDa toxin from strain SSII-1, and the 130-kDa toxin from *B. thuringiensis* subsp. *israelensis* in *Caulobacter crescentus*.

Shows and Andrews (97) found that tetracycline in the medium increased conjugative transposition of the transposon Tn 916 and the transposon-dependent mobilization of the plasmid pC194 in *Bacillus* interspecies matings. When a *B. sphaericus* rifampicin-resistant strain [chr::Tn916] was used as the donor in matings with Bti, no transfer was detected regardless of whether the donor was pregrown with or without tetracycline before matings. Concerns about plasmid stability under conditions of growth and production, as well as after the bacteria are released in the environment, motivated Seyler et al. (95 and 96) to study segregational and structural stability of plasmids in *B. sphaericus*. They followed the fate of pUB110 and its derivatives containing various insert sizes at different locations during growth and sporulation in the mosquito larval cadaver. Structural instability was not detected. However, segregational instability was associated with interruption of the BA4 region (a membrane-binding region) of the plasmids, and this instability occurred primarily during the sporulation phase,

not during vegetative growth.

### **The pAMB1 plasmid**

Clewell et al. (30) reported the isolation and characterization of three plasmids in *Streptococcus* (*Enterococcus*) *faecalis* strain ATCC14508 or DS-5. One of them, the B plasmid, was associated with erythromycin and lincomycin resistance (>1mg/ml). This resistance is representative of the MLS phenotype (macrolides, lincosamides and streptogramin B). The B plasmid's molecular weight was 17 Md (26.5 Kb) and was present in 1 or 2 copies per genome.

Leblanc and Lee (66) studied the genetic determinants that influence plasmid host range, and they used pAMB1 as representative of the broad host range plasmids. They did an extensive physical and genetic analysis of pAMB1. This plasmid is conjugally transferrable to and between a number of other *Streptococcus* (*Enterococcus*) species, *Lactobacillus* species, *Staphylococcus aureus*, *Clostridium acetobutylicum* and various species of *Bacillus*.

Lereclus et al. (68) used the filter mating technique to transfer pAMB1 from *E. faecalis* to several *B. thuringiensis* strains. The same technique was used to obtain conjugal transfer of pAMB1 from *E. faecalis* to *B. sphaericus* 1593 and between derivatives of strain 1593 (84). Plasmid pAMB1 was transferred from *B. subtilis* to *C. acetobutylicum*

and promoted conjugal transfer of a small plasmid, pOD1, by cointegrate formation (85). Van Der Lelie and Venema (113) obtained transfer of pAM $\beta$ 1 from *S. lactis* to *B. subtilis* by conjugation in solid surface matings.

#### **Gene transfer by conjugation in *B. thuringiensis***

The work published by Gonzalez and Carlton (48) constitutes one of the cornerstones in the investigation of the conjugation phenomenon in the genus *Bacillus*. They were first in reporting the use of an efficient conjugation-like system for plasmid transfer among *Bacillus* species. They observed that *B. thuringiensis* can become acrysaliferous and lose toxicity, thus suggesting plasmid curing. Their work led to the discovery of self-transmissible plasmids in *B. thuringiensis* and helped to implicate specific plasmids in the production of delta endotoxin by several strains of *B. thuringiensis*. They obtained plasmid transfer between strains of *B. thuringiensis* and from *B. thuringiensis* to *B. cereus* (47 and 48). They demonstrated that plasmid transmission required direct contact of donor and recipient. The transfer was DNase insensitive. They introduced the idea of the incompatibility-like phenomenon in *Bacillus* and observed restriction of certain plasmids to transfer. In other cases, the entry of a new plasmid into a strain resulted in loss of a native plasmid in that strain. In addition to change in phenotype (to cry+) and gel

electrophoresis, Gonzalez et al. (47) used immunological analysis (Ouchterlony gels) to demonstrate that the structural genes for the delta-endotoxin were plasmid borne.

Klier et al. (63) tried heterospecific mating between *B. subtilis* carrying a recombinant plasmid bearing a toxin (cry) gene and two different *B. thuringiensis* strains as recipients. They also produced a Bti transciptient which produced not only the dipteran-active delta-endotoxin, but also the lepidopteran toxin from a different serotype.

Gonzalez and Carlton (49) reported the presence of eight plasmids in Bti: 3.3, 4.2, 4.9, 10.6, 68, 75, 105 and 135 MDa as well as a plasmid-like linear element of approximately 10 MDa. They implicated the 75-MDa plasmid in crystal production by partially cured mutants and by plasmid transfer experiments. The 75-MDa plasmid was capable of recombining with a 68 MDa Bti plasmid, to which it was partially homologous, and, in one case, the 75-MDa plasmid seemed to have integrated into the chromosome.

Battisti et al. (11) obtained interspecies plasmid transfer by a conjugation-like process in broth matings. Plasmids pOX11 and pOX12 from *B. thuringiensis*, were responsible for pBC16 mobilization to *B. anthracis* and *B. cereus*. The pOX12 plasmid was implicated in crystal production.

Loprasert et al. (72) used protoplast transformation to



obtain a *B. thuringiensis* transformant carrying pC194 and pH33. Protoplast transformation was used since the pC194 plasmid could be introduced into a strain of Bti using the conjugation-like process only after the plasmid was first introduced into another strain of *B. thuringiensis*. The transformed *B. thuringiensis* was used as a donor in conjugation.

Koehler and Thorne (64) reported the presence of a 55 Kb *B. subtilis* plasmid (pLS20) capable of mobilizing small plasmids in interspecies matings. *Bacillus anthracis*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. subtilis* and *B. thuringiensis* acted as recipients. However, transfer was not demonstrated in all the interspecies matings.

Aronson and Beckman (8) detected low frequency transfer of chromosomal genes from *B. thuringiensis* to *B. cereus* by mating. A 29-MDa plasmid present in *B. thuringiensis* serovar. *kurstaki* HD1 seemed to be responsible for chromosome mobilization. These authors suggested that the location of protoxin genes may be dependent on transposons, therefore "their presence in particular plasmids could be fortuitous".

Reddy et al. (91) demonstrated interspecies plasmid transfer by mating *B. thuringiensis* subspecies to *B. cereus* and *B. anthracis*. The *B. thuringiensis* strains contained

self-transmissible plasmids responsible for mobilization of pBC16 in broth mating. Transmission of the selectable plasmid pBC16 helped to indirectly monitor mating ability of the self-transmissibility of the plasmids. The *B. anthracis* transipients, which contained pX01 (the toxin-encoding plasmid in *B. anthracis*) and received the *B. thuringiensis* plasmid, had a variant plasmid. It was suggested that this plasmid variant may have been the result of transposition of sequences from *B. thuringiensis* plasmid to pX01. The pX014 plasmid from *B. thuringiensis* serovar. *tumanoffi* was efficient in mobilizing the toxin and capsule plasmids, pX01 and pX02 respectively, from *B. anthracis* to plasmid-cured *B. anthracis* and *B. cereus*. Reddy et al. (91) reported the occurrence of the "entry exclusion phenomenon". It produced a decrease of 20-100 fold in the frequency of transfer between strains containing the same self-transmissible plasmids. Efficiency was enhanced when donor and recipient cells contained different conjugative plasmids.

Wiwat et al. (116) obtained transfer of plasmids pBC16 and pC194 from Bti to 25 subspecies of *B. thuringiensis* by the conjugation-like process using broth matings. Frequency of transfer within subspecies was higher than between subspecies. Transfer of chromosomal markers was observed, and it was always accompanied by transfer of both pBC16 and pC194.

Andrup et al. (4) conducted matings of *B. thuringiensis* strains in broth using a small, mobilizable plasmid encoding antibiotic resistance to monitor the transfer. These authors found correlation between plasmid transfer with specific protease-sensitive coaggregation between strains of Bti. These authors also observed that some matings were affected when strains with different growth rates or other physiological differences were involved, e.g. a spo- mutant or a strain inhibiting the growth of the other.

*B. thuringiensis* genes have also been transferred by conjugation to bacteria isolated from the field (56, 76, 101, 102). These studies support suggestions that plasmid transfer occurs in nature. This can result in production of new combinations of delta-endotoxin within populations of bacteria. Singh Bora et al. (101) obtained conjugal transfer of a lepidopteran toxin gene of *Bt kurstaki* HD1 into *B. megaterium* RS1, a leaf colonizing bacterium. Toxin expression and survival of transconjugants under field conditions for longer periods of time than the donor strain were observed. Jarret and Stephenson (56) demonstrated plasmid transfer in broth matings between Bt and spore forming bacteria isolated from soil samples. Skot et al (102) cloned the toxin gene from *B. thuringiensis* subsp. *tenebrionis* into *E. coli* and subsequently transferred the gene to *Rhizobium leguminosarum* by conjugation. The 65-kDa

toxin lethal to coleopteran larvae was expressed in the "free living state" of *Rhizobium*. Pea and white clover plants inoculated with *Rhizobium* strains containing the toxin gene suffered less root and nodule damage by *Sitona* larvae.

Transposon mediated conjugation has been demonstrated in *B. thuringiensis*. Nagalich and Andrews (77) introduced the conjugative transposon Tn916 of *Streptococcus faecalis* (16.4 kb, Tet-resistance) into Bti, by filter matings. Transfer frequency of  $7 \times 10^{-7}$  per recipient was obtained. Subsequently, Nagalich and Andrews (78) used Tn916 to induce the mobilization of the non-conjugative plasmids pC194 and pUB110 into Bti from *B. subtilis*. In both cases, transconjugants were obtained only in filter matings not in broth matings. Tn916 inserted in different sites on the chromosome while the plasmids were maintained in the autonomous state.

The possibility of *B. thuringiensis* plasmid transfer in vivo has been tested. Jarret and Stephenson (56) demonstrated plasmid transfer occurring between strains of *B. thuringiensis* in infected larvae of the lepidopteran, *Galleria mellonella* and *Spodoptera littoralis*. Matings were performed in broth and in the larvae and plasmid transfer was analyzed between *B. thuringiensis* strains and between *B. thuringiensis* and soil isolated bacteria. In broth matings

*B. thuringiensis* was able to transfer cry+ and Tet-resistance to sporeforming bacteria isolated from soil samples. The level of transfer of plasmids encoding crystal production and tetracycline resistance were similar to the ones obtained in broth. Transfer was higher in *G. mellonella* larvae than in *S. littoralis*. These results were interpreted as being due to the probability that *B. thuringiensis* has a greater ability to colonize these larvae. Untreated larvae of *G. mellonella* contain lower numbers of gut bacteria, which results in a less competitive environment for *B. thuringiensis* growth and sporulation.

## MATERIALS AND METHODS

### **Bacterial strains and bacteriophage.**

A description of the *Bacillus sphaericus* strains used in the recycling study, and their characteristics is given in Table 1. Table 2 contains the strains used in the conjugation studies. Recombinant strains, *B. sphaericus* NRS 718(pUE382) and *B. subtilis* DB104(pUE382) were obtained from Paul Baumann, *B. sphaericus* 1693(pC35) was obtained from Alan Porter. Bacteriophage 4, lytic for certain *B. sphaericus* strains, was diluted from frozen stocks maintained in this laboratory.

### **Isolation of rifampicin resistant strains**

Rifampicin-resistant mutants were obtained for each strain by growing the bacteria in increasing concentrations of the antibiotic. A loopful of the respective strain was inoculated from a NYSM stock slant into a tube containing 5 ml of NY broth and incubated in a gyratory water bath shaker (model G76, New Brunswick Scientific) at 30°C. After 24 h of incubation, 1 ml of this culture was transferred to a flask containing NY broth and 5 ug/ml of rifampicin (Sigma). After growth was obtained, progressive transfers were carried out to flasks containing increasing concentrations of rifampicin until the strains were resistant to a minimum concentration of 25 ug/ml of the antibiotic.

**Table 1. Bacteria used in recycling studies.**

<b>Strain</b>	<b>DNA homology group</b>	<b>Serotype</b>	<b>Toxin</b>	<b>Source</b>
<i>B. sphaericus</i>				
2362	5a5b	3	Binary	J. Weiser
2362-7 (rif <sup>r</sup> )	5a5b	3	Binary, 100 kDa	S. Singer
IAB 59	6	3	Binary, 100 kDa	J. Ofori
2297	25	4	Binary, 100 kDa	Wickremsinghe
1883	2a2b	2	100 kDa	S. Singer
ATCC 14577	-	-	no toxin	Type strain of species
NRS 1693(pC35)	-	-	100 kDa	A. Porter
NRS 718(pUE382)	-	-	Binary	P. Baumann
NRS 718	-	-	no toxin	P. Baumann
Kellen Q	1a	1	100 kDa	S. Singer
LP12AS	3	8	Binary	F. Priest
<i>B. subtilis</i>				
DB104	-	-	no toxin	P. Baumann
DB104(pUE382)	-	-	Binary	P. Baumann

**Table 2.** Bacteria used in the conjugation experiments

<b>Strain</b>	<b>DNA homology group</b>	<b>Serotype</b>	<b>Source</b>
<i>B. sphaericus</i>			
2362 (pAMB1)	IIA	5a5b	This laboratory
2362a	IIA	5a5b	This laboratory
1593	IIA	5a5b	Dr. S. Singer
1593-P51	IIA	5a5b	This laboratory
1593-4RC	IIA	5a5b	This laboratory
1691	IIA	5a5b	Dr. S. Singer
2297	IIA	25	Dr. Wikremsinghe
31-2	IIA	9a9c	Dr. C. Cokmus
IAB 460	IIA	6	Ms. J. Ofori
SSII-1	IIA	2	Dr. S. Singer
1883	IIA	2	Dr. S. Singer
Kellen Q	IIA	1	Dr. S. Singer
ATCC 14577	I	-	Type of species
NRS 1199	V	-	Dr. R. Gordon
Larval isolate	V	-	This laboratory
<i>B. subtilis</i>			
IG-20 (restrictionless)			
<i>B. thuringiensis</i>			
<i>israelensis</i>			This laboratory
<i>israelensis</i> BIS UM1 (pBC16)			Dr. C. Thorne
<i>B. mycoides</i> (larval isolates)			This laboratory



### **Spore suspension stocks.**

Spore suspensions of strains used in the recycling, germination and conjugation in larvae experiments, were prepared by growing each strain in a 500 ml flask containing 50 ml of NYSM broth. The cultures were incubated at 30°C with shaking at 150 rpm until maximum sporulation was achieved, usually 24-48 h. The sporulated bacteria were harvested by centrifugation, washed three times in sterile distilled water, resuspended in sterile distilled water and stored at 4°C.

### **Treatment of larvae and recycling determinations.**

Assays were run in duplicate. Three hundred, third instar *Culex quinquefasciatus* larvae were placed in 200 ml of sterile tap water containing approximately  $1-5 \times 10^6$  spores/ml. After 15 min of feeding (45 min for strain LP12AS), larvae were removed from the spore suspension, rinsed with sterile tap water and placed in 300 ml of sterile tap water. Twenty five larvae were taken at zero, 6, 12, 24, 48, 72 and 96 h after feeding, rinsed, suspended in 5 ml sterile water and homogenized with a glass tissue grinder. Numbers of spores were determined by heating 2 ml of homogenate at 80°C for 12 min. These samples were sonicated for 30 sec. with the small probe setting at 35 on a Fisher-Sonic Dismembrator (Model 300) to declump spores. They were diluted, plated and incubated at 30°C. Spore and

total viable *B. sphaericus* counts were made in NYSM agar containing 25 ug/ml rifampicin. Total bacterial counts were made in Difco plate count agar.

#### **Surface contamination of the larvae**

To determine the number of spores adhering to the cuticle, one hundred larvae were killed by 2 sec immersions in boiling water. The dead larvae were resuspended in a spore concentration equivalent to that fed to live larvae and held for the same period of time, 15 min. Twenty five larvae were collected, rinsed, homogenized and spores counts performed.

#### **Alkaline extraction of soluble binary toxin from sporulated cultures of *B. sphaericus***

Toxin was extracted as described by Davidson (33) with some modifications. *B. sphaericus* 2362 was grown as described for spore stocks. Spores and adhering parasporal inclusions were harvested by centrifugation and washed once with sterile distilled water. The pellets were resuspended in 0.05 M NaOH. Spores were kept in this suspension for 60 min and removed by centrifugation at 15000xg for 20 min. The supernatant fluid was dialyzed for 24 h against 0.01M MOPS buffer at pH 7.5. The dialyzed toxin solution was filter sterilized, placed in vials and frozen. The protein content of the alkali-solubilized protein toxin was analyzed by Micro BCA Protein Assay Reagent kit (Pierce). To test

activity of the prepared soluble toxin, bioassays were performed as described by Yousten et al. (127).

#### **In vitro spore germination experiments**

*B. sphaericus* strains were tested for the ability of their spores to germinate in bacteriological media or in the presence of defined germinant substances. Spore suspensions of strains 2362-7, 2362(pAMB1), 1883 and Lp12AS were prepared as described for spore stocks. Spores were heat shocked at 80°C for 12 min, suspended in 0.01M MOPS buffer pH 7.5 and adjusted to an absorbance of 0.09 (660 nm). The adjusted spore suspensions were kept cold until all the samples were prepared, put at room temperature and mixed again to start the experiment at time 0 h. Samples were prepared by mixing the germinant substances with the adjusted spore suspensions in a 1:4 proportion. Controls contained sterile distilled water in place of germinant and the spore concentration was also in a ratio of one part water to 4 parts of adjusted spore suspension.

The germinants were used as follows:

1. A combination of 0.3 M alanine and 0.3 M inosine. These substances are known to be effective germinants for a number of aerobic, sporeforming bacteria. The final germinant concentration in the samples was 0.06 M.

2. L-Arginine 0.5M. This amino acid is used as sole carbon source in a defined medium used with *B. sphaericus*.

The final germinant concentration was 0.1 M.

3. Nutrient broth supplemented with 0.05% yeast extract (NY broth). In this case the spores were diluted in NY broth for the germination test, and controls were run by diluting the spores in 0.01 M MOPS buffer at pH 7.5 and set to an absorbance of 0.09 (660 nm). Dilutions were 1:4 (diluent to spore suspension). In all the tests, samples for plating were taken at 0, 0.5, 1, 3, and 6 h; sometimes samples were taken at 10 h and 24 h to determine if counts changed during this period of time. Samples were heated at 80°C for 12 min before plating to eliminate germinated spores.

#### **Conjugation experiments in broth**

To standardize the conditions for in vitro experiments, matings in broth as well as matings on filter membranes were performed. The matings in broth were performed as described by Battisti et al. (11) with some modifications. Donor and recipient cells were separately grown in tubes containing 5 ml of NY broth. When necessary, these tubes were supplemented with the appropriate antibiotic to insure plasmid stability. After inoculation, the cultures were incubated overnight at 30°C in a rotary shaker at 160 rpm. One ml of each culture was transferred to a tube containing fresh sterile medium and incubated under the same conditions for 4 to 5 h. Matings were performed by mixing 2 ml of the donor, strain 2362 (pAMB1), and the recipient 2362a (lys<sup>-</sup>),

in a 125 ml flask containing 25 ml of NY medium. This mating mixture was incubated on a rotary shaker at 30°C for 20 h. The number of donors and recipients were enumerated in NYSM agar containing 10 ug/ml erythromycin or 25 ug/ml rifampicin, respectively. The transconjugants were isolated by plating in NYSM agar containing 10 ug/ml erythromycin and 25 ug/ml rifampicin.

#### **Conjugation experiments on filter membranes.**

The procedure I used was similar to that described by Koheler and Thorne (64) with some modifications. Donor and recipient cells were grown with shaking overnight in NY broth at 30°C. Two-tenths ml of each culture were transferred to 5 ml of NY broth and shaken at 30°C for 5 h. One ml each of donor and recipient were mixed and 0.1 ml of the mixture was spread onto a 45 mm diameter, 0.45 um membrane filter (Millipore. Bedford, MA.) previously placed on NYSM agar. The incubation period was 20 h, at 37°C, as determined in the initial experiments for the standardization of conditions. Cell growth on the membrane was scraped off using a bent glass rod, resuspended in 2 ml of NY broth, diluted and plated. Antibiotic-containing media was used for the selection of donor, recipient and transconjugant cells in the mixture. The selective medium for enumerating transconjugants contained 10 ug/ml erythromycin and 25 ug/ml rifampicin. Plates were incubated

at 30°C and counted after 24 to 48 hours.

#### **In vivo conjugation in larvae**

These experiments were conducted to determine if conjugation occurs in the larval cadaver. Conjugation in vivo was tested by feeding a mixture of spores of the donor bacterium and the recipient bacterium to third instar *Culex quinquefasciatus* larvae. Larvae were homogenized, diluted and plated on appropriate selective media at intervals. Spore counts were determined by diluting and plating heated (80°C, 12 min) samples of the homogenate.

#### **Phage typing**

This technique was used to verify the identity of transconjugants obtained in matings between strains of *B. sphaericus*. *Bacillus sphaericus* strains were grown overnight in 5 ml NY broth. One-tenth ml of the overnight culture was added to 4 ml melted "soft" agar (NY broth supplemented with 0.7% agar) and poured over a plate of NYSM agar to obtain a lawn. The plates were allowed to dry and then spotted with 5 ul of diluted phage. Phage dilutions from frozen stock were done in 0.01 M MOPS buffer, pH 7.0. The parent strain, sensitive to the particular phage, was used as a positive control. Observation of clear plaques on the lawn constituted a positive test. A strain not sensitive to that particular phage was used as the negative control.

#### **Isolation of *B. sphaericus* auxotrophs.**

An auxotrophic variant of *B. sphaericus* 1691 was isolated to facilitate its identification as recipient of conjugative plasmids during mating experiments. *Bacillus sphaericus* was grown overnight with shaking in 5 ml NY broth at 30°C. The tube contents were transferred to a flask containing 20 ml NY broth, and this culture was shaken until reaching a turbidity of 90 Klett units (red filter). A stock solution of 1-methyl-3-nitro-1-nitrosoguanidine (NTG) was added to achieve a final concentration of 20 ug/ml, and this mixture was shaken for 20 min at 30°C. This suspension was centrifuged, the pellet was suspended in 20 ml NY broth, and shaken for 4 h to allow phenotypic expression. The culture was centrifuged, the pellet washed twice in BAT broth (125), suspended in 20 ml BAT broth and shaken for 2 h at 30°C. Ten mg of ampicillin was added, and this mixture was again shaken for 4 h. Cells were centrifuged, washed twice in NY broth, the pellet suspended in NY broth and shaken overnight at 30°C. This culture was diluted, spread on NY agar and incubated at 37°C. Auxotrophs were selected following replica plating on NY and BAT+ agar plates. Specific auxotrophic requirements were determined by transferring auxotrophs to BAT+ (BAT agar supplemented with 0.25% w/v sodium acetate and 0.1% w/v potassium glutamate) supplemented with various individual amino acids.

