Conjugation by Mosquito Pathogenic Strains of *Bacillus sphaericus*

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A mosquito pathogenic strain of Bacillus sphaericus *carried out the conjugal transfer of plasmid pAMß1 to other strains of its own and two other serotypes. However, it was unable to conjugate with mosquito pathogens from three other serotypes, with* B. sphaericus *of other DNA homology groups or with three other species of* Bacillus. *Conjugation frequency was highest with a strain having an altered surface layer (S layer). Conjugal transfer of pAMß1 was not detected in mosquito larval cadavers.* B. sphaericus *2362 was unable to mobilize pUB110 for transfer to strains that had served as recipients of pAMß1. These observations suggest that it is unlikely that genetically engineered* B. sphaericus *carrying a recombinant plasmid could pass that plasmid to other bacteria.*

Key words: *Bacillus sphaericus* - mosquito pathogen - bacterial conjugation

Mosquito pathogenic strains of *Bacillus sphaericus* have been assigned to DNA homology group IIA (Krych et al. 1980). Pathogenicity is caused by the production of one or more of four toxins (Charles et al. 1996, Liu et al. 1996, Thanabalu & Porter 1996). Despite the proven effectiveness of mosquito larvicides based on *B. sphaericus* 2362, it may be useful to introduce genes for additional toxins from other bacteria such as *B. thuringiensis* or to add genes for extracellular protease to enhance manufacturing efficiency on protein substrates. These genes would initially be introduced on plasmid cloning vectors carrying antibiotic resistance genes as markers. The transfer of the antibiotic resistance genes to other bacteria by conjugation in the aquatic environment would be undesirable. It is unknown to what extent *B. sphaericus* is able to transfer genes by conjugation. Grigorova et al. (1988) demonstrated transfer of plasmids from *Streptococcus agalactiae* and *S. pyogenes* to *B. sphaericus* but did not investigate further transfer among *B. sphaericus* strains. Orzech and Burke (1984) reported that *B. sphaericus* 1593 could accept conjugative plasmid pAMß1 from *Enterococcus faecalis* JH2.2 and could transfer pAMß1 to another strain of *B. sphaericus.* Whether *B. sphaericus* could transfer the plasmid to a wider range of *B. sphaericus* strains or to other bacteria was not reported. Also, it is unknown if *B. sphaericus* is capable of conjugation based upon plasmids it normally carries. We addressed these questions by using the broad host range plasmid pAMß1 to investigate the range of bacteria to which a known, conjugative plasmid could be transferred. To determine if the large, cryptic plasmid carried by *B. sphaericus* 2362 is conjugative, the mobilizable plasmid pUB110 was used as a marker to indicate if conjugation took place.

MATERIALS AND METHODS

Bacteria and mosquito larvae - *E. faecalis* JH2.2 [pAMß1] and *B. sphaericus* 2362 [pUB110] were obtained from W Burke. *B. subtilis* PSL1 UM4 [pLS20] and *B. subtilis* IG-20 UM1 were obtained from C Thorne. All other *B. sphaericus* isolates were from the Virginia Tech Culture Collection. Recipients in all matings were rifampicin resistant. *B. sphaericus* 2362 [pAMß1] was constructed by filter mating *E. faecalis* JH2.2 [pAMß1] with *B. sphaericus* 2362 and selecting erythromycin-resistant *B. sphaericus* colonies on BATS agar (Yousten et al. 1985) supplemented with 10 µg/ml erythromycin. The strain 2362 used as recipient was not restriction-deficient. *B. subtilis* PSL1 UM4 [pLS20, pUB110] was constructed by protoplast transformation (Chang & Cohen 1979) of PSL1 UM4 [pLS20] with plasmid DNA extracted from *B. sphaericus* 2362 [pUB110].

Conjugation - Matings in liquid were performed by a modification of the method of Battisti

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et al. (1985). Donor (*B. sphaericus* 2362 [pAMß1]) and recipient (*B. sphaericus* 2362 rif^r,lys^-) were grown with shaking (160 rpm) for 5 hr at 30°C in tubes of NY broth (Difco nutrient broth supplemented with 0.05% yeast extract). The same volume (2 ml) of donor and recipient were added to 25 ml of NY broth and shaken (160 rpm) at 30°C for 20 hr. The number of donors and recipients were enumerated on NY agar containing 10 µg/ml erythromycin (donor) or 25 µg/ml rifampicin (recipient). Transcipients were enumerated on NYSM agar (Myers & Yousten 1978) containing both antibiotics.