### **Preparation of cell walls and S-layer protein**

*Bacillus sphaericus* cultures grown in NY broth were harvested by centrifugation when they reached the early stationary phase. The pellets were resuspended in cold, sterile distilled water, and cells were broken by sonication with a Sonic Dismembrator (model 300; Fisher Scientific Co. Pittsburgh, Pa.). The broken cells were centrifuged at 27,200xg for 20 min, and the pellet was resuspended in cold, sterile distilled water and washed twice by centrifugation. The preparation of the S-layer protein was done as described by Lewis et al. (70). For S-layer protein extraction, broken cells were resuspended in unbuffered 6 M urea, and incubated for 4 h at 37°C with shaking at 250 rpm. After extraction, the broken cells were removed by centrifugation at 27,000xg for 20 min, and the supernatant fluid was dialyzed overnight at 4°C against three changes (3 L each) of 0.01 M Tris hydrochloride pH 8.0. The protein present in the dialyzed fluid was precipitated by adding ammonium sulfate 55% saturation. This precipitate was centrifuged at 27,000xg, the pellet was resuspended in 5 ml of 0.01 M Tris hydrochloride at pH 8.0, and dialyzed overnight against 0.01 M Tris hydrochloride at pH 8.0. The precipitated protein was centrifuged and resolubilized in unbuffered 6 M urea at 37°C with shaking.

### **Sodium dodecyl sulfate polyacrylamide gel electrophoresis**



### **(SDS-PAGE)**

Slab gel electrophoresis was performed under denaturing conditions as described by Lewis et al. (70). Gels were electrophoresed using the discontinuous buffer system with 1.5 mm thick gels consisting of 7.5% acrylamide separating gels and 3% acrylamide stacking gels. Samples were treated with 2% SDS and 5% mercaptoethanol and boiled for 2 min before being loaded onto the gel. After electrophoresis, the gels were stained with Coomassie blue and destained in 40% methanol-10% acetic acid.

### **Plasmid isolation**

The procedure used for plasmid isolation was a modification of the method described by Kado and Liu (58). Two tubes containing 5 ml NY broth were inoculated with cells from stock slants. After incubation for 12-15 h at 30°C with shaking at 150 rpm, the cells were harvested by 10 min centrifugation at 8000xg. Pellets were resuspended in 200 ul of E buffer containing 1 mg/ml of lysozyme. After mixing thoroughly, tubes were incubated for 10 min at room temperature. Four hundred ul of lysis buffer were added, and tubes were mixed gently by wrist action. Samples were incubated at 60°C for 30 min, cooled, and 100 ul of 2 M Tris was added. Samples mixed gently by wrist action were incubated for 15 min at room temperature and extracted by adding 1.2 ml of a phenol-chloroform mixture (50:50,

vol/vol). Samples were mixed for 5 min on a roller drum and centrifuged at 13,000xg for 20 min. The top aqueous layer was removed and stored refrigerated in sterile microfuge tubes. Plasmid precipitation was done to concentrate the plasmid DNA. Precipitation was accomplished by adding one-tenth volume of LiCl<sub>2</sub> buffer pH 8.0, 2 ul (20 mg/ml) of glycogen and 2.5 volume of cold 95% ethanol to the tube containing the sample. After mixing gently, the samples were placed overnight at -20°C. The precipitate was recovered by centrifugation in the cold. The supernatant fluid was discarded, and the pellets were dried under vacuum at room temperature. The pellets were resuspended in 27 ul of TE buffer. Twenty-five ul of each sample were mixed with 5 ul of tracking dye. Agarose gels (0.7%) were electrophoresed at 5 volts/cm until the dye reached the end of the gel. Gels were stained with 0.5 ug/ml ethidium bromide for 15 min.

## Media formulations

### **NYSM broth**

Difco nutrient broth	0.8 g/100 ml
Yeast extract	0.05 g/100 ml
MnCl <sub>2</sub>	5x10 <sup>-5</sup> M
CaCl <sub>2</sub>	7x10 <sup>-4</sup> M
MgCl <sub>2</sub>	1x10 <sup>-3</sup> M

For NYSM agar add 2.0 g agar/100 ml.

### **BAT Medium**

Stock solution 1.

	50ml stock
MgSO <sub>4</sub> ·7H <sub>2</sub> O	25 mg
MnCl <sub>2</sub> ·4H <sub>2</sub> O	4 mg
FeSO <sub>4</sub> ·H <sub>2</sub> O	3 mg
CaCl <sub>2</sub> ·2H <sub>2</sub> O	2 mg
H <sub>2</sub> SO <sub>4</sub> (concentrate)	15 ul

Autoclave

Stock Solution 2

	50ml stock
L-arginine	5 g
biotin	2 ug
thiamine	20 mg

Filter sterilize

**BAT Medium (continued)**

Stock solution 3

	90 ml
Na <sub>2</sub> HPO <sub>4</sub>	0.56 g
KH <sub>2</sub> PO <sub>4</sub>	0.24 g
Agar	2.0 g

Autoclave

5 ml of stock solutions 1 and 2 is added when stock solution 3 is cool. To grow lysine or histidine auxotrophs, 0.2 g L-lysine or histidine were added per 90 ml solution 3.

**Modified BAT medium (BAT+).**

Stock solutions 1 and 2 were the same as in BAT. Stock solution 3 was as follows:

Na <sub>2</sub> HPO <sub>4</sub>	0.56 g
KH <sub>2</sub> PO <sub>4</sub>	0.24 g
Na acetate	0.25 g
L-glutamate (Na or K salt)	0.1 g

Autoclave

Five ml of stock solutions 1 and 2 were added when stock solution 3 was cool.

## RESULTS

### Spore Germination and Recycling

Spontaneous rifampicin-resistant mutants were obtained from the parent strains by growing the bacteria in increasing concentrations of the antibiotic. All strains were resistant to at least 25 ug/ml rifampicin. Spore suspensions of the strains were used to feed the larvae in the recycling and conjugation experiments.

The ability of spores to germinate, grow vegetatively and resporulate with concomitant toxin formation inside the cadaver is known as "recycling". Germination is defined as loss of heat resistance measured by plate counts after heating of the sample at 80°C for 12 min. The ability of various *B. sphaericus* strains to germinate and recycle was investigated. Strains tested for recycling represented different serological and phage groups and produced either or both larvicidal toxins, the binary toxin (51 and 42 kDa) and the 100-kDa toxin. Also, nontoxic strains were tested either as recombinants producing toxin or with soluble, binary toxin.

Larvae were fed spores in a concentration of  $1-5 \times 10^6$  spores/ml. Spore counts at zero h showed that the number of spores taken by the larvae after 15 min of feeding on the spore suspension was ca.  $10^5$  to  $10^6$ /larva. A 15 min feeding

period was enough time for maximum larval feeding capacity. Feeding for a longer period of time resulted in little or no increase in the number of spores found in the larvae after their removal from the spore suspension. This was shown in experiments in which larvae were fed with spores of strain LP12AS for 15 and 45 min. The number of LP12AS spores ingested at 15 min was of  $3.7 \times 10^5$ /larva and the number of spores ingested at 45 min was  $8.4 \times 10^5$ /larva.

In other experiments, larvae were fed in two different concentrations of a 2362-7 spore suspension. Counts at 0 h showed that the number of spores taken by the larvae after 15 min of feeding on a spore suspension of ca.  $4.0 \times 10^6$  spores/ml was of  $9.9 \times 10^5$ /larva. In the lower concentration,  $4.0 \times 10^5$  spores/ml, the number of spores ingested at 0 h was  $2.7 \times 10^5$ /larva, but the recycling pattern was not altered (Fig. 1).

In the recycling experiments, the number of spores ingested was determined by homogenizing the larvae. The number of spores adhering to the outside of the cuticle of the dead larvae was estimated to determine if these spores significantly affected counts of total number of spores ingested. These counts of spores adhering to larval surface ranged between  $10^1$  and  $10^2$ /larva and therefore represented an insignificant fraction of the total number of spores associated with larvae.

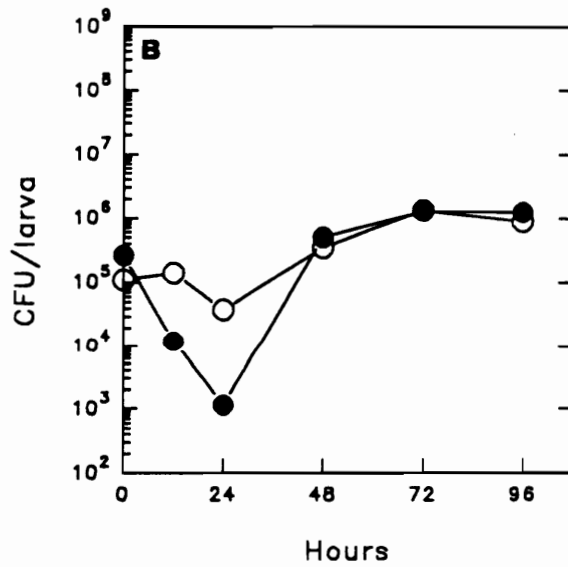
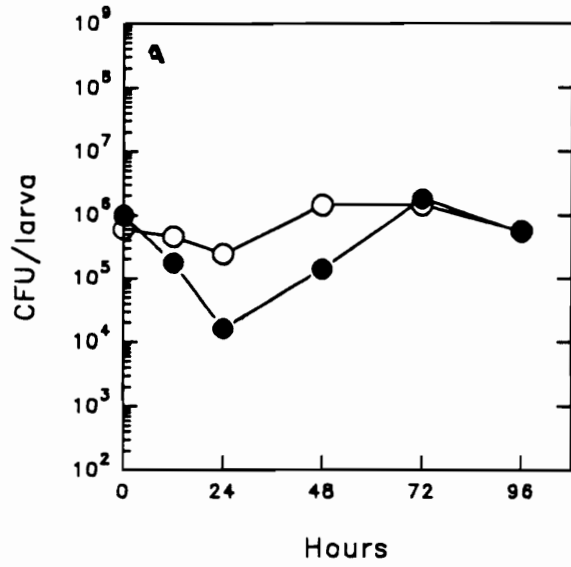


FIG. 1. Recycling of *B. sphaericus* 2362-7 in larvae fed in two different concentrations of spores. A:  $4.0 \times 10^6$  spores/ml; B:  $4.0 \times 10^5$  spores/ml. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells).

Larvae fed with the high toxicity strains 2362-7 or IAB59 died within the 12-24 h following ingestion of spores and attached paraspores. Rapid larval death was also observed when normally nontoxic strains carrying the binary toxin genes on a recombinant plasmid were fed, or if soluble binary toxin extracted from strain 2362 was fed along with nontoxic or low-toxicity strains. Although strains 2297 and LP12AS produced binary toxin, larvae died somewhat more slowly than when fed 2362-7 or IAB59. As a result, larvae still living after 24 h were removed from pans. Larvae fed the low toxicity strains 1883 or Q or the recombinant NRS 1693(pC35) producing the 100-kDa toxin died slowly, usually during the 24-48 h following feeding. Larvae remaining alive after 48 h were removed from pans. When non-pathogenic strains were fed to larvae, no mortality occurred. In this case the larvae were changed to fresh water every 24 h to avoid the possibility that the larvae would re-ingest the spores if they were gradually eliminated.

Spores of the high toxicity strains were initially tested for recycling ability. Upon ingestion of 2362-7 (serotype 5a5b) spores, a high percentage of the spores germinated rapidly during the 12 h after their ingestion. This was evidenced by the considerable decrease in heat resistant spore numbers during this period of time (Fig. 2). During the next 48 h sporulation occurred with an increase



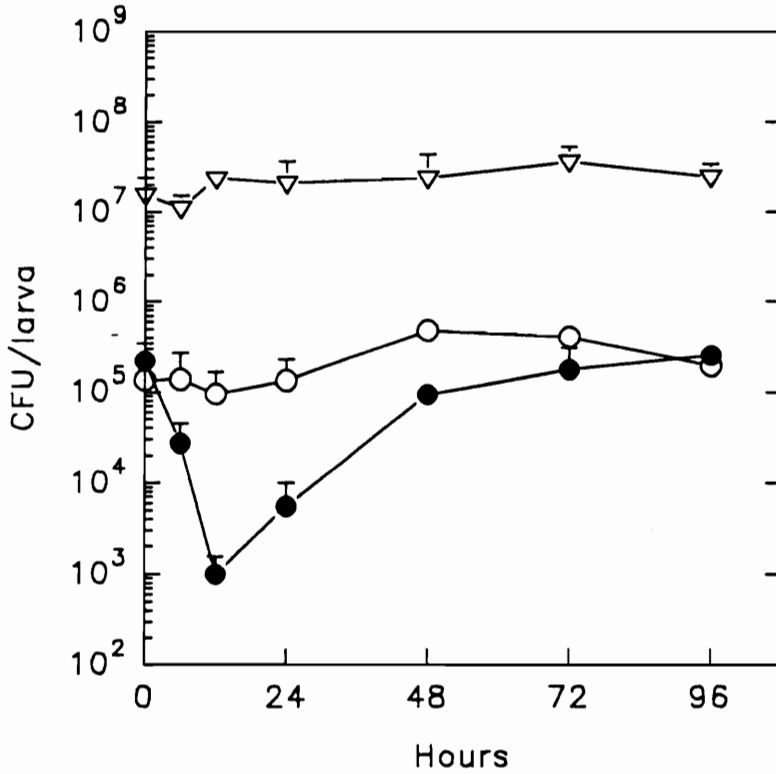


FIG. 2. Recycling of *B. sphaericus* 2362-7 in larval cadavers. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells); (▽) Total bacteria associated with cadavers.

in the spore number until 72 or 96 h. The final number of spores was between  $10^5$  to  $10^6$  spores/larva. To test if new toxin was being produced in larval cadavers, equal numbers of 24 h and 72 h cadavers were homogenized and used in bioassays. Larval mortality was higher with homogenates of 72 h cadavers indicating production of toxin within the cadavers during the 24 h to 72 h time frame.

The same general pattern of spore germination, growth and resporulation was observed with other high toxicity strains. Strain IAB 59, serotype 6 (Fig. 3) and 2297, serotype 25 (Fig. 4), produce both the binary toxin and the 100-kDa toxin, as does 2362. In experiments with IAB59 and 2297 the final number of spores varied, although, in general, it was about the same as the number of spores initially ingested or slightly higher.

The total number of *B. sphaericus* (spores plus vegetative cells) in cadavers was monitored by plating dilutions of non-heated samples in NYSM medium containing 25 ug/ml rifampicin. The total number of *B. sphaericus* remained high in all the trials except for strain IAB59 (Fig. 4). With this strain there was some decrease during the 12 h after ingestion, but this decrease was less than the spore count decrease. Decrease in the spore count with little or no decrease in the total *B. sphaericus* is indication of spore germination rather than loss due to defecation or loss

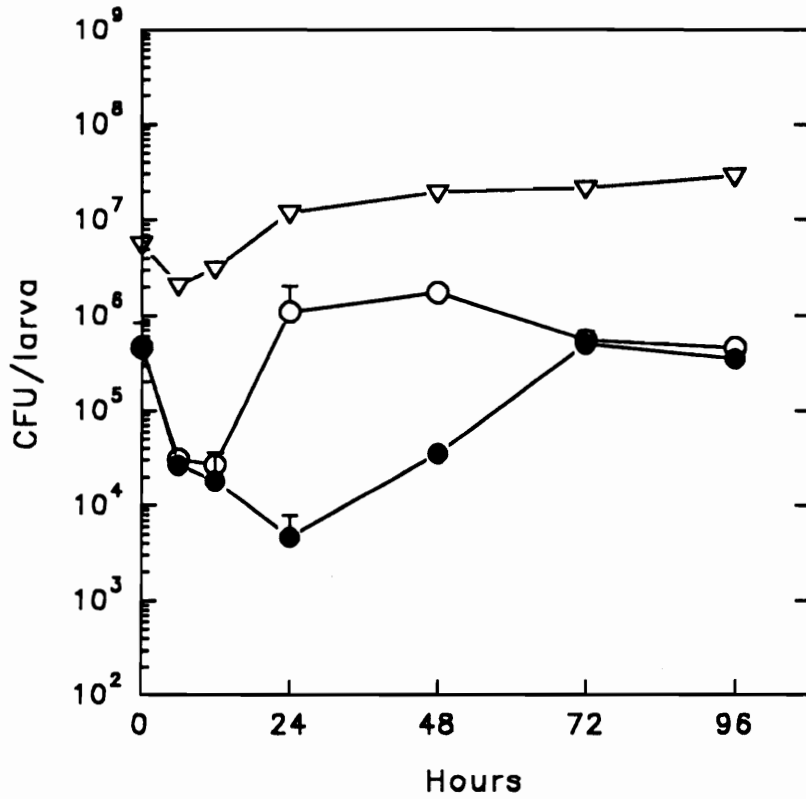


FIG. 3. Recycling of *B. sphaericus* IAB 59 in larval cadavers. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells); (▽) Total bacteria associated with cadavers.

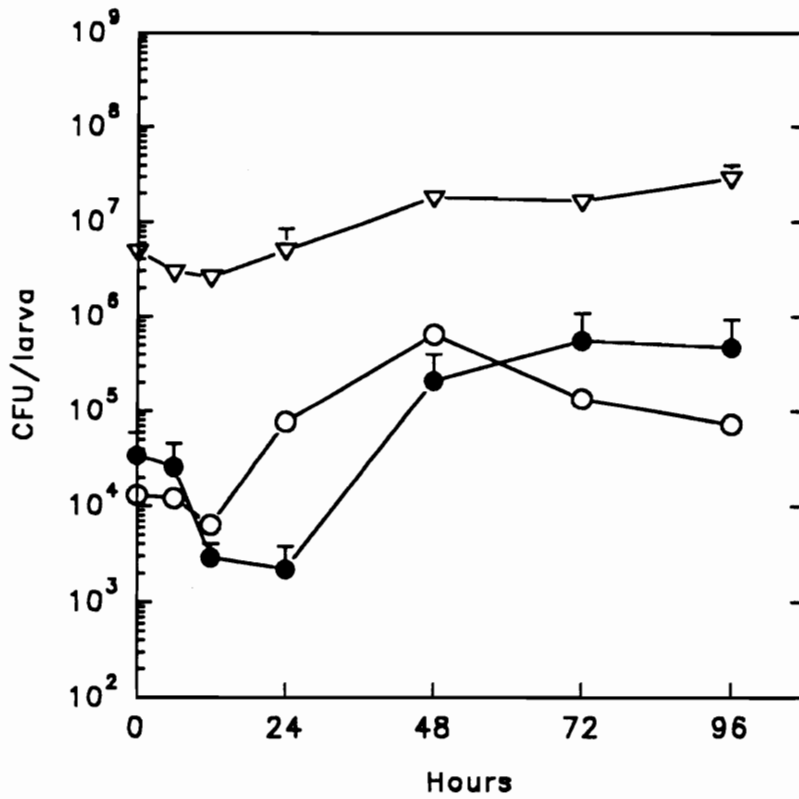


FIG. 4. Recycling of *B. sphaericus* 2297 in larval cadavers. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells); (▽) Total bacteria associated with cadavers.

of cell viability. It was generally observed that the number of total bacterial flora in the larvae increased after death of the larvae. The highly toxic LP12AS strain, which contains the binary toxin and lacks the gene for the 100-kDa toxin, showed very poor germination and little decrease or increase in the number of spores (Fig. 5).

To determine if presence of a particular toxin, binary or 100-kDa, had an effect on spore germination and recycling, strains producing various toxin combinations were tested. In addition to high toxicity strains, non-toxic and low toxicity strains were fed to larvae alone or in combination with soluble binary toxin from strain 2362. Also nontoxic bacteria carrying recombinant plasmids expressing larvicidal toxin were tested.

The recycling ability of strains producing only the 100-kDa toxin was tested with strain 1883, serotype 2 (Fig. 6). The number of spores and total *B. sphaericus* cells decreased during the initial 24 h. Subsequently, there was a small increase in spores and total *B. sphaericus* cells, reaching a number similar to that originally consumed. The decrease in spore number was less than that observed for the highly toxic strains producing both toxins. To test if adding the binary toxin promoted the recycling of this strain, spores of strain 1883 were fed to larvae in combination with soluble 2362 binary toxin (Fig. 7).

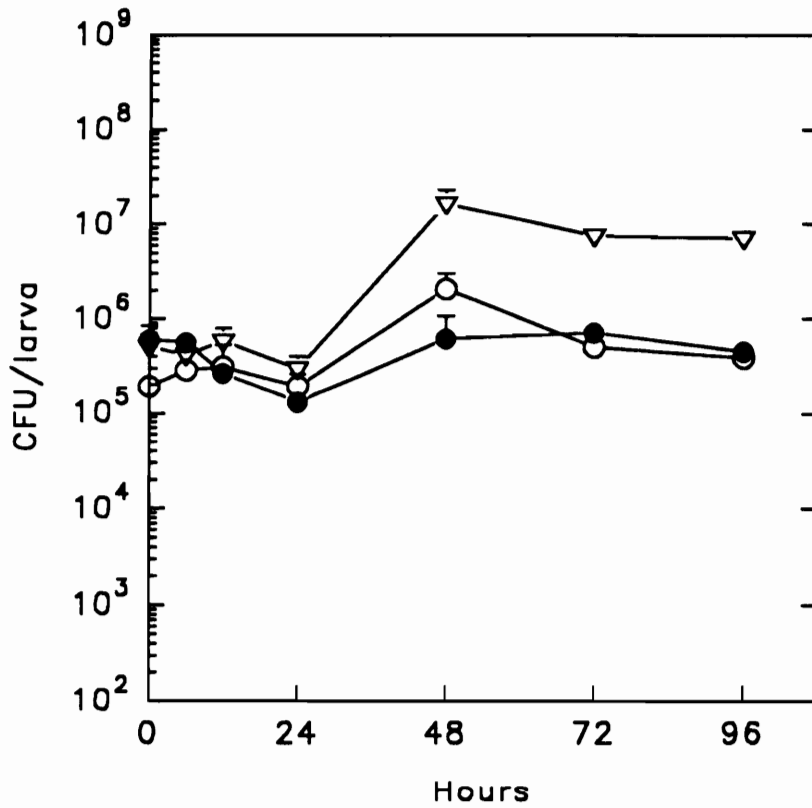


FIG. 5. Recycling of *B. sphaericus* LP12AS in larval cadavers. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells); (▽) Total bacteria associated with cadavers.

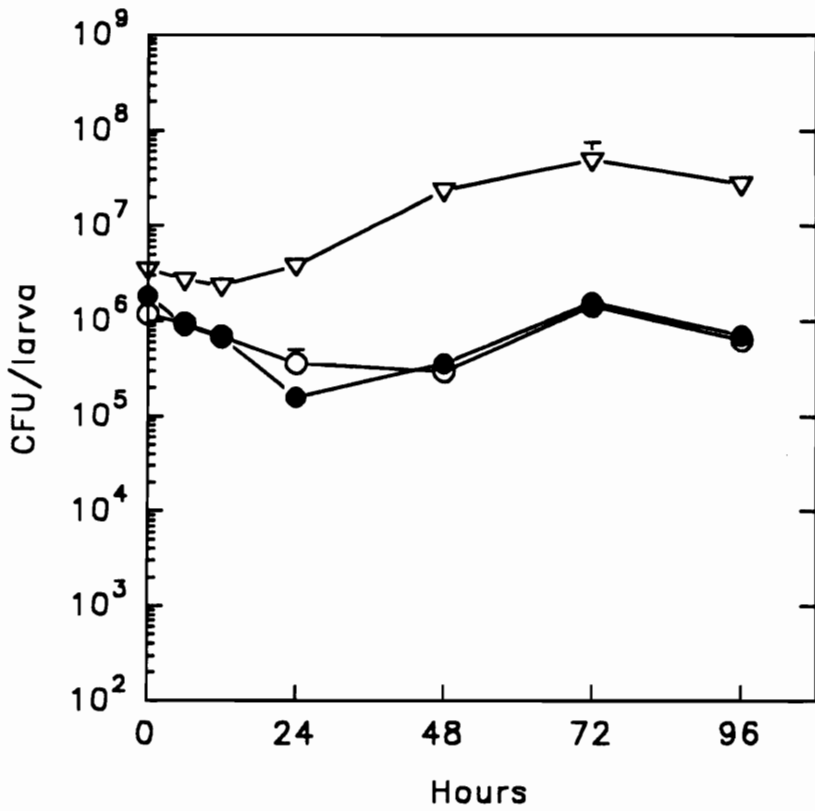


FIG. 6. Recycling of *B. sphaericus* 1883 in larval cadavers. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells); (▽) Total bacteria associated with cadavers.

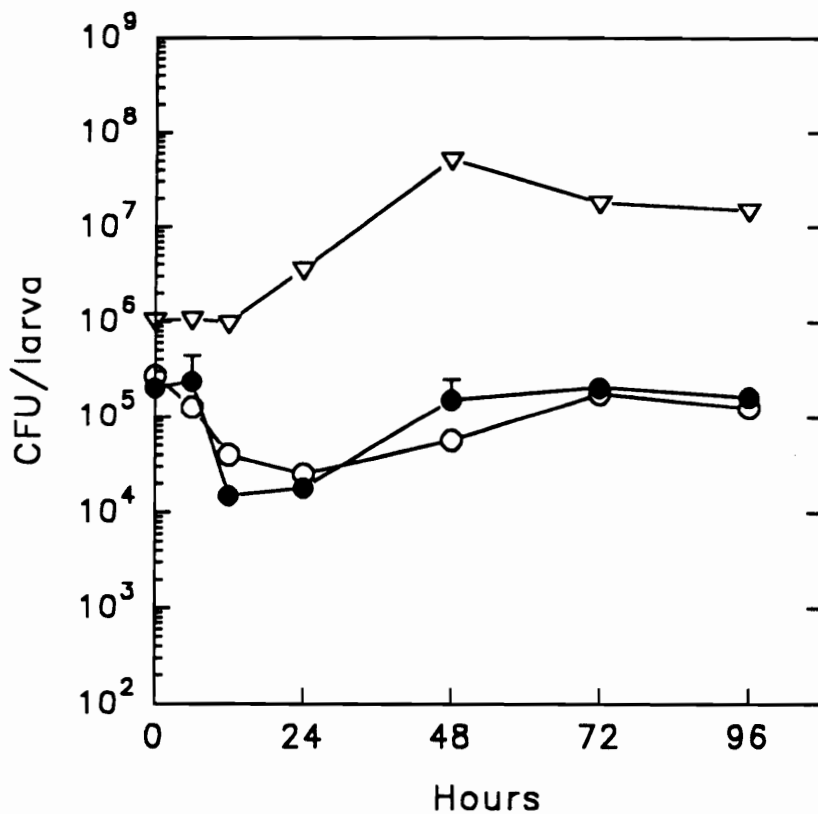


FIG. 7. Recycling of *B. sphaericus* 1883 in larval cadavers. Spores were fed along with strain 2362 soluble binary toxin. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells); (▽) Total bacteria associated with cadavers.



Although the larvae died faster and germination occurred more rapidly, the extent of germination was the same as when binary toxin was absent. A decrease in the number of total *B. sphaericus* cells paralleled the decrease in spores. In both cases, germination and recycling ability were lower than observed for the high toxicity strains.

*Bacillus sphaericus* Q (serotype 1), a low toxicity strain producing only the 100-kDa toxin was tested for its ability to recycle (Fig. 8). Spores and total cell number of *B. sphaericus* Q decreased up to 24 h. After this time the number of spores kept decreasing while the total cell number stabilized. There was no indication of vegetative growth. Probably vegetative forms predominated after larval death but were unable to resporulate.

Spores of the nontoxic *B. sphaericus* ATCC 14577, the type strain of the species, were fed to larvae along with soluble binary toxin (Fig. 9). Although larvae died rapidly due to the effect of the toxin, the number of spores decreased only slightly during the sampling period. In experiments with strain 14577, the spore counts were usually higher than the total *B. sphaericus* count.

Another non-pathogenic *B. sphaericus*, strain NRS 718, that produces neither toxin was tested alone (Figure 10), in combination with soluble binary toxin from 2362 (Figure 11) and as a recombinant carrying on a plasmid (pUE382) the

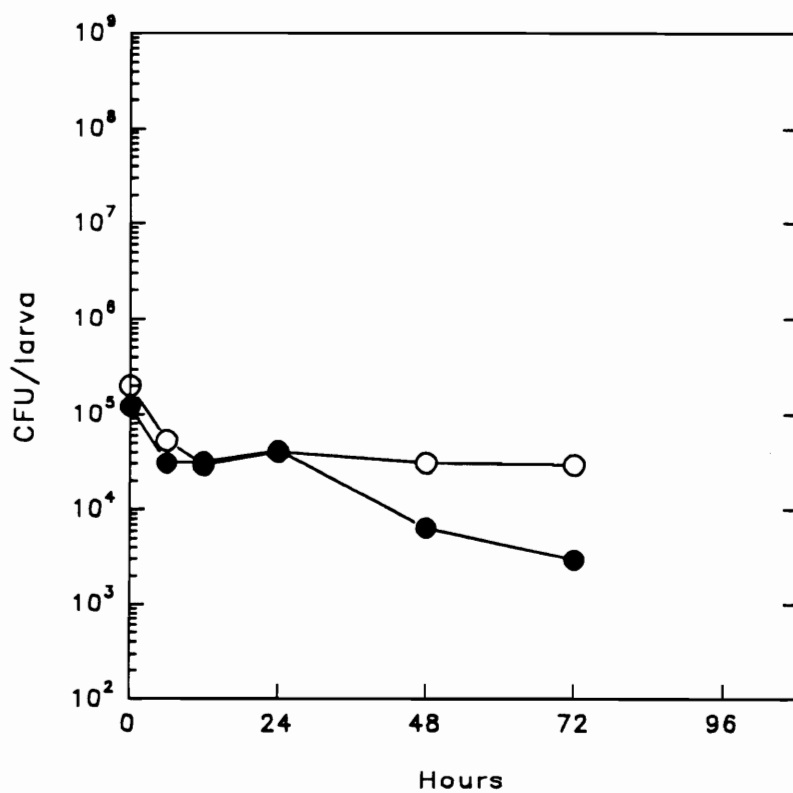


FIG. 8. Recycling of *B. sphaericus* Q in larval cadavers. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells).

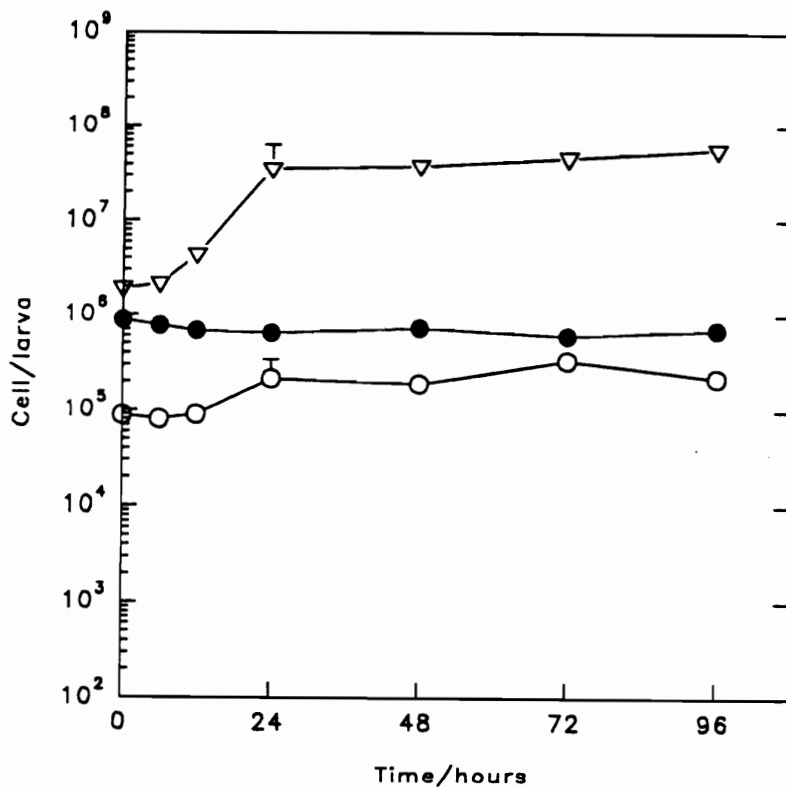


FIG. 9. Recycling of the nontoxic *B. sphaericus* ATCC 14577 in larval cadavers. Spores were fed along with the soluble binary toxin from strain 2362. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells); (▽) Total bacteria associated with cadavers.

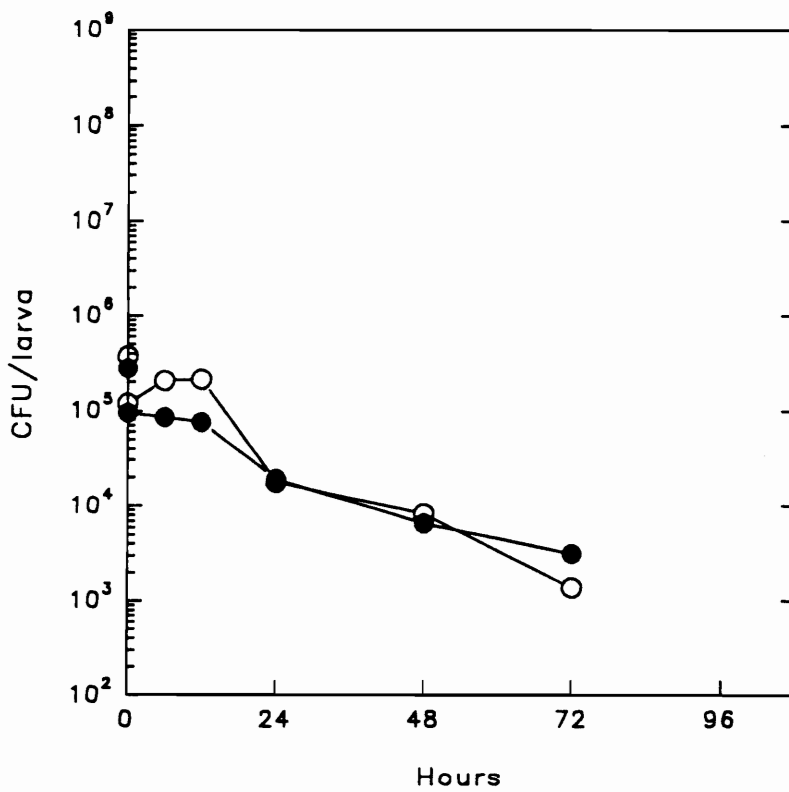


FIG. 10. Recycling of *B. sphaericus* NRS 718 in larvae. (○) *B. sphaericus* spores; (●) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells).

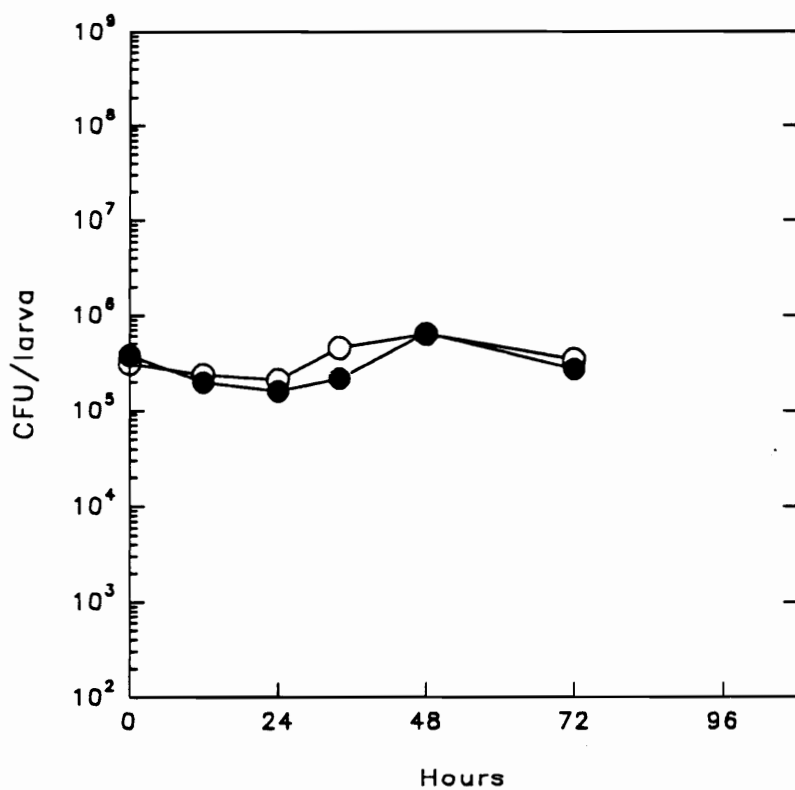


FIG. 11. Recycling of *B. sphaericus* NRS 718 in larval cadavers. Spores were fed along with strain 2362 soluble binary toxin. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells).

genes for the binary toxin (Figure 12). Spores of the Spores of the nonpathogenic 718 did not kill the larvae. It was of interest to follow the fate of the spores when the larvae remained alive for the length of the experiment. The results showed that both spores and total *B. sphaericus* cells decreased in number through time (Fig. 10). Spores were probably eliminated by defecation after ingestion. In other experiments, larvae were fed with NRS 718 spores and the soluble binary toxin (Fig. 11) or with the recombinant strain (Fig. 12). In both cases there was little spore germination or increase in the spore number in cadavers.

*Bacillus subtilis* DB104 (pUE382), a recombinant carrying binary toxin genes, was tested for its ability to germinate and recycle in the larval cadaver. *B. subtilis* produces spores but is not closely related to *B. sphaericus*. However, it was genetically transformed to produce the binary toxin and therefore behave as a mosquito pathogen. The recombinant strain (Fig. 13) was compared to the parent, nontoxic strain, *B. subtilis* DB104 (Fig. 14). The recombinant killed the larvae but the parent did not. The lack of spore germination observed in larvae killed by *B. subtilis* DB104 (pUE382) was very similar to the pattern seen with *B. sphaericus* NRS 718 (pUE382) (Fig. 12).

The effect of the 100-kDa toxin was also tested using the nonpathogen *B. sphaericus* 1693 which expressed the

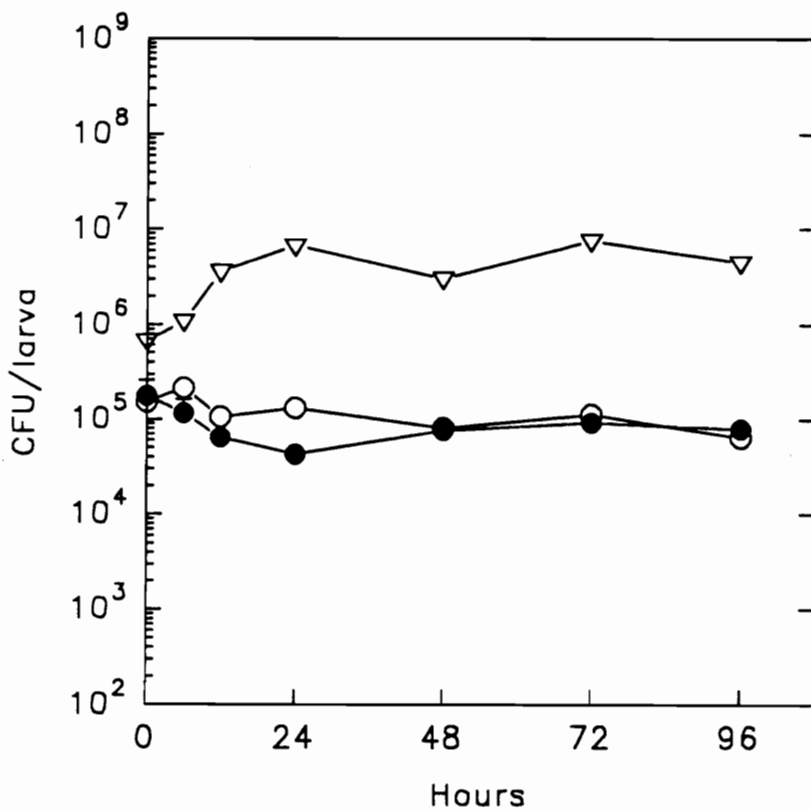


FIG. 12. Recycling of *B. sphaericus* NRS 718 (pUE382) in larval cadavers. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells); (▽) Total bacteria associated with cadavers.

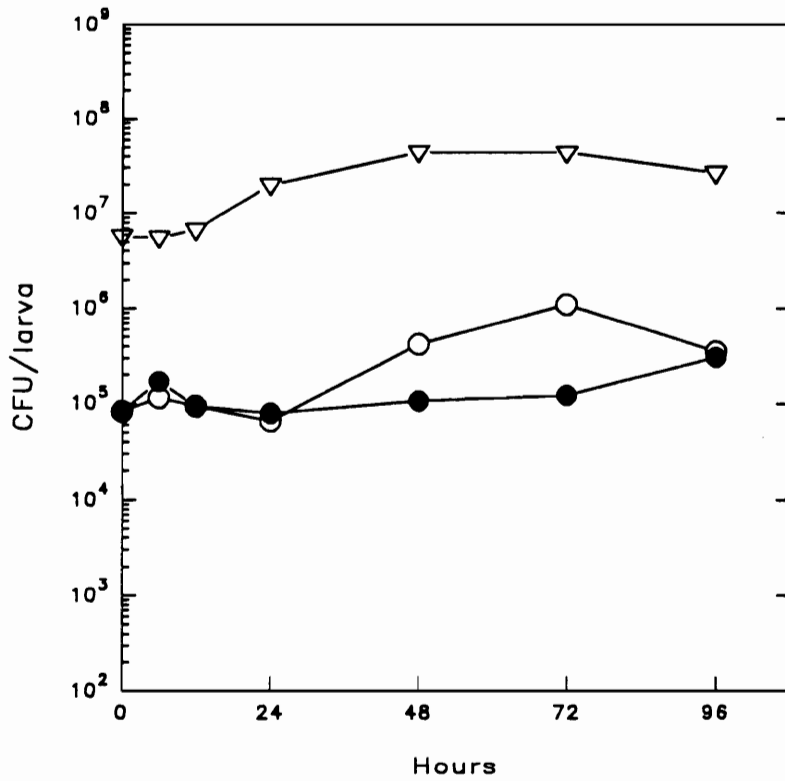


FIG. 13. Recycling of *B. subtilis* DB104 (pUE382) in larval cadavers. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells); (▽) Total bacteria associated with cadavers.



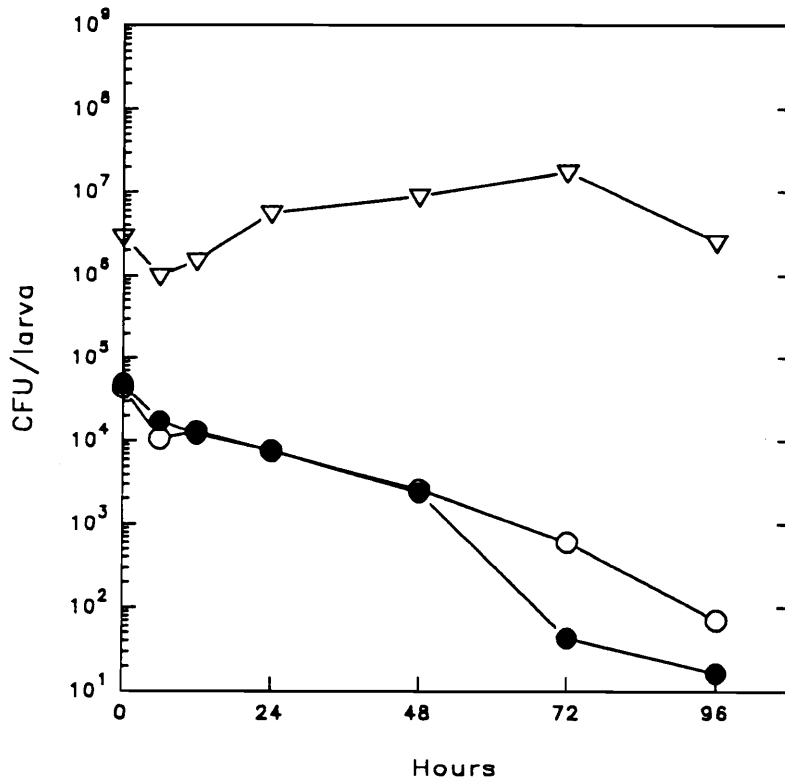


FIG. 14. Recycling of *B. subtilis* DB104 in larvae.  
 (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells  
 (spores, germinated spores and vegetative cells);  
 (▽) Total bacteria associated with larvae.

100-kDa toxin from recombinant plasmid (pC35). These spores germinated poorly in the larval cadaver, and there was no increase in the number of spores (Fig. 15). To complete the scheme of possible combinations of toxins and finally determine the influence or role of the toxins in spore germination and recycling, one more toxin combination was assayed. Spores of 1693(pC35) were fed to larvae in combination with soluble toxin (Fig. 16). The same pattern and extent of spore germination observed with strain 1693(pC35) alone as was observed when adding the soluble toxin. An overall decrease in the number of spores was observed 96 h after ingestion (Fig. 15).