Conjugation on membranes was done by a modification of the method of Koehler and Thorne (1987). Donors and recipients were grown with shaking for 5 hr at 30°C. One ml each of donor and recipient were mixed and 0.1 ml of the mixture spread onto a 45 mm diameter, 0.45 µm membrane filter (Millipore, Bedford, MA) placed on NYSM agar. Bacteria were incubated for 20 hr at 37°C. Growth was washed off the membrane into 2 ml NY broth, diluted and plated. Donors, recipients, and transcipients were enumerated as described above using 25 µg/ml rifampicin and either 10 µg/ml erythromycin (pAMß1) or 5 µg/ml neomycin (pUB110).

Conjugation in mosquito larval cadavers was tested by feeding third instar larvae of *Culex quinquefasciatus*, mixtures containing equal numbers of donor and recipient spores as previously described (Correa & Yousten 1995). The larvae were removed from the spore suspension, rinsed, and placed in clean water lacking spores. At 0 hr (immediately upon completion of feeding), at 48 hr and at 72 hr, 75 larvae were removed, rinsed in sterile water, and homogenized in a sterile glass tissue grinder. At 48 hr and at 72 hr, all the larvae removed were dead. The homogenate was divided, and part heated (80°C, 12 min) and part held unheated prior to plating on NYSM agar containing erythromycin and rifampicin. Conjugation frequency in all experiments was the number of transcipients divided by the number of potential recipients.

Verification of transcipient identity and plasmid transfer - To verify that the putative transcipients were derived from the recipients, recipients were either amino acid auxotrophs (strain 2362 lys^- and 1691 his^-) or possessed different sensitivity to bacteriophages (Yousten et al. 1980) than the donors. Representative transcipients were selected and tested for these traits. Plasmid extraction and electrophoresis was done as described by Seyler et al. (1993).

RESULTS

The effect of incubation time on membranes on conjugation frequency was tested by mating *B. sphaericus* 2362 [pAMß1] with *B. sphaericus* 2362 lys^- and plating after 3, 6, 9 and 20 hr. There was an approximate 20-fold increase in frequency as the incubation was extended from 3 to 20 hr. The same pair of strains was used in 20 hr filter mating to demonstrate an approximate doubling in conjugation frequency as the temperature of membrane incubation was increased from 25°C to 30°C and another doubling with an increase from 30°C to 37°C (data not shown). Subsequent filter matings were carried out for 20 hr at 37°C. Conjugation frequency in broth and on membranes was compared by mating *B. sphaericus* 2362 [pAMß1] with *B. sphaericus* 2362 lys^-. Conjugation frequencies in broth were much lower ($7.2X10^{-7}$ $\pm 1.7X10^{-7}$) than on membranes ($1.9X10^{-4} \pm 1.1X10^{-4}$).

B. sphaericus 2362 and other mosquito pathogens belong to DNA homology group IIA whereas the type strain of the species, ATCC 14577, is a nonpathogen and belongs to homology group I. Additional nonpathogens, all presently referred to as *B. sphaericus*, are found in four other homology groups. The pathogens of group IIA have been subdivided by serotyping. To determine the ability of mosquito pathogenic strain 2362 to transfer pAMß1 to other bacteria, filter matings were performed and the results are reported in Table. In addition to being able to transfer the plasmid to 2362 at a mean frequency of $1.9X10^{-4}$, 2362 [pAMß1] successfully transferred pAMß1 to strains 1593 and 1691 of the same serotype (5a5b) as the donor and to strains 2297 and 31-2 of serotypes 25 and 9a9c respectively. However, no transfer was detected to mosquito pathogens IAB460 (serotype 6), SSII-1 (serotype 2),or Kellen Q (serotype 1). Also, pAMß1 was not transferred to nonpathogenic *B. sphaericus* strain ATCC 14577 (homology group I), to NRS 1199 (homology group V) or to a *B. sphaericus* (homology group V) isolated from mud in a local mosquito breeding site. To test for the possibility of interspecies conjugation, *B. subtilis* IG-20, a restrictionless variant, as well as *B. mycoides* and *B. thuringiensis* var. *israelensis* were used as recipients. No transfer of pAMß1 was detected.