The ability of *B. thuringiensis* subsp. *israelensis* (Bti) to recycle was also studied (Fig. 17). Although there was an increase in the final number of spores per cadaver, the initial spore germination was poor.

#### **In vitro spore germination**

It was of interest to determine if good (or poor) spore germination in cadavers was reflected in similar germination pattern in vitro.

Three strains representing members of each group of toxin expression were tested. Strain 2362-7 produces the binary and 100-kDa toxin, strain 1883 produces only the 100-kDa toxin and LP12AS only produces the binary toxin.

Data from trials using three different germinants

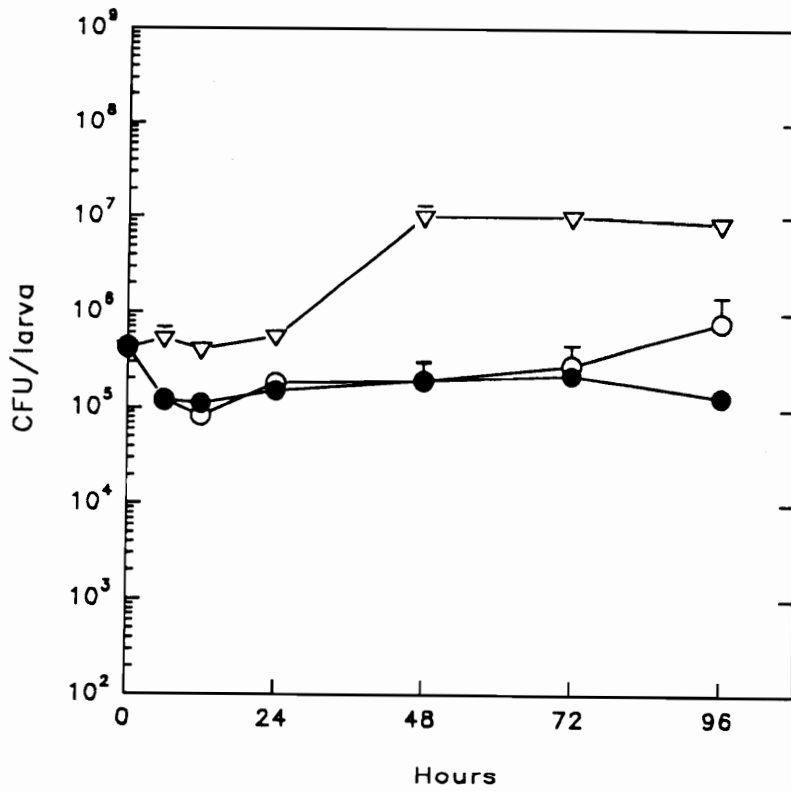


FIG. 15. Recycling of *B. sphaericus* 1693 (pC35) in larval cadavers. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells); (▽) Total bacteria associated with cadavers.

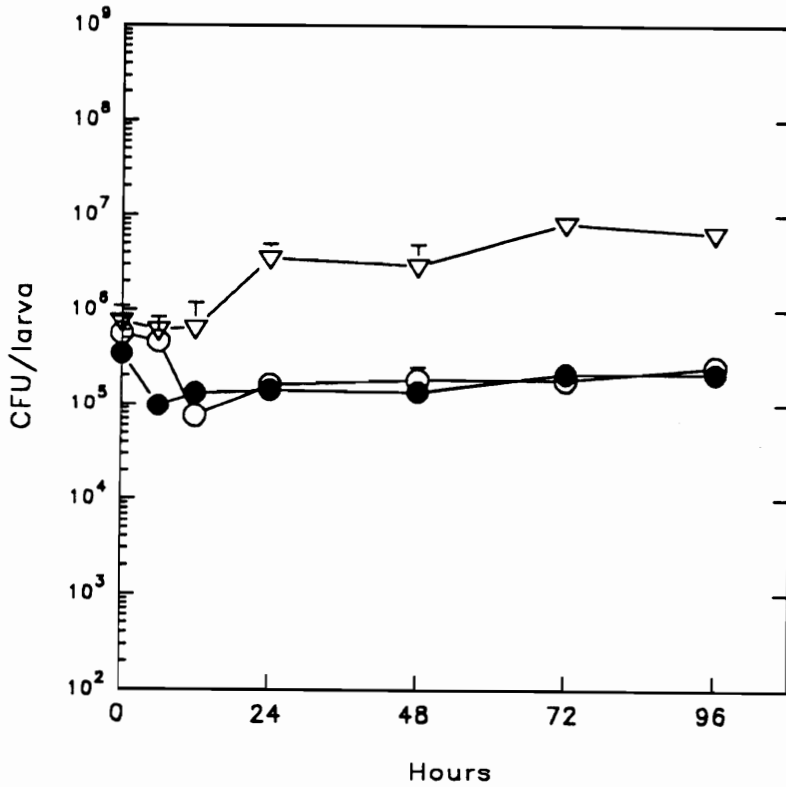


FIG. 16. Recycling of *B. sphaericus* 1693 (pC35) in larval cadavers. Spores were fed along with the soluble binary toxin from strain 2362. (●) *B. sphaericus* spores; (○) Total *B. sphaericus* cells (spores, germinated spores and vegetative cells); (▽) Total bacteria associated with cadavers.

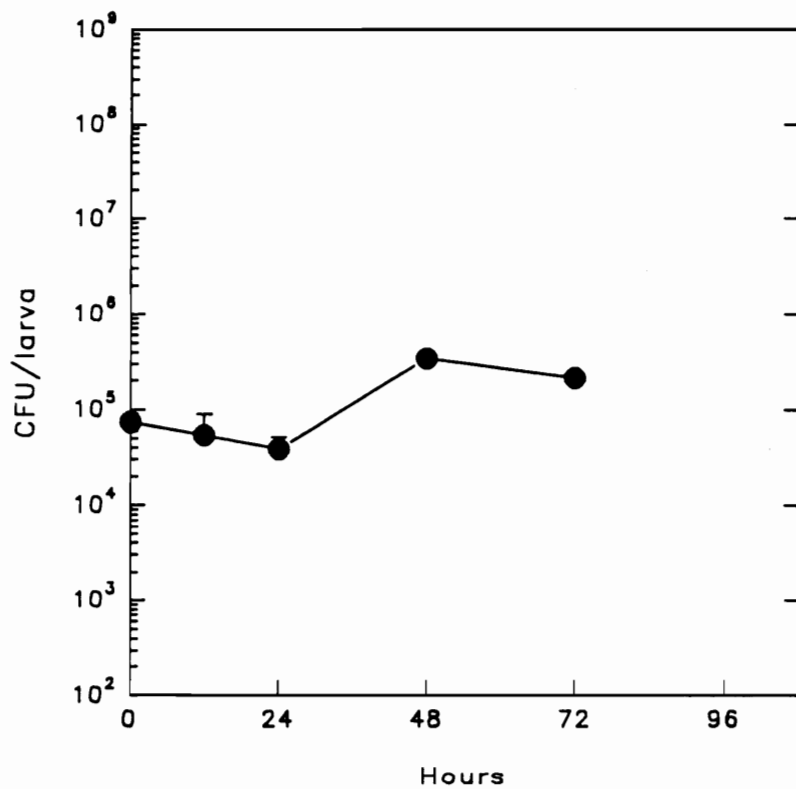


FIG. 17. Recycling of *B. thuringiensis* serovar. *israelensis* (Bti) in larval cadavers. (●) Bti spores.

(alanine/inosine, arginine, and NY broth) were plotted in a single figure for each strain tested. Data from spore germination in larvae was plotted in the same figure for comparison. This data was taken from experiments presented in previous figures. The highly toxic 2362-7 strain which produces both types of toxins, germinated in alanine/inosine more rapidly than in larvae (compared at 6 h). However at 12 h the extent of germination was about the same in vitro and in vivo. In NY broth or in arginine, germination was very low (Fig. 18). Strain 1883 (Fig. 19) which only expresses the 100-kDa toxin, also germinated more rapidly in alanine/inosine than in the larval cadaver. The spore counts decreased about ten fold. When tested in NY broth or mixed with arginine, germination was very low. Strain LP12AS (Fig. 20) produces the binary toxin but not the 100-kDa toxin. This strain showed low germination in larvae and also germinated poorly when mixed with alanine/inosine and with arginine. When the spores were suspended in NY broth, the spore counts remained constant.

### **Conjugation**

#### ***In vitro* conjugation experiments using plasmid pAMB1**

There is interest in expanding the spectrum of activity, increasing the level of toxicity and improving manufacturing efficiency of the mosquito pathogens. This might be done by introducing certain genes on a plasmid

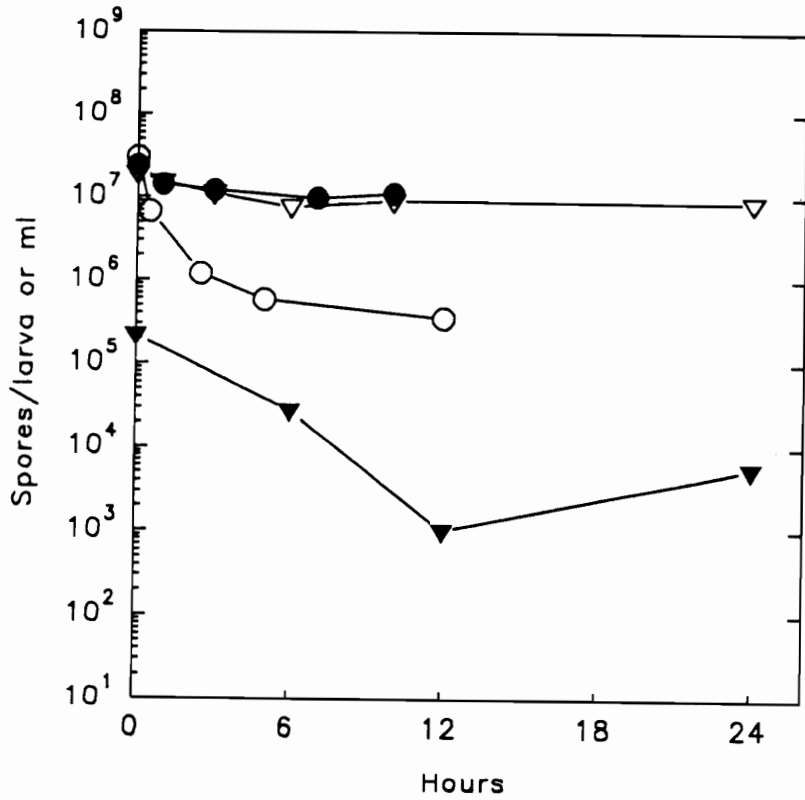


FIG. 18. Comparison of in vivo and in vitro spore germination by *B. sphaericus* 2362-7. (O) 0.06M alanine/0.06M inosine; (●) 0.1M arginine; (▽) NY broth; (▼) larval cadaver.

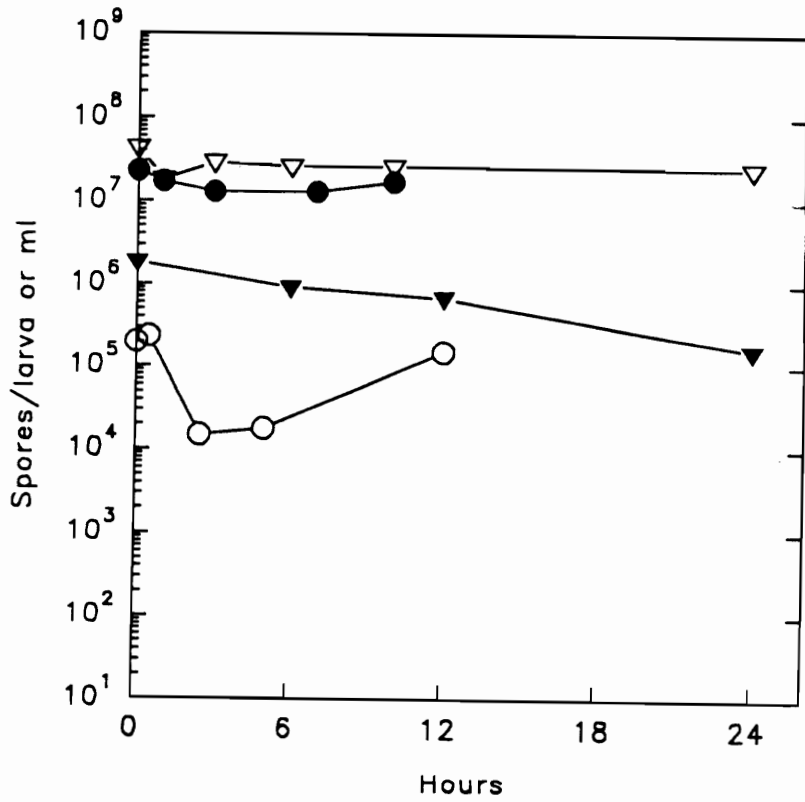


FIG. 19. Comparison of in vivo and in vitro spore germination by *B. sphaericus* 1883. (○) 0.06M alanine/0.06M inosine; (●) 0.1M arginine; (▽) NY broth; (▼) larval cadaver.



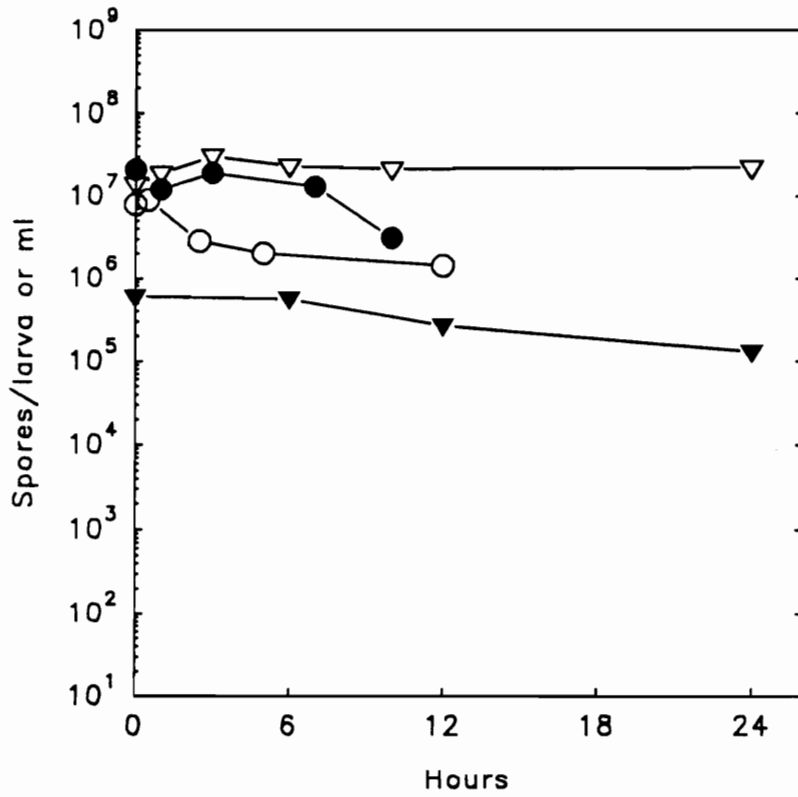


FIG. 20. Comparison of in vivo and in vitro spore germination by *B. sphaericus* LP12AS. (○) 0.06M alanine/0.06M inosine; (●) 0.1M arginine; (▽) NY broth; (▼) larval cadaver.

vector. Such a vector is also likely to carry an antibiotic resistance gene as a marker. The possibility of conjugal transfer of genetic information between environmental flora and recombinant mosquito pathogens might be favored by the close proximity in which these bacteria are found inside larval cadavers. While recycling of mosquito pathogens is considered a desirable feature, the conjugal transfer of genetic information contained in germinated spores may be undesirable, especially if that information involves antibiotic resistance.

The second part of this project was designed to assess the possibility that conjugal transfer of genetic information could occur between *B. sphaericus* and other related and non-related bacteria. The first step was the establishment of a set of conditions under which conjugation was most likely to be observed. This included the placement of a known conjugal plasmid into strain 2362 and determination of optimal mating conditions. The data obtained here would help to set the parameters to achieve our ultimate goal, to determine if *B. sphaericus* conjugally transfers genetic information to other bacteria *in vivo* when recycling in the larval cadaver.

Initial experiments were designed to establish an *in vitro* mating system with *B. sphaericus* that could be later used to test *in vivo* conjugation. A donor strain was

obtained by moving the promiscuous plasmid pAMB1 from *Enterococcus faecalis* JH2.2 into *B. sphaericus* 2362. The resulting strain was used as donor to optimize the *in vitro* mating experiments. In subsequent experiments the first strain tested as a recipient was *B. sphaericus* 2362a (lysine auxotroph and rifampicin resistant). Donor viable cell counts were performed in NYSM supplemented with 10 ug/ml of erythromycin (resistance conferred by pAMB1) and recipient counts were done in NYSM containing 25 ug/ml of rifampicin. Selection of transconjugants was done by plating in NYSM containing 25 ug/ml of rifampicin and 10 ug/ml of erythromycin. Donor and recipient cells were always plated in their respective opposite media and in selection media to test for the appearance of spontaneous mutants. Confirmation that colonies obtained in the selection medium were real transconjugants and not donors or recipients that were spontaneous mutants resistant to the antibiotics was done by plating these colonies on BAT<sup>+</sup> medium with and without lysine. The donor and recipient strains were also plated to serve as controls. The donor grew in both media whereas the recipient and transconjugants only grew in BAT<sup>+</sup> medium supplemented with lysine.

#### **Effect of incubation time on conjugation frequencies.**

Equal volumes of 5 h cultures of donor and recipient were mixed, and 0.1 ml of the mixture applied to the

membranes. Membranes were incubated at 30°C on non selective medium. Cells were washed off membranes with 2 ml NY broth at 3, 6, 9 and 20 h. These suspensions were diluted and plated on the selective medium for growth and expression of transconjugants. Results showed that conjugation could be detected as early as 3 h (Table 3), however high numbers of transconjugants were obtained after 9 h. Conjugation frequency increased less between 9 and 24 h than between 3 h and 6 h or between 6 h and 9 h.

**Effect of incubation temperature on conjugation frequencies.**

In these experiments, the conditions were as previously described. The membranes were incubated for 20 h at 25°C, 30°C and 37°C on nonselective medium. Frequencies of conjugation at 30°C were ca. 2-fold the frequencies observed at 25°C, and frequencies at 37°C were almost twice the frequencies obtained at 30°C (Table 4).

**Broth matings vs. mating on filter membrane.**

Conjugation in broth was performed by mixing 2 ml each of donor and recipient in a 125 ml flask containing 10 ml of fresh NY broth. Mating mixtures were incubated at 30°C for 20 h with shaking (100 rpm). The number of transconjugants was very low compared to numbers obtained in matings performed on membranes under similar conditions (Table 5).

From the above results, the conditions for further experiments were established. Matings were done on a solid

Table 3  
Effect of Mating/Expression Time on Frequency of Conjugation  
between *B. sphaericus* 2362 [pAMB1] and *B. sphaericus* 2362a<sup>1</sup>

Mating/expression time (hours)	Donor 2362[pAMB1] <sup>2</sup>	Recipient 2362a <sup>2</sup>	Transconjugants <sup>2</sup>	Frequency <sup>3</sup>
3	1.4x10 <sup>7</sup>	1.9x10 <sup>7</sup>	7.0x10 <sup>1</sup>	3.7x10 <sup>-6</sup>
6	2.0x10 <sup>8</sup>	2.3x10 <sup>8</sup>	1.9x10 <sup>3</sup>	8.3x10 <sup>-6</sup>
9	2.8x10 <sup>8</sup>	7.3x10 <sup>8</sup>	1.7x10 <sup>4</sup>	2.3x10 <sup>-5</sup>
20	3.6x10 <sup>8</sup>	4.6x10 <sup>8</sup>	3.2x10 <sup>4</sup>	7.0x10 <sup>-5</sup>

1 Equal volumes of 5h cultures were mixed and 0.1 ml applied to the membrane surface. *B. sphaericus* 2362 [pAMB1] (1.8x10<sup>8</sup> cells) and *B. sphaericus* 2362a (7.3x10<sup>8</sup> cells) were applied to the membranes. Membranes were incubated for indicated times at 30°C on nonselective medium. Cells were washed off membranes into 2 ml NY broth, diluted in NY broth, and plated on selective medium (25 ug/ml rifampin, 10 ug/ml erythromycin). No erythromycin resistant cells were detected among recipients plated prior to mixing with the donor.

2 cfu/ml in the NY broth used to wash the mating mixture off the membrane.

3 Number of transconjugants divided by number of recipients.

Table 4.  
Effect of Temperature on Frequency of Conjugation  
between *B. sphaericus* 2362 [pAMB1] and *B. sphaericus* 2362a<sup>1</sup>

Temperature (°C)	Donor 2362 [pAMB1] <sup>2</sup>	Recipient 2362a <sup>2</sup>	Transconjugants <sup>2</sup>	Frequency <sup>3</sup>
25 (Trial 1)	5.2x10 <sup>8</sup>	1.2x10 <sup>9</sup>	1.6x10 <sup>4</sup>	1.3x10 <sup>-5</sup>
(Trial 2)	2.4x10 <sup>8</sup>	1.4x10 <sup>9</sup>	3.8x10 <sup>4</sup>	2.7x10 <sup>-5</sup>
30 (Trial 1)	5.0x10 <sup>8</sup>	2.2x10 <sup>9</sup>	4.3x10 <sup>4</sup>	2.0x10 <sup>-5</sup>
(Trial 2)	1.3x10 <sup>8</sup>	4.9x10 <sup>8</sup>	2.2x10 <sup>4</sup>	4.5x10 <sup>-5</sup>
37 (Trial 1)	5.6x10 <sup>8</sup>	8.8x10 <sup>8</sup>	3.9x10 <sup>4</sup>	4.4x10 <sup>-5</sup>
(Trial 2)	2.4x10 <sup>8</sup>	2.5x10 <sup>8</sup>	2.3x10 <sup>4</sup>	9.2x10 <sup>-5</sup>

<sup>1</sup> Equal volumes of 5h cultures were mixed and 0.1 ml applied to the membrane surface. In trial 1, 2.5x10<sup>6</sup> cells of donor and 2.2x10<sup>6</sup> cells of recipient were placed onto the membranes. In trial 2, 1.2x10<sup>7</sup> cells of donor and 6.5x10<sup>6</sup> cells of recipient were placed onto the membranes. Membranes were incubated for 20 hours at the indicated temperatures on nonselective medium. Cells were washed off membranes into 2 ml NY broth, diluted in NY broth, and plated on selective medium (25 ug/ml rifampin, 10 ug/ml erythromycin). No erythromycin resistant cells were detected among recipients plated prior to mixing with the donor.