The highest conjugation frequency detected in this series of experiments was found with *B. sphaericus* 1593-5-1 as recipient. The mean frequency was about 15-fold higher than with 2362 as recipient and about 7-fold higher than with the parental 1593 as recipient. Strain 1593-5-1 is the same homology group and serotype as the donor, 2362 [pAMß1], but differs by possessing a sur-

TABLE

Conjugation frequencies between *Bacillus sphaericus* 2362 [pAMß1] and other strains of *B. sphaericus*[a]

Recipient strain	Serotype	Conjugation frequency (mean ± SD)
2362	5a5b	$1.90 \times 10^{-4} \pm 1.13 \times 10^{-4}$
1593	5a5b	$4.03 \times 10^{-4} \pm 3.07 \times 10^{-4}$
1593-5-1	5a5b	$2.93 \times 10^{-3} \pm 2.56 \times 10^{-4}$
1691	5a5b	$1.80 \times 10^{-5} \pm 7.07 \times 10^{-6}$
2297	25	$2.20 \times 10^{-4} \pm 1.41 \times 10^{-4}$
31-2	9a9c	$8.85 \times 10^{-5} \pm 3.04 \times 10^{-5}$

a: donor strain was neomycin resistant and all recipient strains were rifampicin resistant. Transcipients were selected on medium containing both antibiotics.

face protein layer, S layer, of lower molecular weight than the parent strain 1593 (Lewis & Yousten 1988). The mutant was isolated based on its resistance to phage 4, a lytic phage for both 2362 and the parental strain 1593. The phage resistance was used as positive identification of the transcipients.

Evidence against transformation or transduction being involved in pAMß1 transfer was provided by carrying out the mating on a membrane incubated on medium containing 100 µg/ml DNase and by substituting filter sterilized culture supernatant from the donor for the donor bacteria themselves. DNase did not affect the frequency of recovering erythromycin resistant colonies and no erythromycin resistant bacteria were found when culture supernatant was incubated in place of the donor bacteria.

Several large, self-transmissible, usually cryptic plasmids detected in *B. thuringiensis* and *B. subtilis* can mobilize the transfer of smaller plasmids (Battisti et al. 1985, Koehler & Thorne 1987, Reddy et al. 1987). It was unknown if the large, cryptic plasmid residing in *B. sphaericus* 2362 was conjugative and if it might be capable of mobilizing plasmids. This was tested using strain 2362 carrying the large, cryptic plasmid as well as the 4.5 kb pUB110.

The pUB110 present in the strain 2362 that was to be used as donor was proven to be mobilizable by transferring the plasmid to *B. subtilis* PSL1 UM4 [pLS20] by protoplast transformation. pLS20 had been shown to promote the transfer of pUB110 (Battisti et al. 1985). *B. subtilis* PSL1 UM4 [pLS20, pUB110] was filter mated with *B. subtilis* IG-20 UM1 and transcipients selected on a medium containing 10 µg/ml rifampicin and 5 µg/ml neomycin. Conjugation frequency obtained in this mating was equal to that reported by Koehler and Thorne (1987) who used the same *B. subtilis* strains. This demonstrated that the pUB110 used in experiments with *B. sphaericus* was capable of being mobilized.

B. sphaericus 2362 [pUB110] was filter mated with *B. sphaericus* 2362, 1691, 2297 and 31-2, four strains that had been shown to be effective recipients for pAMß1. It was also mated with strains Kellen Q and 1883 (serotype 2). In none of these experiments were transcipients recovered.

If *B. sphaericus* was able to transfer recombinant plasmids by conjugation following its dispersal as a larvicide in the aquatic environment, the most likely site for this to take place would be in the larval cadaver. In this site the spores of *B. sphaericus* are known to germinate and grow vegetatively in the presence of large numbers of bacteria indigenous to the larvae (Correa & Yousten 1995). To test the suitability of the larval cadaver as a site for conjugation, spores of strain 2362 [pAMß1] were fed with spores of either 2362 lys^- or 1593-5-1 to third instar mosquito larvae. Larvae accumulated between 10^5 and 10^6 spores of each strain per larva. In two trials with each recipient strain, no transcipients were recovered at either 48 hr or 72 hr after feeding, although these same combinations of bacteria had been shown to conjugate when filter mated. To test whether soluble substances in the larval cadaver might interfere with conjugation, larval homogenate was mixed with cells of 2362 [pAMß1] and 1593-5-1 when they were placed on filter membranes for mating. There was no decrease in conjugation frequency in the presence of homogenate.