<sup>2</sup> cfu/ml in the NY broth used to wash the mating mixture off the membrane.

<sup>3</sup> Number of transconjugants divided by number of recipients.

Table 5

*B. sphaericus* 2362 [pAMB1] conjugation frequencies  
on membranes and in broth

Conjugation in/on	Conjugation frequency (mean)	n =
broth	$7.20 \times 10^{-7} \pm 1.70 \times 10^{-7}$	2
membrane	$1.90 \times 10^{-4} \pm 1.13 \times 10^{-4}$	6

Mating: *B. sphaericus* 2362 [pAMB1] X *B. sphaericus* 2362a

surface (membrane filter) for 20 h at 37°C.

**Effect of donor/recipient ratio on conjugation frequency.**

In these experiments the conditions were as described above except that the proportion of donor to recipient was varied. The results shown in Table 6 indicated that conjugation frequency was higher when the donor was present in higher number than the recipient.

**Range of recipient strains.**

Following optimization of conjugation conditions, *B. sphaericus* 2362 (pAMB1) (DNA homology group IIA, serotype 5a5b) was used as donor in conjugation experiments with other bacteria. Recipients were rifampicin-resistant strains and included strains of *B. sphaericus* from different serotypes within homology group IIA. Recipients also included the type strain of the species (ATCC 14577) of homology group I and two representatives of homology group 5, one of which was a strain isolated from field collected mosquito larvae. *Bacillus mycoides* isolated from field-collected mosquito larvae as well as *B. subtilis* IG20, a restrictionless mutant, were also tested as recipients.

Strain 2362 (pAMB1) used as donor in filter mating experiments successfully transferred the pAMB1 plasmid to strains 2362a, 1593, 1593-4RC, 1593-P51, 1691 (all of serotype 5a5b), 31-2 (serotype 9a9c) and 2297 (serotype 25), (Table 7). Agarose gel electrophoresis verified the presence



Table 6

Effect of donor : recipient ratio on frequency of conjugation between *B. sphaericus* 2362 [pAMB1] and *B. sphaericus* 2362-a<sup>1</sup>

Ratio	Donor 2362 [pAMB1] Applied	Removed <sup>2</sup>	Recipient 2362-a Applied	Removed <sup>2</sup>	Trans conjugants <sup>3</sup>	Frequency <sup>3</sup>
14.3 : 1	1.2x10 <sup>7</sup>	6.6x10 <sup>4</sup>	8.4x10 <sup>5</sup>	8.3x10 <sup>7</sup>	3.5x10 <sup>4</sup>	4.2x10 <sup>4</sup>
1.5 : 1	6.5x10 <sup>6</sup>	2.0x10 <sup>4</sup>	4.2x10 <sup>6</sup>	2.0x10 <sup>6</sup>	3.0x10 <sup>4</sup>	1.5x10 <sup>4</sup>
0.2 : 1	1.3x10 <sup>6</sup>	9.0x10 <sup>7</sup>	7.6x10 <sup>6</sup>	3.6x10 <sup>6</sup>	2.1x10 <sup>4</sup>	5.8x10 <sup>3</sup>

83

<sup>1</sup> Final whole cultures of donor and recipient were mixed to achieve the donor:recipient ratios shown in the first column. "Applied" refers to the total number of cells applied to the membrane, "removed" refers to the cells/ml in the 2 ml NY broth used to rinse the mating mixture off the membrane. membranes were incubated for 20 hours at 37°C on nonselective medium. Cells were washed off membranes into 2 ml NY broth, diluted in NY broth, and plated on selective medium (25 ug/ml rifampicin, 10 ug/ml erythromycin). No erythromycin resistant cells were detected among recipients plated prior to mixing with the donor.

<sup>2</sup> Cfu/ml in the NY broth used to wash the mating mixture off the membrane.  
<sup>3</sup> Number of transconjugants divided by number of recipients

Table 7

Conjugation frequencies between *B. sphaericus* 2362 [pAMB1] and other bacteria<sup>1</sup>

Trial	Donor <sup>2</sup>	Recipient <sup>2</sup>	Recipient DNA homology group	Recipient serotype	Transconjugants <sup>1</sup>	Frequency <sup>4</sup>
<b><i>B. sphaericus</i> 2362</b>						
1	(6.0x10 <sup>6</sup> ) 8.8x10 <sup>8</sup>	<i>B. sphaericus</i> 2362 (1.3x10 <sup>7</sup> ) 4.9x10 <sup>8</sup>	IIA	5a5b	1.3x10 <sup>5</sup>	2.7x10 <sup>4</sup>
2	(6.0x10 <sup>6</sup> ) 4.2x10 <sup>8</sup>	(1.3x10 <sup>7</sup> ) 1.3x10 <sup>8</sup>			4.4x10 <sup>5</sup>	3.4x10 <sup>4</sup>
3	(8.5x10 <sup>6</sup> ) 2.8x10 <sup>8</sup>	(3.7x10 <sup>6</sup> ) 1.7x10 <sup>8</sup>			1.1x10 <sup>4</sup>	6.5x10 <sup>5</sup>
4	(8.5x10 <sup>6</sup> ) 1.5x10 <sup>8</sup>	(3.7x10 <sup>6</sup> ) 1.5x10 <sup>8</sup>			3.4x10 <sup>4</sup>	2.3x10 <sup>4</sup>
5	(1.0x10 <sup>7</sup> ) 3.0x10 <sup>8</sup>	(7.0x10 <sup>6</sup> ) 2.8x10 <sup>8</sup>			1.6x10 <sup>4</sup>	5.7x10 <sup>5</sup>
6	(1.0x10 <sup>7</sup> ) 1.6x10 <sup>8</sup>	(7.0x10 <sup>6</sup> ) 2.1x10 <sup>8</sup>			3.8x10 <sup>4</sup>	1.8x10 <sup>4</sup>
<b><i>B. sphaericus</i> 2362</b>						
1	(4.3x10 <sup>6</sup> ) 3.0x10 <sup>8</sup>	<i>B. sphaericus</i> 1593 (9.0x10 <sup>6</sup> ) 2.6x10 <sup>8</sup>	IIA	5a5b	3.6x10 <sup>4</sup>	1.4x10 <sup>4</sup>
2	(4.3x10 <sup>6</sup> ) 5.0x10 <sup>8</sup>	(9.0x10 <sup>6</sup> ) 2.4x10 <sup>8</sup>			7.8x10 <sup>4</sup>	3.3x10 <sup>4</sup>
3	(5.5x10 <sup>6</sup> ) 4.2x10 <sup>8</sup>	(8.5x10 <sup>6</sup> ) 5.4x10 <sup>8</sup>			4.0x10 <sup>5</sup>	7.4x10 <sup>4</sup>
<b><i>B. sphaericus</i> 1593-4RC</b>						
1	(3.4x10 <sup>6</sup> ) 1.9x10 <sup>8</sup>	<i>B. sphaericus</i> 1593-4RC (2.9x10 <sup>6</sup> ) 4.2x10 <sup>8</sup>	IIA	5a5b	9.6x10 <sup>4</sup>	2.3x10 <sup>4</sup>
2	(3.4x10 <sup>6</sup> ) 1.1x10 <sup>8</sup>	(2.9x10 <sup>6</sup> ) 4.6x10 <sup>8</sup>			1.7x10 <sup>5</sup>	3.7x10 <sup>4</sup>
3	(5.5x10 <sup>6</sup> ) 1.4x10 <sup>8</sup>	(2.7x10 <sup>6</sup> ) 2.4x10 <sup>8</sup>			6.8x10 <sup>4</sup>	2.8x10 <sup>4</sup>
4	(5.5x10 <sup>6</sup> ) 1.7x10 <sup>8</sup>	(2.7x10 <sup>6</sup> ) 1.7x10 <sup>8</sup>			2.2x10 <sup>4</sup>	1.3x10 <sup>4</sup>
<b><i>B. sphaericus</i> 1593-P51</b>						
1	(9.0x10 <sup>5</sup> ) 5.2x10 <sup>8</sup>	<i>B. sphaericus</i> 1593-P51 (4.1x10 <sup>5</sup> ) 2.0x10 <sup>8</sup>	IIA	5a5b	2.2x10 <sup>5</sup>	1.1x10 <sup>3</sup>
2	(9.0x10 <sup>5</sup> ) 4.0x10 <sup>8</sup>	(4.1x10 <sup>5</sup> ) 1.9x10 <sup>8</sup>			4.4x10 <sup>5</sup>	2.3x10 <sup>3</sup>
3	(5.5x10 <sup>5</sup> ) 6.0x10 <sup>8</sup>	(2.4x10 <sup>5</sup> ) 9.0x10 <sup>7</sup>			6.0x10 <sup>5</sup>	6.7x10 <sup>3</sup>
4	(5.5x10 <sup>5</sup> ) 4.6x10 <sup>8</sup>	(2.4x10 <sup>5</sup> ) 2.8x10 <sup>8</sup>			4.4x10 <sup>5</sup>	1.6x10 <sup>3</sup>
<b><i>B. sphaericus</i> 1691</b>						
1	(8.5x10 <sup>6</sup> ) 3.8x10 <sup>8</sup>	<i>B. sphaericus</i> 1691 (2.7x10 <sup>6</sup> ) 2.4x10 <sup>8</sup>	IIA	5a5b	5.6x10 <sup>3</sup>	2.3x10 <sup>5</sup>
2	(8.5x10 <sup>6</sup> ) 5.4x10 <sup>8</sup>	(2.7x10 <sup>6</sup> ) 3.2x10 <sup>8</sup>			4.0x10 <sup>3</sup>	1.3x10 <sup>5</sup>

Table 7 (Continued)

Conjugation frequencies between *B. sphaericus* 2362 [pAMB1] and other bacteria<sup>1</sup>

Trial	Donor <sup>2</sup>	Recipient <sup>2</sup>	Recipient DNA homology group	Recipient serotype	Transconjugants <sup>3</sup>	Frequency <sup>4</sup>
1	(2.3x10 <sup>6</sup> )	<i>B. sphaericus</i> 2297 (1.9x10 <sup>6</sup> )	IIA	25	7.6x10 <sup>4</sup>	1.2x10 <sup>4</sup>
2	(2.3x10 <sup>6</sup> )	(1.9x10 <sup>6</sup> )			1.9x10 <sup>5</sup>	3.2x10 <sup>4</sup>
1	(1.2x10 <sup>6</sup> )	<i>B. sphaericus</i> 31-2 (6.6x10 <sup>6</sup> )	IIA	9a9c	1.6x10 <sup>4</sup>	6.7x10 <sup>5</sup>
2	(1.2x10 <sup>6</sup> )	(6.6x10 <sup>6</sup> )			2.6x10 <sup>4</sup>	1.1x10 <sup>4</sup>
1	(4.3x10 <sup>6</sup> )	<i>B. sphaericus</i> IAB460 (9.5x10 <sup>6</sup> )	IIA	6	0	<1.1x10 <sup>8</sup>
2	(4.3x10 <sup>6</sup> )	(9.5x10 <sup>6</sup> )			0	<1.1x10 <sup>8</sup>
1	(1.1x10 <sup>7</sup> )	<i>B. sphaericus</i> SSII-1 (1.1x10 <sup>7</sup> )	IIA	2	0	<2.5x10 <sup>8</sup>
2	(1.1x10 <sup>7</sup> )	(1.1x10 <sup>7</sup> )			0	<2.5x10 <sup>8</sup>
1	(4.3x10 <sup>6</sup> )	<i>B. sphaericus</i> Q (7.0x10 <sup>6</sup> )	IIA	1	0	<2.0x10 <sup>7</sup>
2	(4.3x10 <sup>6</sup> )	(7.0x10 <sup>6</sup> )			0	<2.1x10 <sup>8</sup>
1	(4.1x10 <sup>6</sup> )	<i>B. sphaericus</i> 14577 (2.8x10 <sup>6</sup> )	I		0	<6.6x10 <sup>7</sup>
2	(1.0x10 <sup>7</sup> )	(6.5x10 <sup>6</sup> )			0	<3.0x10 <sup>6</sup>
1	(1.1x10 <sup>7</sup> )	<i>B. sphaericus</i> NRS1199 (4.0x10 <sup>5</sup> )	V		0	<3.6x10 <sup>7</sup>
2	(1.1x10 <sup>7</sup> )	(4.0x10 <sup>5</sup> )			0	<4.8x10 <sup>7</sup>

Table 7 (Continued)

Conjugation frequencies between *B. sphaericus* 2362 [pAMB1] and other bacteria<sup>1</sup>

Trial	Donor <sup>2</sup>	Recipient <sup>2</sup>	Recipient DNA homology group	Recipient serotype	Transconjugants <sup>3</sup>	Frequency <sup>4</sup>
			<i>B. sphaericus</i> larval isolate	V		
1	(7.5x10 <sup>6</sup> )	1.6x10 <sup>8</sup>	(4.3x10 <sup>6</sup> )	1.0x10 <sup>5</sup>	0	<4.0x10 <sup>5</sup>
2	(7.5x10 <sup>6</sup> )	1.0x10 <sup>9</sup>	(4.3x10 <sup>6</sup> )	2.0x10 <sup>4</sup>	0	<2.0x10 <sup>4</sup>
			<i>B. subtilis</i> IG-20			
1	(1/9x10 <sup>6</sup> )	7.6x10 <sup>8</sup>	(2.7x10 <sup>6</sup> )	3.2x10 <sup>8</sup>	0	<1.2x10 <sup>8</sup>

<sup>1</sup> *B. sphaericus* 2362 is DNA homology group IIA, serotype 5a5b. Equal volumes of 5 h donor (ery<sup>r</sup>) and recipient (rif<sup>r</sup>) broth cultures were mixed and 0.1 ml applied to membranes. Membranes were incubated at 37°C for 20 h. Cells were washed off membranes into 2 ml NY broth, diluted in NY broth, and plated on selective medium (NY agar, 25 ug/ml rifampicin, 10 ug/ml erythromycin). No erythromycin-resistant cells were detected among recipients plated prior to mixing with the donor. In those experiments where conjugation frequencies are indicated by the "<" symbol, a small number of potential transconjugants may have been observed. These were each tested for auxotrophic requirement or bacteriophage sensitivity to verify that they were actually the donor strain and not transconjugants.

<sup>2</sup> Numbers in parenthesis are the total cfu applied to the membrane and the numbers outside parenthesis are the total cfu present in the 2 ml used to wash bacteria off the membrane.

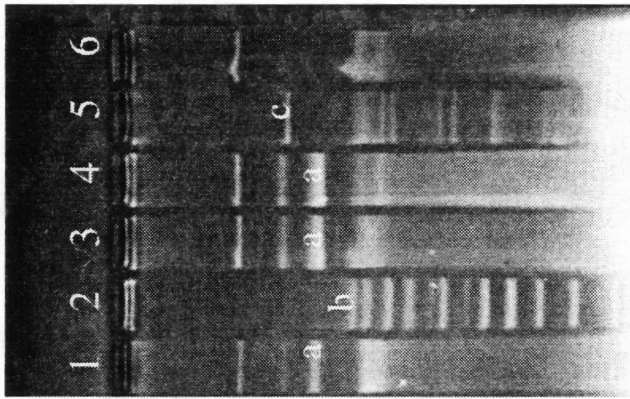
<sup>3</sup> Total number of transconjugants present in the 2 ml used to wash bacteria off the membrane.

<sup>4</sup> Number of transconjugants divided by the number of recipients in the 2 ml used to wash the membrane. When no transconjugants were detected, the level of sensitivity of detection is indicated.

of pAMB1 in transconjugants of 2362a (lane 3 and 4, Fig. 21) and other recipients. Frequencies were about 10-fold higher in matings with 1593-P51 (about  $10^{-3}$ ) as the recipient than with the other strains (in the range of  $10^{-4}$  to  $10^{-5}$ ). Strains 1593-P51 and 1593-4RC were isolated based on resistance to a lytic bacteriophage. Strain 1593-P51 has a protein surface layer (S layer) of lower molecular weight than the parent strain (Fig. 22) whereas strain 1593-4RC has an S-layer of the same molecular weight as the parental strain.

To verify the identity of the putative transconjugants of 1593-4RC and 1593-P51, the transconjugants were tested for resistance to phage 4. The donor and recipient strains were used as controls. Transconjugants and the recipient strains were resistant to the phage but the donor, 2362 (pAMB1) was sensitive (Fig. 23). Gel electrophoresis of the transconjugants did not allow unambiguous identification of pAMB1 due to the presence of two resident plasmids of about the same size as pAMB1 (lanes 1, 3 and 5, Fig. 24). However, when strain 1593-4RC appeared to receive pAMB1 (gained erythromycin resistance), it appeared to have lost one or both of the two resident plasmids of about the same size as pAMB1 (lane 4, Fig. 24). Although the presence of pAMB1 was not verified by electrophoresis, the high conjugation frequencies make it very unlikely that the transconjugants

Figure 21



Lane 1: 2362 [pAMB1] donor; a = pAMB1

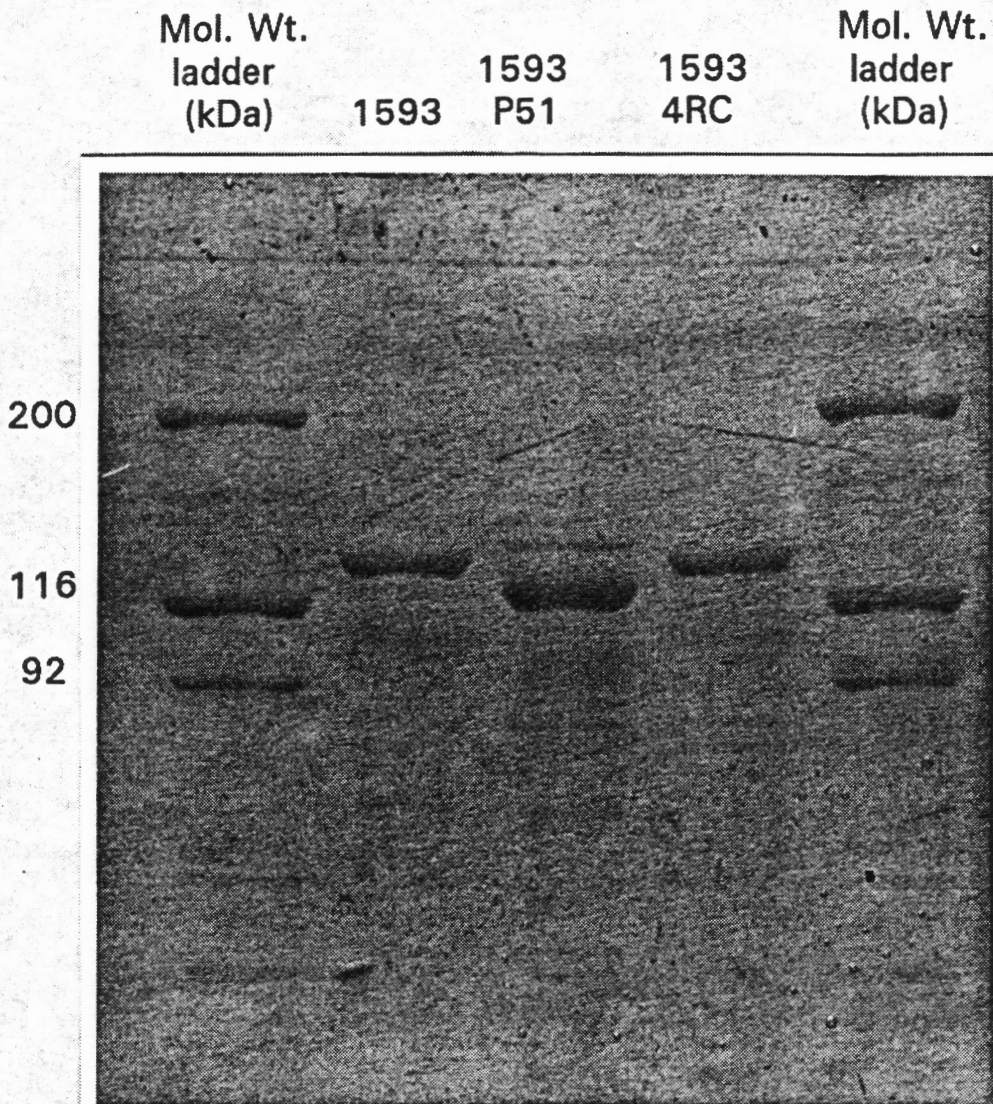
Lane 2: ccc ladder; b = 16.2 kb

Lane 3, 4: 2362 transconjugants

Lane 5: *E. coli* V517; c = 54.3 kb

Lane 6: 2362a recipient

FIG. 21. Agarose gel electrophoresis of plasmid DNA from the relevant strains used to demonstrate transfer of pAMB1 to *B. sphaericus* 2362-a recipients.



*B. sphaericus* 1593 and phage resistant mutant 4RC have surface layer proteins of apparent molecular weight 136,000. Phage resistant mutant P51 has a surface layer protein of apparent molecular weight 121,000.

FIG. 22. SDS-PAGE patterns of surface layer proteins of *B. sphaericus* 1593 and bacteriophage resistant mutants.

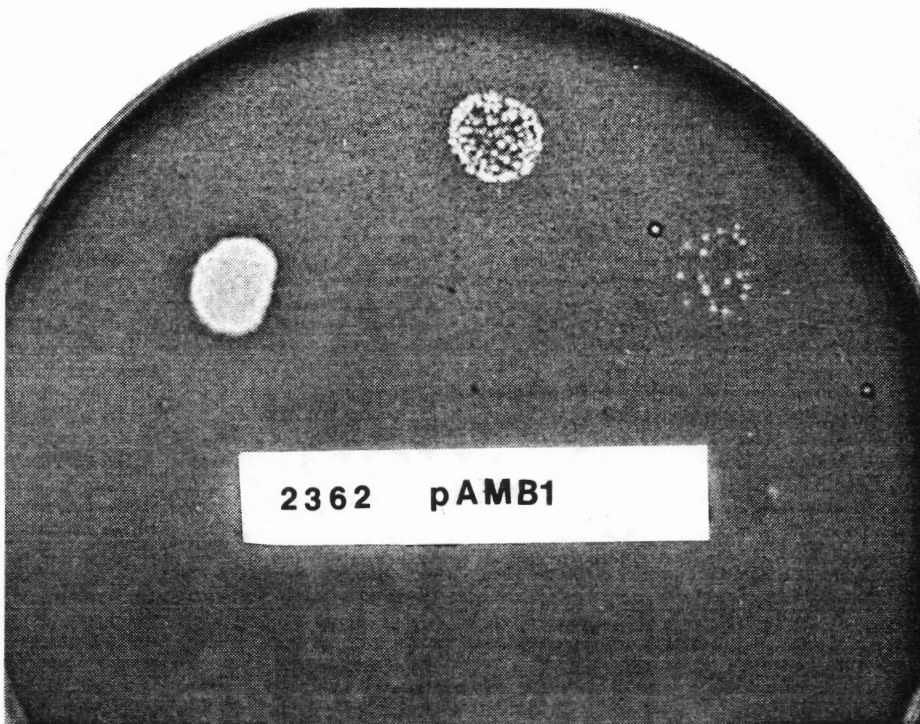
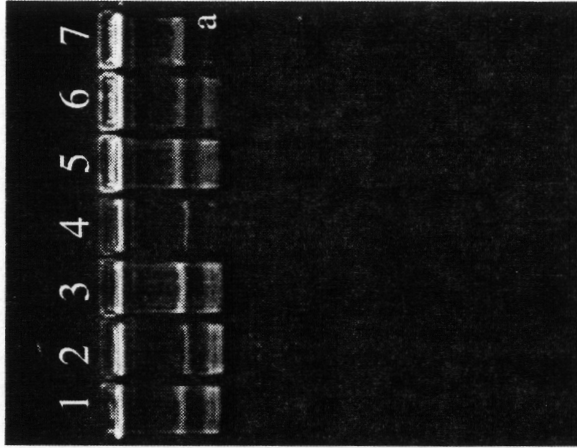


FIG. 23. Bacteriophage sensitivity of *B. sphaericus* 2362 (pAMB1) donor.



Figure 24



Lane 1: 1593 recipient

Lane 2: 1593 transconjugant

Lane 3: 1593-4RC recipient

Lane 4: 1593-4RC transconjugant

Lane 5: 1593-P51 recipient

Lane 6: 1593-P51 transconjugant

Lane 7: 2362 [pAMB1] donor; a = pAMB1

FIG. 24. Agarose gel electrophoresis of plasmid DNA from the relevant strains used to demonstrate transfer of pAMB1 to *B. sphaericus* 1593 recipients.

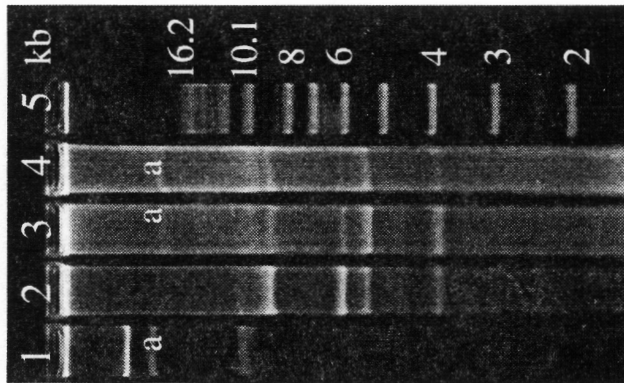
were rifampicin-resistant mutants of the donor. Also, unlike the donor, strains 1593-4RC and 1593-p51 were resistant to phage 4. No rifampicin-resistant mutants of the donor were detected among the cells originally placed on the membrane. Transconjugants from matings with strains 2297 and 31-2 as recipients were also confirmed by phage testing and gel electrophoresis (Fig. 25 and 26).

Conjugation was not detected when the recipient strains were IAB 460 (serotype 6), SSII-1 (serotype 2), Q (serotype 1), ATCC 14577 (type strain of the species), NRS 1199, and a *B. sphaericus* non-pathogen isolated from field collected larvae (Table 7). In some cases a few colonies were obtained in the selection medium, but these corresponded to spontaneous antibiotic resistant mutants of the donor strain. For nontoxic or low toxicity strains this could be determined by microscopic observation of the cells which revealed the presence of parasporal bodies carried only by the donor strains but absent in the recipients. For nontoxic recipients, it was also determined by the toxicity to mosquito larvae of the putative transconjugants. Sensitivity to lytic bacteriophage was also used to verify the identity of putative transconjugants.

**Interspecies conjugation: Bti, *B. subtilis* and *B. mycoides* as recipients of pAMB1.**

Interspecies matings were carried out using *B. subtilis*

Figure 25



Lane 1: 2362 [pAMB1] donor; a = pAMB1

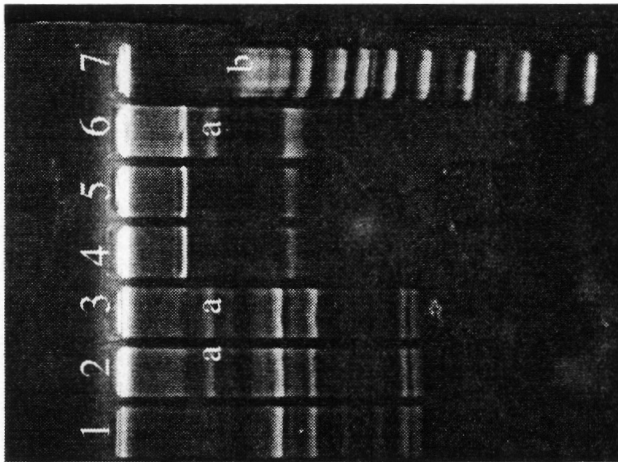
Lane 2: 2297 recipient

Lane 3, 4: 2297 transconjugants

Lane 5: ccc ladder

FIG. 25. Agarose gel electrophoresis of plasmid DNA from the relevant strains used to demonstrate transfer of pAMB1 to *B. sphaericus* 2297 recipients.

Figure 26



Lane 1: 31-2 recipient

Lane 2, 3: 31-2 transconjugants; a = pAMB1

Lane 4: 2362

Lane 5: 2362a

Lane 6: 2362 [pAMB1] donor

Lane 7: ccc ladder; b = 16.2 kb

FIG. 26. Agarose gel electrophoresis of plasmid DNA from the relevant strains used to demonstrate transfer of pAMB1 to *B. sphaericus* 31-2 recipients.

IG-20 (a restrictionless mutant), Bti and three strains of *B. mycoides* isolated from field collected mosquito larvae as recipients. The donor was *B. sphaericus* 2362(pAMB1).

Transfer of pAMB1 into the restrictionless *B. subtilis* IG-20 was not detected (Table 7). Also no conjugation took place with Bti or with *B. mycoides*. A few colonies grew in the selection medium but microscopic observation of cell morphology demonstrated that they corresponded to the donor. In conjugation experiments with Bti or the *B. mycoides* strains, the number of recipients washed off the membrane was always lower than the number originally added to the membrane. The number of recipients decreased dramatically while the donor cell number increased. In an experiment with Bti as recipient, mating mixtures were incubated for 6 and 20 h. Results showed that counts of Bti recovered from membranes decreased as incubation time increased.

#### **In vitro conjugation experiments to detect mobilization of pUB110**

Entomopathogenic *B. thuringiensis* contains self-transmissible, large, cryptic plasmids that can mobilize small plasmids. Many strains of *B. sphaericus* (e.g. 2362) also contain cryptic plasmids, some of them as large as 180 kb. It was of interest to find out if the large plasmid present in strain 2362 was able to promote its own transfer. Since it lacks selectable markers, mobilization of the

small, nonconjugative plasmid pUB110 was used as a detection system. Rifampicin-resistant mutants were isolated for all the recipient strains. Transfer of pUB110 would confer neomycin resistance on the transconjugants. Transconjugants receiving pUB110 were selected in media containing both 25 ug/ml rifampicin and 5 ug/ml neomycin. The experimental conditions were as described before and as determined by the condition optimization experiments.

Recipient strains used in this series of conjugation experiments were 2362a (lysine auxotroph), 1691 (histidine auxotroph), 2297, Q, 1883 and 31-2 (Table 8). The use of auxotrophy for identification of transconjugants of the same serotype and having the same phage sensitivity as the donor, 2362 (pUB110), is illustrated in Figs. 27, 28 and 29 for strain 1691. In Fig. 27 and 28, the bacteria were grown on a defined medium (BAT+) lacking histidine. In Fig. 29, the medium was supplemented with histidine and the auxotrophic mutant grew. Strain 1691 lacked the large plasmid carried by 2362 (lane 4, Fig. 30) and therefore might be a suitable recipient if an exclusion phenomenon was present as found in *B. thuringiensis*. Auxotrophy and phage sensitivity were used for unambiguous identification of potential transconjugants. No verified transconjugants were isolated in any of the filter mating experiments.

Table 8

Conjugation frequencies between *B. sphaericus* 2362 [PUB110] and other bacteria

Trial	Donor <sup>2</sup>	Recipient <sup>2</sup>	Recipient DNA homology group	Recipient serotype	Transconjugants <sup>3</sup>	Frequency <sup>4</sup>
<b><i>B. sphaericus</i> 2362</b>						
1	(6.0x10 <sup>5</sup> ) 4.0x10 <sup>8</sup>	<b><i>B. sphaericus</i> 2362</b> (1.6x10 <sup>6</sup> ) 3.6x10 <sup>8</sup>	IIA	5a5b	0	<1.1x10 <sup>8</sup>
2	(6.0x10 <sup>5</sup> ) 3.6x10 <sup>8</sup>	(1.6x10 <sup>6</sup> ) 3.8x10 <sup>8</sup>			0	<1.0x10 <sup>8</sup>
<b><i>B. sphaericus</i> 1691</b>						
1	(6.0x10 <sup>5</sup> ) 2.6x10 <sup>8</sup>	(3.5x10 <sup>5</sup> ) 1.2x10 <sup>8</sup>	IIA	5a5b	0	<3.3x10 <sup>8</sup>
2	(6.0x10 <sup>5</sup> ) 2.8x10 <sup>8</sup>	(3.5x10 <sup>5</sup> ) 9.6x10 <sup>7</sup>			0	<4.2x10 <sup>8</sup>
<b><i>B. sphaericus</i> 2297</b>						
1	(3.2x10 <sup>6</sup> ) 9.0x10 <sup>8</sup>	(2.6x10 <sup>6</sup> ) 4.2x10 <sup>9</sup>	IIA	25	0	<9.5x10 <sup>10</sup>
<b><i>B. sphaericus</i> Q</b>						
1	(1.2x10 <sup>5</sup> ) 2.4x10 <sup>8</sup>	(1.1x10 <sup>5</sup> ) 4.0x10 <sup>8</sup>	IIA	1	0	<1.0x10 <sup>8</sup>
2	(1.2x10 <sup>5</sup> ) 3.4x10 <sup>8</sup>	(1.1x10 <sup>7</sup> ) 2.2x10 <sup>8</sup>			0	<1.8x10 <sup>8</sup>
<b><i>B. sphaericus</i> 1883</b>						
1	(1.2x10 <sup>5</sup> ) 2.8x10 <sup>8</sup>	(1.5x10 <sup>6</sup> ) 5.2x10 <sup>8</sup>	IIA	2	0	<7.7x10 <sup>9</sup>
2	(1.2x10 <sup>5</sup> ) 5.2x10 <sup>8</sup>	(1.5x10 <sup>6</sup> ) 2.8x10 <sup>8</sup>			0	<1.4x10 <sup>8</sup>
<b><i>B. sphaericus</i> 31-2</b>						
1	(3.4x10 <sup>5</sup> ) 1.9x10 <sup>7</sup>	(5.0x10 <sup>6</sup> ) 1.3x10 <sup>9</sup>	IIA	9a9c	0	<3.1x10 <sup>9</sup>
2	(3.4x10 <sup>5</sup> ) 1.7x10 <sup>7</sup>	(5.0x10 <sup>6</sup> ) 1.3x10 <sup>9</sup>			0	<3.1x10 <sup>9</sup>

<sup>1</sup> *B. sphaericus* 2362 is DNA homology group IIA, serotype 5a5b. Equal volumes of 5 h donor (ne<sup>r</sup>) and recipient (rif<sup>r</sup>) broth cultures were mixed and 0.1 ml applied to membranes. Membranes were incubated at 37°C for 20 h. Cells were washed off membranes into 2 ml NY broth, diluted in NY broth, and plated on selective medium (NY agar, 25 µg/ml rifampicin, 5 µg/ml neomycin). No neomycin-resistant cells were detected among recipients plated prior to mixing with the donor. In those experiments where conjugation frequencies are indicated by the "<" symbol, a small number of potential transconjugants may have been observed. These were each tested for auxotrophic requirement or bacteriophage sensitivity to verify that they were actually the donor strain and not transconjugants.

<sup>2</sup> Numbers in parenthesis are the total cfu applied to the membrane and the numbers outside parenthesis are the total cfu present in the 2 ml used to wash bacteria off the membrane.

<sup>3</sup> Total number of transconjugants present in the 2 ml used to wash bacteria off the membrane.

<sup>4</sup> Number of transconjugants divided by the number of recipients in the 2 ml used to wash the membrane. When no transconjugants were detected, the level of sensitivity of detection is indicated.



FIG. 27. Growth of *B. sphaericus* 1691 histidine auxotroph and 2362 (pUB110) on the defined medium BAT+.



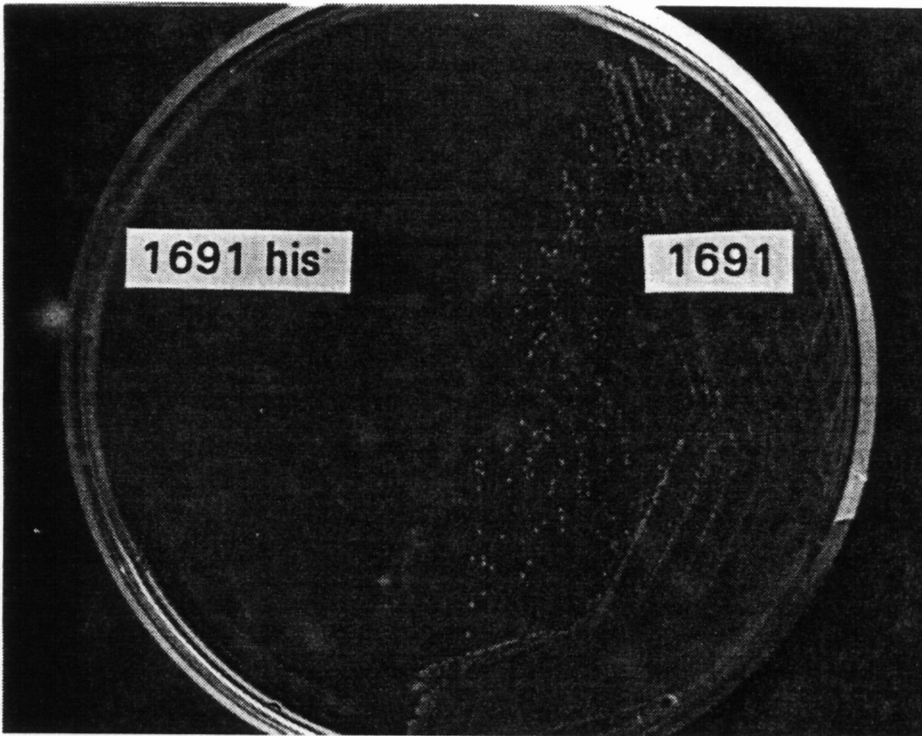


FIG. 28. Growth of *B. sphaericus* 1691 and 1691 histidine auxotroph on the defined medium BAT+.

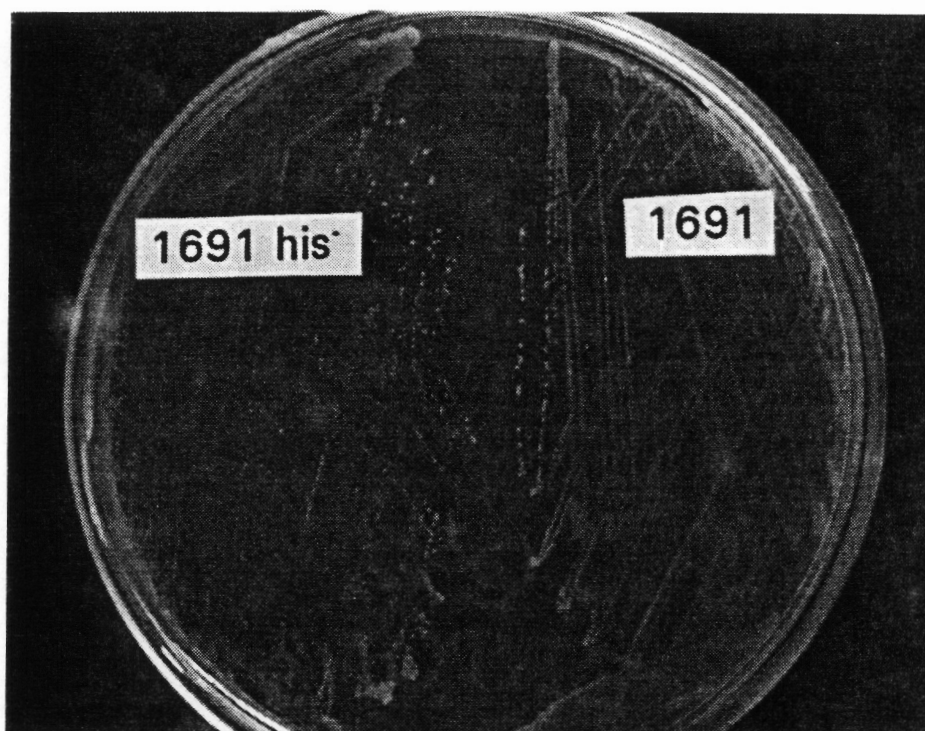
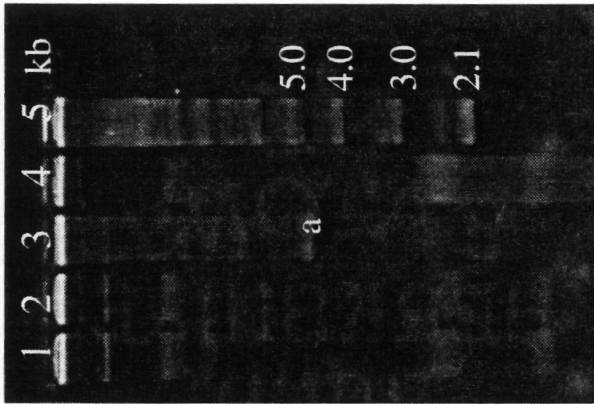


FIG. 29. Growth of *B. sphaericus* 1691 and 1691 histidine auxotroph on the defined medium BAT+ supplemented with histidine.

Figure 30



Lane 1: 2362

Lane 2: 2362a

Lane 3: 2362 [pUB110]; a = pUB110

Lane 4: 1691

Lane 5: ccc ladder

FIG. 30. Agarose gel electrophoresis of plasmid DNA from some of the relevant strains used to attempt transfer of pUB110 to other *B. sphaericus* recipients.

### **Mating experiments with Bti carrying the pBC16 plasmid.**

Some isolates of *B. thuringiensis* from various serotypes conjugate with the closely related bacilli, *B. cereus* and *B. anthracis*. Bti has been shown to conjugate in this way (though the plasmid carrying the toxin genes is not conjugative). Another series of experiments involved mating between *B. thuringiensis* serovar. *israelensis* (Bti) carrying pBC16 conferring tetracycline resistance and *B. sphaericus* rifampicin resistant strains 2362a (lys<sup>-</sup>) and 1691 (his<sup>-</sup>) as recipients (Table 9). No transconjugants were recovered on the tetracycline/rifampicin selective medium.

### **In vivo conjugation in larval cadavers**

The ultimate goal of this work was to determine if conjugation occurs in the larval cadavers. In vivo conjugation experiments were done by feeding larvae with spores of donor 2362(pAMB1) together with spores of strains that had been successful recipients of pAMB1 in membrane matings. Third instar *Culex quinquefasciatus* larvae were fed with these mixtures. Strains used as recipients were 2362a (lysine auxotroph), and 1593-P51 (defective surface protein). Larvae died rapidly. They were homogenized and plated at intervals on appropriate selective media. Although spore germination was observed, no transconjugants were detected in cadavers up to 72 hours after feeding spores (Table 10 and 11). It is possible that conjugal transfer

Table 9

Conjugation frequencies between *B. thuringiensis* serovar. israelensis (pBC16) and *B. sphaericus*

Trial	Donor <sup>2</sup>	Recipient <sup>2</sup>	Transconjugants <sup>1</sup>	Frequency <sup>4</sup>
<b><i>B. thuringiensis</i> B. sphaericus 2362</b>				
1	(4.6x10 <sup>6</sup> )	2.0x10 <sup>6</sup> (2.0x10 <sup>6</sup> )	1.3x10 <sup>9</sup>	0
2	(4.6x10 <sup>6</sup> )	1.1x10 <sup>6</sup> (2.0x10 <sup>6</sup> )	1.2x10 <sup>9</sup>	0
<b><i>B. sphaericus</i> 1691</b>				
1	(4.6x10 <sup>6</sup> )	2.2x10 <sup>6</sup> (1.5x10 <sup>7</sup> )	5.8x10 <sup>6</sup>	0
2	(4.6x10 <sup>6</sup> )	2.2x10 <sup>6</sup> (1.5x10 <sup>7</sup> )	5.6x10 <sup>6</sup>	0

<sup>1</sup> Equal volumes of 5 h donor (tet<sup>r</sup>) and recipient (rif<sup>r</sup>) broth cultures were mixed and 0.1 ml applied to membranes. Membranes were incubated at 37°C for 20 h. Cells were washed off membranes into 2 ml NY broth, diluted in NY broth, and plated on selective medium (NY agar, 25 ug/ml rifampicin, 10 ug/ml tetracycline). No tetracycline-resistant cells were detected among recipients plated prior to mixing with the donor.

<sup>2</sup> Numbers in parenthesis are the total cfu applied to the membrane and the numbers outside parenthesis are the total cfu present in the 2 ml used to wash bacteria off the membrane.

<sup>3</sup> Total number of transconjugants present in the 2 ml used to wash bacteria off the membrane.

<sup>4</sup> Number of transconjugants divided by the number of recipients in the 2 ml used to wash the membrane. When no transconjugants were detected, the level of sensitivity of detection is indicated.