DISCUSSION

The broad host range, conjugative plasmid pAMß1 was used to determine favorable conditions for plasmid donation by *B. sphaericus* 2362. With the same strain as recipient, conjugation frequency increased both with increase in temperature and time of incubation. The frequency was much higher when mating was done on a membrane surface than when done in broth, a common observation among gram positive bacteria.

Transfer of pAMß1 was successful when strains of the same serotype (5a5b) as the 2362 donor were used as recipients. pAMß1 was also transferred to two other pathogens, strains 2297 and 31-2, of different serotypes. However, not all strains of DNA homology group IIA (mosquito pathogens) functioned as recipients. Strains IAB460, SSII-1, and Q yielded no transcipients. Also, strains outside homology group IIA as well as three other species of *Bacillus* did not yield transcipients. Successful recipients of serotype 5a5b are known to have restriction endonucleases, but these are likely to have

DNA modified in the same way as the donor (2362) of that serotype. Strains 2297 and 31-2 of different serotypes than the donor lack restriction endonuclease activity and this may explain their success as recipients. Kellen K and SSII-1 have restriction endonuclease of different specificity than 2362 and this may explain their failure as recipients (Zahner & Priest, pers. comm.). Also, *B. sphaericus* possesses a surface protein layer (S layer) that is presumably the point of contact between cells at the initiation of conjugation. The protein of this layer is known to differ immunologically and in peptide maps between serotypes. It also differs in arrangement of subunits between homology groups (Lewis et al. 1987). It is possible that differences in S-layer protein create physical barriers that prevent plasmid transfer. The observation that the conjugation frequency was higher with a mutant strain (1593-5-1) having a modified S-layer protein than with the parent strain, suggests that the S layer may be involved in conjugation in this species.

The existence of cryptic, conjugal plasmids in *B. thuringiensis* was demonstrated by detecting the mobilization of pUB110 and pBC16 (Battisti et al. 1985, Reddy et al. 1987). However, no transfer of pUB110 was detected from *B. sphaericus* 2362 to strains that had been shown to be recipients for transfer of pAMß1. This indicates that the large, cryptic plasmid of *B. sphaericus* 2362 is not capable of mobilizing smaller plasmids.

Jarrett and Stephenson (1990) demonstrated plasmid transfer between strains of *B. thuringiensis* in cadavers of lepidopteran larvae killed by the toxins of that bacterium. No transcipients were recovered from mosquito larvae killed by the mixture of *B. sphaericus* spores fed to them although the same strains were capable of transferring pAMß1 on membranes. It is possible that the conditions in the larval cadavers are not suitable for conjugation or that the failure to detect transcipients is related to the frequency of conjugation and the number of bacteria available in the cadavers. If the frequency of conjugation was similar to that found on membranes (about 10^{-3} to 10^{-4} for the recipients tested in larvae), a few transcipients should have been recovered from among the recipient cells present in each cadaver. However, conjugation frequency was much lower in broth than on membranes, and the conditions in the decomposing cadaver may have resembled broth more than membrane. A lower conjugation frequency similar to that in broth would have produced too few transcipients to detect unless several hundred or thousand larval cadavers had been examined. The number of *B. thuringiensis* cells in the lepidopteran larvae used by Jarrett and Stephenson (1990) was higher (about 10^6 to 10^7 per larva) than the number present in smaller mosquito larvae. Different conditions in the lepidopteran cadaver and a higher number of bacteria may have been responsible for conjugation in that site. If conjugation occurred in mosquito larval cadavers, it was a rare event below the level of our detection sensitivity in these experiments.

The failure of *B. sphaericus* 2362 to mobilize transfer of pUB110 and the apparent low (undetectable) conjugation frequency of a known conjugative plasmid in larval cadavers, indicates that it is unlikely that genetically engineered *B. sphaericus* would pass recombinant plasmids to other bacteria in the larval cadaver.

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