Table 10

Conjugation in mosquito larval cadavers<sup>1</sup>

Trial	Donor	Recipient	Transconjugants <sup>2</sup>	Frequency <sup>3</sup>
<b>B. sphaericus 2362</b>				
<b>at 0 hours</b>				
1	1.1x10 <sup>6</sup> cfu/larva	1.2x10 <sup>6</sup> cfu/larva		
2	8.4x10 <sup>5</sup> spores/larva	7.2x10 <sup>5</sup> spores/larva		
1	1.7x10 <sup>6</sup> cfu/larva	1.4x10 <sup>6</sup> cfu/larva		
2	7.8x10 <sup>5</sup> spores/larva	4.0x10 <sup>5</sup> spore/larva		
<b>at 48 hours</b>				
1	8.6x10 <sup>5</sup> cfu/larva	3.2x10 <sup>4</sup> cfu/larva	0	<5.7x10 <sup>6</sup>
2	1.6x10 <sup>6</sup> spores/larva	9.3x10 <sup>5</sup> spores/larva	0	<1.4x10 <sup>5</sup>
1	1.4x10 <sup>6</sup> cfu/larva	1.2x10 <sup>4</sup> cfu/larva	0	<1.1x10 <sup>5</sup>
2	4.4x10 <sup>5</sup> spores/larva	1.2x10 <sup>4</sup> spores/larva	0	<1.1x10 <sup>5</sup>
<b>at 72 hours</b>				
1	8.3x10 <sup>5</sup> spores/larva	5.1x10 <sup>4</sup> spores/larva	0	<3.9x10 <sup>6</sup>
2	4.9x10 <sup>5</sup> spores/larva	1.8x10 <sup>4</sup> spores/larva	0	<1.1x10 <sup>5</sup>

<sup>1</sup> Fourth instar larvae were allowed to feed for 15 min in a suspension containing 2.2x10<sup>6</sup> spores/ml of *B. sphaericus* 2362 [pAMβ1] and 1.1x10<sup>6</sup> spores/ml of *B. sphaericus* 2362 (lysine auxotroph, rif<sup>r</sup>). Larvae were removed from spore suspension, rinsed, and placed in clear water. Larval homogenate plated immediately after feeding did not produce any colonies resistant to both erythromycin and rifampicin. At 48 and at 72 hours, 75 and 50 larval cadavers respectively were homogenized and plated with or without heating at for 12 min at 80°C. Counts derived from homogenates plated without heating are reported as cfu/larva and those plated after heating as spores/larva.

<sup>2</sup> Transconjugants (erythromycin resistant, rifampicin resistant, lysine auxotrophs) per ml of larval homogenate.

<sup>3</sup> When no transconjugants were detected, the number represents the level of sensitivity of detection.

Table 11

Conjugation in mosquito larval cadavers<sup>1</sup>

Trial	Donor	Recipient	Transconjugants <sup>2</sup>	Frequency <sup>3</sup>
<b><i>B. sphaericus</i> 2362</b> <b><i>B. sphaericus</i> 1593p51</b>				
<b>at 0 hours</b>				
1	4.2x10 <sup>5</sup> cfu/larva	3.4x10 <sup>5</sup> cfu/larva		
2	2.8x10 <sup>5</sup> spores/larva	1.5x10 <sup>5</sup> spores/larva		
2	4.8x10 <sup>5</sup> cfu/larva	3.0x10 <sup>5</sup> cfu/larva		
	3.4x10 <sup>5</sup> spores/larva	2.6x10 <sup>5</sup> spores/larva		
<b>at 48 hours</b>				
1	1.6x10 <sup>6</sup> cfu/larva	1.0x10 <sup>4</sup> cfu/larva	0	<1.3x10 <sup>5</sup>
	1.1x10 <sup>6</sup> spores/larva	9.3x10 <sup>3</sup> spores/larva	0	<1.4x10 <sup>5</sup>
2	2.1x10 <sup>6</sup> cfu/larva	2.4x10 <sup>4</sup> spores/larva	0	<5.6x10 <sup>6</sup>
	3.3x10 <sup>6</sup> spores/larva	3.1x10 <sup>4</sup> spores/larva	0	<4.3x10 <sup>6</sup>
<b>at 72 hours</b>				
1	6.9x10 <sup>6</sup> spores/larva	7.6x10 <sup>4</sup> spores/larva	0	<2.6x10 <sup>6</sup>
2	4.4x10 <sup>6</sup> spores/larva	9.3x10 <sup>4</sup> spores/larva	0	<2.1x10 <sup>5</sup>

<sup>1</sup> Fourth instar larvae were allowed to feed for 15 min in a suspension containing 2.2x10<sup>6</sup> spores/ml of *B. sphaericus* 2362 [pAM51] and 4.5x10<sup>6</sup> spores/ml of *B. sphaericus* 1593p51 (rif). Larvae were removed from spore suspension, rinsed, and placed in clear water. Larval homogenate plated immediately after feeding did not produce any colonies resistant to both erythromycin and rifampicin. At 48 and at 72 hours, 75 and 50 larval cadavers respectively were homogenized and plated with or without heating at for 12 min at 80°C. Counts derived from homogenates plated without heating are reported as cfu/larva and those plated after heating as spores/larva.

<sup>2</sup> Transconjugants (erythromycin resistant, rifampicin resistant) per ml of larval homogenate.

<sup>3</sup> When no transconjugants were detected, the number represents the level of sensitivity of detection.

does not occur in the larval cadaver. However, it is also possible that the frequency of conjugation was too low and could not be detected.

There is also the possibility that the larval cadaver was not a suitable place for conjugation, and some factor in the larvae may act to inhibit conjugation. To rule out this possibility, larval homogenate was incubated with the bacterial mixture before adding it to the membranes. In this experiment the conjugation frequencies for 2362 (pAMB1) with 1593-P51 was  $2.0 \times 10^{-4}$  and for the same bacteria with larval homogenate the frequency was  $1.7 \times 10^{-4}$ ,  $\pm 6.0 \times 10^{-5}$  (duplicate matings). No inhibition of conjugation was observed.



## DISCUSSION

Studies of *B. sphaericus*, both in the laboratory and in the field, have progressed to the point where this bacterium is likely to be produced as a commercial product for mosquito control during 1995 or 1996. Although strain 2362, the strain to be included in a commercial product, is safe and effective, research is being carried out to improve the bacterium by changing its toxicity, spectrum of activity and manufacturing characteristics. The genes conferring these changes are initially to be introduced on small recombinant plasmids. It is important to know if these plasmids, usually carrying antibiotic resistance genes, can be mobilized and transferred by conjugation to other bacteria in the environment. This question was approached in two phases. In the first phase, the ability of *B. sphaericus* spores to germinate and produce vegetative cells that could serve as potential donors for conjugation in mosquito larval cadavers was determined for a number of mosquito pathogenic strains. In the second phase, the ability of *B. sphaericus* to conjugate was investigated.

### Spore Germination and Recycling

This study showed that only spores of the highly toxic *B. sphaericus* strains producing both the binary and the 100-kDa toxins were able to germinate in high percentage and to

recycle. These strains were 2362-7, 2297 and IAB 59, serotypes 5a5b, 25 and 6 respectively.

After spores from these strains germinated, vegetative forms were able to multiply using the nutrients in the larval cadaver and produced more spores and toxin. The final number of spores present in cadavers was usually slightly higher than the number originally ingested. During sporulation in cadavers, new toxin is produced, an advantage for this microbial pesticide in the field. The toxin will be available for other larvae feeding on these cadavers, extending the effect in the habitat. This phenomenon enhances persistence in the field.

These results are in agreement with previous studies that demonstrated the ability of strains 2362 and 1593 to recycle under laboratory and field conditions. These two strains belong to serotype 5a5b and produce both types of toxin (27, 38, 95). This study was expanded to compare recycling activity among strains belonging to other serotypes and representing different toxin combinations.

Aly et al. (2) demonstrated that Bti recycled in *Aedes aegypti* and *Anopheles albimanus* larvae with the number of spores at 72 h equivalent to  $10^5$  spores/larva. In our study we also demonstrated that Bti as well as *B. sphaericus* can use the larval cadaver for growth and sporulation. Initial recycling experiments with Bti and *B. sphaericus* 2362 not

only confirmed previous results (27, 38, 90, 95) but also helped to set the conditions for testing a wider range of strains. In one experiment, 24 and 72 h cadavers were used in bioassays to show that new toxin was being produced.

The maximum number of spores ingested by the larvae with any of the strains under the feeding conditions of these experiments was  $10^5$  to  $10^6$  spores/larva. This seems to be the maximum number of spores that a larva can harbor. This conclusion is supported by reports of Charles and Nicolas (27), Davidson et al. (38) and Ramoska and Hopkins (90) who found similar results. If the larvae consumed fewer spores, e.g. the trial with strain 2297 (Fig. 4), the final number of spores/larva increased to  $10^5$  to  $10^6$ /cadaver.

An overall increase in the number of spores was further evidence that the bacteria recycled. At least some of the original spores must have germinated, and the resulting vegetative cells multiplied to result in an increased final spore number. A second type of evidence that the cells recycled was to find the same pattern of spore germination, growth and resporulation when the larvae were fed either on a spore suspension of  $10^5$  or of  $10^6$  spores/ml. The final number of spores was the same regardless of the spore concentration in which the larvae were feeding.

The decrease in the number of heat-resistant spores while the total number of *B. sphaericus* cells remained high

indicates that the decrease in spores was caused by spore germination and not by destruction or elimination of spores. In some cases, for instance with strains 1883 and Q, the total number of *B. sphaericus* decreased in parallel to the decrease in number of spores before larval death. This indicated that some *B. sphaericus* cells were eliminated or destroyed while the larvae were still alive. This was probably caused by the slower death of larvae after consuming the low toxicity strains. Davidson (32) studied the ultrastructural changes in larvae fed with the low toxicity strain *B. sphaericus* SSII-1. She found that vegetative cells of strain SSII-1 dropped sharply during the first hour after feeding and progressive loss of bacterial structures was observed, probably removed by digestion. Strain SII-1, as Q and 1883, only produces the 100-kDa toxin and belongs to the low toxicity group.

The number of total bacteria (*B. sphaericus* and all others that grew under the culture conditions) usually increased after larval death. This was expected since deterioration of larval body provides the appropriate environment and nutrients for many bacteria to colonize and grow.

The spores of strains that had the ability to germinate in the larvae probably require a period of time to be activated, start germination and take advantage of the

nutrient-rich larval environment. The results seem to indicate that activation and germination occur as a result of availability of substances released by the larval tissues. This idea is supported by comparison of the times in which spore germination occurred during the recycling experiments and reports about the timing of events and the pathology occurring in the larvae after the ingestion of *B. sphaericus* toxin. According to Davidson (34), after ingestion of a high dose of *B. sphaericus*, symptoms can appear as early as 30 to 60 min. Changes in the midgut lead to the appearance of cytolysosomes in the midgut cells until they separate at their base. Bacteria were confined within the peritrophic membrane until after larval death (32). Death can occur as early as 4 h after bacterial injection but with some strains may take 48 h.

Spores of strains that naturally produce both type of toxins have evolved the ability to germinate in the larval cadaver and to recycle. In general, these strains kill the larvae rapidly. Rapid death of the larvae would represent an advantage to those strains able to recycle. It would decrease the possibility of their spores being eliminated from the gut by the normal digestive process. Spores that germinate well can take advantage of the nutrients provided by larval tissues after the toxin has exerted its lytic effect.

Larvae fed with the low toxicity strains Q, 1883 and recombinant 1693(pC35) died between 24 and 48h following feeding. These strains only produce the 100 kDa-toxin. This toxin has been cloned from SSII-1 and has been shown to be as toxic as the binary toxin when produced in *Escherichia coli* (107). Therefore, the low toxicity of strain SSII-1 was not due to the nature of the toxin but was probably due to low expression or low stability of the toxin protein.

My experiments showed that if the larvae do not die quickly, some of the ingested spores appear to be eliminated or destroyed during the normal digestive process, leaving fewer spores present to germinate in the cadaver. However if elimination was occurring, it was not complete and the number of remaining spores should have been enough to go through the recycling process if these strains had this ability. This was the case with strain IAB 59 which showed an initial decrease in the number of total *B. sphaericus* cells, however, the remaining spores were able to recycle.

Microcycle sporulation, the phenomenon in which spores germinate and resporulate with no increase in number, is somewhat different from recycling. It is possible that microcycling could be occurring in those strains in which germination was low and the final number of spores was similar to the one initially ingested.

No mortality was produced among larvae fed with non-

toxic strains, NRS 718 or *B. subtilis* DB104. The decrease in spore number was parallel to the decrease in total bacilli. This indicates that the spores were eliminated by defecation or digestion after ingestion. The water was changed periodically to ensure no reingestion of the spores. It appeared that these spores could not germinate under the conditions present in the healthy larval gut and that they might need the nutrients released from the tissues after larval death for their activation and germination. I studied this possibility by testing these non-pathogenic strains as either recombinants (NRS 718 and DB104) or with added soluble binary toxin (ATCC 14577). Despite rapid larval kill, spores of all 3 strains germinated poorly. Apparently the spores of these strains cannot take advantage of the larval cadaver as a medium in which to grow. The factors present in the larvae that could trigger germination of other strains were not effective for them. *Bacillus subtilis* DB104 and *B. sphaericus* strains NRS 718 and ATCC 14577 are not mosquito pathogens and therefore these bacteria may have not evolved mechanisms to germinate and take advantage of the nutrients present in the larval tissues.

In the recycling experiments spores were not heat activated before feeding to larvae but part of the homogenate sample was heated at 80°C for 12 min. Sublethal heat treatment or extreme pH are effective in activating

spores for germination but the mechanism is unclear. Alteration in the permeability properties of the spore is probably involved since changes in spore coat ultrastructure and inner membrane fluidity have been reported (76). Heat treatment allowed the enumeration of just spores by the elimination of vegetative forms present in the sample. Heat pretreatment of the spores before plating might have also caused enhanced subsequent germination response. Results obtained with strain ATCC 14577 (larvae killed with soluble binary toxin), the type strain of the species, seem to indicate that the spores of this strain may be highly sensitive to activation by heat pretreatment. During recycling experiments in the larvae, spore counts of strain ATCC 14577 showed little variation but the number of total *B. sphaericus* was always somewhat lower than the number of spores. This seems to indicate that the spores in the heated samples had been activated while some of the spores in the sample not heated were unable to break the dormancy resulting in lower counts. With strain ATCC 14577, spores appeared to respond dramatically to heat activation.

Results of all these experiments demonstrated that just providing soluble toxin or a plasmid for expression of either toxin is not enough to enable low toxic or non-toxic strains that do not normally germinate, multiply and recycle, to do so. This also indicates that the toxins



themselves are not acting directly as germinant substances. It also suggests that only those strains that possess the ability to produce both toxins have also evolved the ability to recycle and take advantage of the larval nutrient environment to grow.

It is not yet known what exactly is triggering germination and enabling these strains to recycle. In these strains binary toxin production is linked to sporulation. It is likely that these strains evolved their ability to produce toxins before the ability of their spores to germinate in mosquito larvae and to recycle. Strains like 1883 that only produce the 100-kDa toxin which is produced during vegetative growth probably would not have been under selection pressure to evolve the ability to germinate in larvae since toxin production did not require going through the sporulation process.

During the course of this study, it was observed that strains of the low toxicity group (e.g. Q) sporulate poorly in NYSM broth. Strain LP12AS, despite producing the binary toxin, sporulated poorly in this medium and in the larvae did not show recycling ability. To obtain spores of LP12AS and Q for recycling studies, the bacteria had to be spread on NYSM plates where sporulation was improved. This indicates the difficulty that these strains have sporulating under certain conditions. There seems to be a correlation

between poor germination and poor sporulation, but there is not an obvious mechanism to account for this.

Although Bti recycling is similar to *B. sphaericus*, it is well known that *B. sphaericus* can persist for a longer time in the aquatic environment. It has been speculated that persistence is favored by recycling, however other factors may play a role in enhancing the longer persistence of *B. sphaericus*. DesRoches and Garcia (42) have reported that presence of cadavers in the aquatic environment contributes to maintenance and higher levels of the pathogen. These cadavers provide the source of bacteria for other larvae and protect the bacteria from the deleterious effects of sunlight. Arthropods, non-target invertebrates and predator species may have a role in persistence as well. Kratch et al. (61) found that these organisms help to redistribute the bacteria in the water column. This is important since the sinking of the spores and toxin to the bottom of ponds renders the bacteria inaccessible to the larvae. The adherence properties of this bacterium also influence the degree and speed of sinking (126). Yousten et al. (122) also reported that exposed non-target invertebrates might transport microbial control agents to untreated sites by carrying spores in/on the adult body. Although recycling may not be the only condition influencing persistence, it is indeed an important characteristic of the microbial

pesticides and should be taken into account when developing microbial control agents. For example, if toxin genes are moved to other bacilli, the spores of those strains should be tested for their ability to recycle. The failure of *B. subtilis* to recycle is a good illustration of the fact that this event cannot be assumed to occur.

#### **Effects of rifampicin resistance on sporulation**

The strains used in this study were spontaneous rifampicin resistant mutants from which spore suspensions were obtained. Sometimes it was desirable to select mutants resistant to a different antibiotic or to have a second antibiotic marker. Isolation of other antibiotic resistant strains proved to be more difficult than isolation of rifampicin-resistant mutants. Resistance to some antibiotics produced growth or sporulation problems, and if mutants were obtained they were unstable. This was the problem found when trying to obtain mutants resistant to antibiotics such as vancomycin, tetracycline and ampicillin. Even rifampicin resistance produced sporulation problems in some *B. sphaericus* strains.

In some cases spores were not obtained by growing the cells in NYSM broth with shaking, but they could be produced on NYSM plates. However some rifampicin-resistant strains did not sporulate under any of the conditions tried. These strains could not be used in the recycling work since it was

necessary to start with spores to feed the larvae. These strains were replaced by strains representing the same serotype or toxin group. However, rifampicin-resistant mutants were easily isolated from most of the strains selected for this study.

Yurks and Singer (129) conducted studies to isolate *B. sphaericus* strains resistant to different antibiotics for use as environmental marker strains. They found that *B. sphaericus* strains are naturally resistant to antibiotics such as chloramphenicol and streptomycin. Other studies to screen for naturally occurring *B. sphaericus* resistant strains confirmed these results (1, 60). In Yurks and Singer's study, rifampicin resistant strains were isolated often as a one-step acquisition. Since this study involved a greater variety of strains, some of them more refractory to mutation, our isolation required a step-wise procedure. In addition, the goal was to obtain mutants resistant to at least 25 ug/ml, while the Yurks and Singer target was to get resistance to a minimum inhibitory concentration (MIC) that ranged between 0.39 to 25 ug/ml for six strains. These authors found as we did, that vancomycin and ampicillin-resistant mutants usually lost viability. This seems to occur frequently, not only with the bacilli but also with clinical isolates.

The rifampicin resistance mutation is probably

chromosomal because resistance persisted indefinitely without selection pressure. The strains did not have to be grown with the antibiotic added to the medium to retain resistance.

#### **In vitro spore germination**

Germination, the conversion of dormant spores into vegetative cells is the first stage of the recycling phenomenon. This step leads to breaking of dormancy, reactivation of metabolism and a variety of degradative biochemical reactions (76, 87, 88). Typical spore properties such as refractility by phase microscopy, resistance to ordinary stains, and heat resistance measured by plate counts are lost at this time.

It is known that different *Bacillus* species respond differently to a variety of germinant substances. Results of the in vitro germination experiments with strains 1883, LP12AS, and 2362-7 showed that the L-alanine-inosine combination favored germination of spores of strains 2362-7 and 1883. L-alanine-inosine has been reported to be a powerful germinant in *B. subtilis* and several other species. Other species are known to germinate in sugars plus inorganic cations or cations alone (76, 87) The extent of germination of strains 2362-7 and 1883 in alanine-inosine was comparable to the extent of germination in the larval cadaver though more rapid. This may reflect the time

required to release germinants from tissues of dying larvae.

None of the strains assayed responded to arginine, although this amino acid serves as a sole carbon and nitrogen source for growth of the bacteria. Failure of these strains to germinate well in NY broth was somewhat surprising. Perhaps the concentration of free (not bound in protein or peptide) L-alanine is too low to cause germination or there is little or no inosine in the NY broth.

Strain LP12AS did not germinate well with any of the germinants used and it also did not germinate well in the larval cadaver. This strain may respond to some completely different germinant or it may have a defective germination system. It would be interesting to examine the germination and recycling response in other isolates of this serotype.

The ability of bacterial spores to germinate quickly and in high percentage with any germinant would allow that bacterium to leave the dormant state, grow rapidly and to successfully compete for the nutrients in the surrounding medium. This feature is certainly an ecological advantage and it may contribute to the ability of such a strain to recycle and to persist in the environment.

The wide distribution of *B. sphaericus* in soil and sites suitable for larval growth such as pools, lakes and drainage ditches, suggests that the ability of these strains

to recycle has proved an ecological advantage. Spores could remain dormant for long periods under unfavorable conditions and germinate in conditions conducive to growth, such as the presence of larvae in the ponds. If conditions remain constantly favorable, the vegetative forms will predominate, increasing the number of the population.

A large number of spores of a particular type in a habitat is strongly indicative of previous or continuous growth and metabolism in the habitat. For *B. sphaericus*, those strains that are able to rapidly kill the larvae before being eliminated from the gut, and whose spores germinate and grow using the larval cadaver as the medium, would have a competitive advantage. Thus recycling may be responsible for maintaining high populations of these spores in the habitat.

#### Conjugation

*B. sphaericus* 2362 has been registered with the U.S EPA and will be commercialized soon. It is one of the strains found able to recycle in the larval cadavers. There is continuing interest in expanding the spectrum of activity, increasing the level of toxicity and improving manufacturing efficiency of these bacteria. Some attempts to achieve these modifications have already been made (10, 86, 112). These genetically engineered strains are usually produced by introduction of the specific gene on a plasmid. The plasmids

commonly used as vectors carry an antibiotic resistance gene as a marker and lack the functions for self-transfer. However, these nonself-transmissible plasmids can sometimes be mobilized by larger plasmids capable of self-transfer. The likelihood of gene exchange in nature and the possibility of these genes being spread in the environment is undesirable. In this section I report the result of investigations on the possibility of gene transfer in *B. sphaericus*.

Gene exchange in the environment seems to be relatively common in some species. It has led to the spread of antibiotic resistance among bacteria, but may also lead to extensive reorganization of the genotypic structure of the populations. For example, *B. subtilis* growing together in soil have been shown to exchange blocks of linked genes by transformation (87). However, conjugation is probably the prevailing mechanism for natural dissemination of plasmids. Conjugational transfer has been demonstrated in nutrient media in laboratory, in microcosms representing natural habitats (93), in soil (89), in wastewater (46, 74), and in drinking water (94).

If *B. sphaericus* is able to conjugate in nature, the most probable place for conjugation to occur will be the larval cadaver. Here, germinating spores of *B. sphaericus* will produce large numbers of vegetative cells that will be



in close proximity with larval flora or bacteria ingested by the larvae during the feeding process. *B. sphaericus* strains that would more likely conjugate would be those whose spores can germinate and grow in the nutritious environment provided by the larval cadaver. Results from the recycling experiments suggested that only high toxicity strains expressing both toxins have evolved the ability to readily germinate in high percentage and take advantage of the rich environment created by nutrients released by the larvae. These strains would also be the candidates for genetic manipulations and commercialization.

The cellular and molecular basis of conjugal transfer by the self-transmissible plasmids in *Bacillus* is not completely understood. The mechanism that leads to effective cell to cell contact between donors and recipients and transmission of the genetic material is not known. Even less is known about the *B. sphaericus* plasmids and their ability to transfer to other *B. sphaericus* strains or other species. Therefore, it is hypothesized that the *B. sphaericus* system is similar to that found in more explored and better known systems, for example, *B. subtilis* and *B. thuringiensis*.

Results from experiments directed to establish the conditions in which conjugation of *B. sphaericus* was most likely to be observed showed that there were some similarities with the requirements of other bacilli.

Conjugation frequencies were higher with increased time of incubation in filter matings on nonselective medium. These results are similar to those reported by Gonzalez and Carlton (48) in *B. thuringiensis* conjugation experiments. They found that transconjugants appeared as early as 90 min, but high frequencies were obtained between 6 and 25 h.

Incubation at 37°C produced higher conjugation frequencies than at the lower temperatures. *B. sphaericus* is a mesophilic bacterium which grows well at 37°C. The ability to grow well is an important feature and affects conjugation frequencies in strains able to conjugate.

Broth mating for 20 h resulted in markedly lower frequencies than mating on filter membranes. It has been reported that broad host range plasmid transfer, in this case pAMB1, requires that the donor and recipient cells be co-cultivated on a solid surface for most efficient plasmid transfer (73).

That conjugation frequencies were higher when the donor was present in higher number than the recipient may indicate that a high availability of self-transferable plasmid favors conjugation.

Addition of DNase did not reduce the frequency of transformants obtained, which was very similar to the frequency obtained in the control with no addition of DNase. DNase destroys free DNA and no transformants would have been

obtained if the mechanism were transformation. If transduction or phage mediated transformation were the mechanism, phage particles present in the supernatant of the donor would have produced transformants. No transformants were obtained in this test. These tests are typically used to eliminate the possibility that transformation or transduction are the mechanisms for DNA transfer (8, 11, 48, 64).

Results from conjugation of strain 2362(pAMB1) with other bacteria varied. The donor 2362(pAMB1) successfully transferred the pAMB1 plasmid to closely related strains belonging to its own serotype (5a5b) and to two other strains of serotypes 9a9c and 25. pAMB1 is a broad host range plasmid that can promote its own transfer to a variety of species, such as *Streptococcus*, *Staphylococcus*, *Clostridium*, *Lactobacillus* and *Bacillus* (66, 68, 73, 84, 85, 113). Therefore, it was expected that this would be a good system to demonstrate plasmid transfer between *B. sphaericus* and other bacteria. pAMB1 encodes resistance to the macrolides, lincosamides and streptogramin B antibiotics (MLS<sup>r</sup>). These determinants encode an RNA methylase capable of N<sup>6</sup> dimethylation of specific adenine residues in the ribosomal RNA, which prevents the binding of MLS antibiotics to the bacterial ribosome (73). The use of erythromycin as the marker for selection of transconjugants inheriting the

plasmid in addition to the rifampicin resistance conferred by the recipient allowed initial identification of transformants. Confirmation that plasmid transfer had occurred and that the colonies obtained were not donor or recipient cells that had become resistant to the other antibiotic was done by gel electrophoresis, auxotrophic identification and phage typing. To rule out the appearance of spontaneous resistant mutants in each experiment, the donor and recipient cells were always plated first on media containing both antibiotics as well as the antibiotic to which they were resistant. When spontaneous mutants arose, their frequencies were very low compared to the high frequencies of real conjugation.

High frequencies of conjugation among *B. sphaericus* that are more closely related was similar to results obtained by Gonzalez and Carlton (49) with Bti. They reported high frequencies of conjugation among closely related Bti variants. Wiwat et al (116) obtained plasmid transfer from Bti to 25 subspecies of *B. thuringiensis*. They found that frequencies of conjugation were higher within subspecies than between subspecies.

Frequency of pAMB1 transfer was higher with strain 1593-P51 than with the parent 1593 as the recipient. Strain 1593-P51 has a modified cell surface protein layer. This suggests that composition and structure of the outermost

layer of cell walls may have an effect on plasmid transfer in *B. sphaericus*. Aronson and Beckman (8) have suggested that changes in surface properties of the donor affect the efficiency of transfer. They found strains with altered plasmid profiles that had surface properties different from the parental strains. The changes could be detected by observing different colony morphology and clumping in suspension.

The S layer or surface layer protein present in many bacteria has been suggested to have a role in conjugation, perhaps acting as a "channel for gene transfer". Wiwat et al. (117) demonstrated that antibody produced against the S-layer of Bti reduced frequencies of plasmid transfer by conjugation in broth. *B. sphaericus* also possess an S layer. Strains 1593-4RC and 1593-P51, derivatives of strain 1593, were isolated based on their resistance to a lytic bacteriophage. Strain 1593-P51 has a protein surface layer of lower molecular weight than the parental strain, whereas strain 1593-4RC has an S-layer protein of the same molecular weight as the parental strain. Although I do not have evidence that the S layer in *B. sphaericus* has a specific role in conjugation, it is interesting that strain 1593-P51 possessing a modified S layer also presented the highest frequency of conjugation among any of the *B. sphaericus* strains tested.

Strain 1593 and its derivatives carry two large plasmids that migrate on the gel to the same point as pAMB1. When 1593-4RC transconjugants were examined by agarose gel electrophoresis, loss of one or both of the pair of plasmids normally seen in the recipient strain was observed. A single plasmid was present in the position previously occupied by the pair of plasmids. It was not determined if this was pAMB1 (replacing the pair) or if it was one of the pair. The presence of erythromycin resistance indicates that the single plasmid was pAMB1. Plasmid loss in strain 1593-4RC suggests that this may be a case of incompatibility-like phenomenon like the one described by Gonzalez and Carlton (48). Manifestations of this incompatibility are restriction of entry for certain plasmids in some strains or entry of a plasmid destabilizes the inheritance of the resident plasmid. This produces the loss of the resident plasmid in that strain. The phenomenon of plasmid incompatibility occurs in plasmids that are genetically related, for instances, two plasmids sharing common elements responsible for plasmid maintenance, such as replication control or partitioning systems (62). However, the fact that transfer of pAMB1 to 1593 and 1593-P51 did not eliminate in any of them the resident plasmids, argues against this hypothesis.

Because pAMB1 was not unambiguously identified on gels of 1593 and its derivatives, antibiotic resistance and phage

typing were used in recognition of transconjugants. The two 1593 derivatives (1593-4RC and 1593-P51) were recognized based on their resistance to lytic phage to which the donor, 2362(pAMB1), was sensitive.

In this work conjugation was obtained with *B. sphaericus* strains within the same serotype as the donor and with two other serotypes. However, conjugation was not detected when the recipient strains were *B. sphaericus* belonging to other serotypes or a non-pathogenic *B. sphaericus* strain isolated from field collected larvae. Several reports suggest that mating may be the mechanism for natural dissemination of plasmids within the *Bacillus* species (8, 11, 47, 48, 64), however, some strain specificity is involved. Strain specificity was reported by Loprasert et al. (72) in conjugation experiments involving *B. thuringiensis* strains. Reddy et al. (91) described the occurrence of "entry exclusion phenomenon" in interspecies matings using *B. thuringiensis* subspecies as donors of their self-transmissible plasmids. They found that a resident plasmid may interfere with the entry of genetic material in strains containing the same or a related self-transfer plasmid. Aronson and Beckman (8) also found that not all *B. thuringiensis* strains efficiently transfer their plasmids. For example, no detectable plasmid transfer occurred from *B. thuringiensis* serovar. *kurstaki* HD1 to a plasmid-free

*thuringiensis* serovar. *kurstaki* HD1 to a plasmid-free derivative of the same strain.

Cokmus and Yousten (31) reported that some strains of *B. sphaericus* produce high molecular weight substances thought to be bacteriocins that inhibit other *B. sphaericus*. It is possible that bacteriocin produced by the donor, 2362(pAMB1) may have affected conjugation with some of the recipients (e.g. ATCC 14577, NRS 1199, the *B. sphaericus* larval isolate). Evidence for the bacteriocin effect on conjugation was given by Aronson and Beckman (8). They found that efficiency of transfer may be affected by the presence of plasmids encoding bacteriocins. When the plasmid encoding bacteriocin was lost, efficiency of transfer was increased. In the case of *B. sphaericus*, bacteriocin production has not been linked to a specific plasmid.

The decrease in number of recipient cells following mating has also been observed in Bti conjugations. Andrup et al. (4) found that frequencies of transfer were affected when transfer involved strains with different growth rates or a strain inhibiting the growth of other, for instance, Bti to *B. thuringiensis* serovar. *kurstaki*.

In conjugation experiments with *B. sphaericus* (pAMB1) as the donor, transfer was not obtained when recipients were Bti, *B. subtilis* or isolates of *B. mycoides* (the latter obtained from field collected mosquito larvae). There are



*Bacillus* (8, 11, 47, 48, 64, 72, 101, 102). All of these reports refer to species belonging to members of the same taxa within the genus *Bacillus*. *B. thuringiensis*, *B. mycoides*, *B. subtilis*, *B. megaterium*, *B. pumilus* and *B. licheniformis*, among others, belong to group II. This is a traditional or natural grouping that is consistent with phylogenetic observations and that allocates many *Bacillus* species into six taxa (88). Members belonging to these groups have distinguishable physiologies and fit the division based on spore morphologies.

Members of group II produce ellipsoidal spores that do not swell the mother cell, produce acid from variety of sugars and most are able to grow at least weakly in absence of oxygen, especially if nitrate is present. *B. sphaericus* belongs to group IV which is characterized by production of spherical spores that usually swell the mother cell. Most members of this group do not contain meso-A<sub>2</sub>pm, diaminopimelic acid, as do all the other members in the *Bacillus*. Instead, they contain L-lysine or ornithine in the cell wall and although all the species are strictly aerobic, some have limited ability to produce acid from sugars.

As is implied by phylogenetic divisions, *B. sphaericus* is not closely related to members of group II. This may explain at least in part, why they do not conjugate. But other factors may be interfering. One of these is the nature

other factors may be interfering. One of these is the nature of the plasmid. In the case of the *B. thuringiensis* and *B. subtilis* reports of interspecies conjugation, the plasmids involved were self-transferable plasmids native to the donor strains. In the case of pAMB1, even though it is a promiscuous plasmid it is not native to *B. sphaericus*. In this respect, Orzech and Burke (84) who transferred pAMB1 from *E. faecalis* to *B. sphaericus* found that frequencies of conjugation were better with *E. faecalis* than with *B. sphaericus* transconjugants as donors. Among the possible reasons given to explain these results was the possibility that proteins involved in mobilization and transfer were not as efficiently transcribed and translated in *B. sphaericus*. Also, composition and structure of cell walls may have had an effect on plasmid transfer.

It is still not known if pAMB1 encodes surface components on donors and recipients that are involved in conjugation (73). It has been reported that small variations such as type and pore size of filter, or filter side can produce variations in transfer frequencies of pAMB1. However, it is known that pAMB1 possesses considerable latitude in the range of its transfer and inheritance. pAMB1 can conjugally transfer from *E. faecalis* to a number of species, including various species of *Bacillus*. It is also possible that impairment of pAMB1 transfer was due to

factors related to donor specificity. Shows and Andrews (97) obtained conjugative transposition of Tn 916 and the transposon-dependant mobilization of pC194 in matings between *B. subtilis* and Bti, and between *E. faecalis* and Bti. However, transconjugants were not detected when the donor was *B. sphaericus* carrying Tn916 in matings with Bti. Van Der Lelie and Venema (113) obtained transfer of pAMB1 from *E. faecalis* to *B. subtilis*, however no transfer was obtained when *B. subtilis* was used as the donor and *E. faecalis* as the recipient. Aronson and Beckman (8) in matings between Bt strains and *B. cereus* found that sometimes strains receiving a specific plasmid did not act as donors. This was the case of *B. cereus* transciipients of the 50 MDa plasmid from *B. thuringiensis* HD73 which failed to transfer the acquired plasmid to other strains. Battisti et al. (11) used *B. thuringiensis* as donor in interspecific matings with *B. anthracis* and *B. cereus*. Although transfer occurred, some specificity in the transfer among various donors was observed. Some *B. thuringiensis* donors were more effective as donors with *B. cereus* than with *B. anthracis*. One possible reason is that *B. cereus* is more closely related to *B. thuringiensis* than is *B. anthracis*.

Results from matings between *B. sphaericus* 2362(pAMB1) and the field collected larval isolates (*B. sphaericus* and *B. mycoides*) were in contrast with reports of conjugal

transfer between *B. thuringiensis* and field isolates. Singh Bora (101) obtained gene transfer from *B. thuringiensis* serovar. *kurstaki* HD1 into a leaf colonizing *B. megaterium*.

The entry of genetic material into a bacterial cell can be affected by the presence of a restriction system in the recipient cell. Koehler and Thorne (64) demonstrated that the *B. subtilis* (*natto*) plasmid pLS20 was self transmissible. pLS20 does not carry a known selectable marker that made it possible to select for transipients. Therefore, cotransfer of pBC16 made it possible to select for transconjugants. Cotransfer was only observed in intraspecies matings and in matings with a restriction-deficient *B. subtilis* strain. There was indication that pLS20 was transferred to various recipient strains but was subject to restriction.

In these experiments, the possibility was tested that failure to detect transfer of pAMB1 in interspecies matings was due to the presence of restriction modification systems. The restriction deficient *B. subtilis* IG-20 was used as recipient in matings with 2362(pAMB1) as the donor. pAMB1 transfer to the restrictionless strain was not obtained. This does not eliminate the possibility that restriction interferes with transfer in matings with other recipient strains.

Many large self-transmissible plasmids in gram negative

nonconjugative plasmids. This mobilization can happen in two ways. In the mobilization called in *trans* or *donation*, the conjugative plasmid and the mobilized DNA remain physically separated through the mobilization process. The other form of mobilization occurs with the formation of a *cointegrate* between the conjugative plasmid and the replicon to be mobilized. This mobilization is in *cis* and the two plasmids recombine resulting in a covalent link between them (92).

Large, self-transferable plasmids that can mobilize small, non-conjugative plasmids, have been described in *B. thuringiensis* (11, 72, 91, 116) and in *B. subtilis* (64). In the entomopathogenic Bti, a 75-MDa plasmid has been shown to encode the toxin, and a self-transmissible plasmid of 135 MDa (pXO16) encodes the plasmid transfer functions (49 and 91). Jensen et al. (57) correlated this plasmid with the presence of an aggregation phenotype in Bti. This aggregation phenomenon occurs when small plasmids are mobilized and is non-pheromone-induced and protease-sensitive.

The large plasmid in *B. sphaericus* 2362 is cryptic, it lacks selectable markers. Cotransfer of the large plasmid with pUB110 from a donor such as 2362 carrying only these two plasmids would have demonstrated that the large plasmid is self-transmissible. In this series of experiments no verifiable transconjugants were obtained. Mobilization of

verifiable transconjugants were obtained. Mobilization of pUB110 was not detected.

These results suggest that the large plasmid (aprox. 180 kb) present in strain 2362 is not able to transfer or promote mobilization of this small, nonself-transmissible plasmid.

It is possible that the large plasmids found in *B. sphaericus* strains show some degree of incompatibility and that entry of the plasmid into the recipient carrying the same plasmid was excluded. This would be similar to the incompatibility-like phenomenon in *Bt* described by Gonzalez and Carlton (48). This could have been the case when strain 2362a (*lys*<sup>-</sup>) was used as the recipient. This strain was used as a recipient due to its demonstrated ability to conjugate in membrane mating experiments where 2362(pAMB1) was the donor. To avoid the possibility of plasmid incompatibility, other recipients used in this series of experiments were *B. sphaericus* strains whose plasmid profiles were markedly different from the donor (2297, 31-2, Q, 1883 and 1691). Strain 1691 does not harbor any plasmid and therefore incompatibility should not have been a problem. Strains 1691, 2297 and 31-2 had been shown to be able to conjugate with strain 2362(pAMB1) on membranes.

It could be possible that the large *B. sphaericus* plasmid was capable of self-transfer, however it could not

plasmids can be mobilized in either of two general ways. The first is by *cointegrate* formation (sometimes termed *conduction*). This is mediated by recombination between homologous sequences in the two plasmids or by the association of the two plasmids during the translocation of a transposable element. The second is by *donation* in which the two plasmids are independently transferred, therefore, there is no need for homologous sequences (92). If the mechanism for pUB110 transfer were cointegrate formation, both plasmids should have had some degree of homology and system-specific proteins to mediate recombination. The sequence of the 180 kb cryptic plasmid is unknown so it cannot be compared to pUB110 to look for sequence homology. Because of its dependence on specific interactions, frequencies of transfer are generally low when conduction is the mechanism of transfer.

Reports of *B. thuringiensis* plasmids that promote their own transfer and are capable of mobilizing pBC16 and other small nonconjugative plasmids are well documented (11, 64, 72, 91, 116). *B. sphaericus* and Bti are already used in many parts of the world as mosquito control agents and it would not be surprising if they can be recovered from the same mosquito breeding site. In an attempt to determine if the two entomopathogenic bacilli could transfer genetic information, matings were carried out between Bti carrying

information, matings were carried out between Bti carrying pBC16 ( $tet^r$ ) and two *B. sphaericus* strains, 2362a ( $lys^-$ ) and 1691 ( $his^-$ ). No transciipients were recovered in any case. Results from these experiments and the ones outlined before involving matings between *B. sphaericus* and bacilli members of group II, seem to indicate that these particular bacilli are not able to transfer genetic material among themselves, at least under the conditions and with the plasmids tested.

The main reason for performing the various conjugation experiments was to reach the final goal, to determine if conjugational transfer of genetic material occurs in the mosquito larval cadaver. No transconjugants were detected in larval cadavers up to 72 h after feeding spores of donor 2362(pAMB1) together with either 2362a or 1593-P51 as recipients. It is possible that conjugation does not occur in the larval cadaver. However it is also possible that the frequency of conjugation was too low to be detected. The cadaver may be similar to mating in liquid medium where the frequency of conjugation was about  $7 \times 10^{-7}$ . In the trials with both potential recipients, the number of recipient cells in cadavers was about  $10^4$  to  $10^5$  per cadaver at 48 and 72 h. If the frequency was similar to that found in broth, one could expect about 1 transconjugant per 100 to 1000 cadavers examined. In these tests, 75 cadavers were examined at 48 h and 50 cadavers at 72 h. If conjugation was



occurring, it may be too infrequent to detect in these experiments.

Another factor that may have complicated the in vivo conjugation experiments is the apparent ability of strain 2362(pAMB1) to germinate very efficiently. This was observed in experiments using chemical germinants (alanine-inosine, arginine, NY broth). In the conjugation experiments in larvae, the number of recipient cells dropped after 72 h of feeding while the number of donor cells increased. This ability of donor spores to germinate quickly and in high percentage would allow the bacterium to leave the dormant state, grow rapidly and to successfully compete for the nutrients in the surrounding medium. During the in vivo experiments, this competition phenomenon could interfere with conjugation. The decrease in the number of recipient cells could reduce the frequency of conjugation with that particular recipient.

Since conjugation between strains of *B. thuringiensis* in infected lepidopteran larvae has been demonstrated (56), one more possibility was tested. This was the possibility that the larval cadaver was not suitable for plasmid transfer due to substances released from the tissues that could also inhibit conjugation on membrane. Larval homogenate incubated with bacterial mixtures did not affect frequencies of conjugation on membranes. These results

suggest lack of detection of conjugation in the larval cadaver was probably not due to inhibitors present in the larvae. It also supports the suggestion that frequency of conjugation in the larvae was too low to be detected. The physical and chemical environment present in dead and dying mosquito larvae is undoubtedly very different from that on the surface of a membrane in contact with a bacteriological medium.

## CONCLUSIONS

### Spore germination and recycling

Spores of highly pathogenic *B. sphaericus* strains of three serotypes which produce the binary and 100-kDa toxins were able to recycle in larval cadavers. These spores germinated rapidly and in high percentage, and the resulting vegetative cells multiplied and sporulated again which resulted in a higher number of spores than originally ingested.

Spores of low toxicity strains, and non-toxic strains supplemented with binary toxin or carrying a gene expressing the binary or 100-kDa toxin, germinated poorly and did not recycle.

Neither the binary toxin, the 100-kDa toxin or the combination of both seem to act as direct spore germinants in larval cadavers.

Spore germination of *B. sphaericus* strains in the larval cadaver proved to be as efficient as when spores were mixed with substances known to stimulate spore germination in other bacilli.

## Conjugation

Conjugation and transfer of the broad host range plasmid pAMB1 in filter matings occurred between closely related *B. sphaericus* strains, such as strains within the same serotype (5a5b) and two other serotypes.

The large plasmid (180 kb) present in some *B. sphaericus* strains seems not to be capable of self-transfer or of mobilization of a small non-transferable plasmid.

Conjugation was not detected in the larval cadaver between strains that were shown to conjugate on filters. It is possible that the cadaver is not suitable for conjugation or that the frequency is too low to be detected.

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### PROFESSIONAL EXPERIENCE

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#### Clinical Laboratory

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#### Internships

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### PROFESSIONAL MEMBERSHIPS

- \*American Society for Microbiology (ASM)
- \*Society for Invertebrate Pathology (SIP)
- \*Phi Sigma, Biology Honor Society, Virginia Tech.
- \*Phi Kappa Phi, Graduate Student Honor Society
- \*Sigma Xi, The Scientific Research Society

### NON PROFESSIONAL MEMBERSHIPS AND SERVICES

- \*Biology Graduate Student Association, Representative
- \*Graduate Student Assembly, Delegate
- \*Council of International Students Organizations (CISO)

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- \*President, International Club VA Tech, 1992-1993
- \*Commission of Student Affairs, GSA delegate
- \*Patrulla Aerea Colombiana (Colombian Air Patrol),  
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**GRANT AND HONOR AWARDS**

- \*USAID Scholarship. Epidemiology, statistics  
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**PUBLICATIONS**

Correa, M. and A. Yousten. 1995. *Bacillus sphaericus* Spore Germination and Recycling in Mosquito Larval Cadavers Strains. J. Invertebr. Pathol. 66:76-81.

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**PRESENTATIONS AT PROFESSIONAL MEETINGS**

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