Microbiology, Vol. 72, No. 6, 2003, pp. 690–693. Translated from Mikrobiologiya, Vol. 72, No. 6, 2003, pp. 780–784.

EXPERIMENTAL ARTICLES

Conjugal Plasmid Transfer in *Bacillus subtilis* under Conditions of Soil Microcosms

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Received December 3, 2002; in final form; March 24, 2003

Abstract—Conjugal transfer of the small plasmid pUB110 between *Bacillus subtilis* strains was studied under conditions of microcosms with sterile and nonsterile soil. Plasmid transfer proved to be possible after soil inoculation with vegetative partner cells or with their spores. Plasmid transfer occurred at temperatures of 30 and 22–23°C.

Key words: plasmid, *Bacillus subtilis*, microcosm.

In the last 10–20 years, both direct and indirect evidence has accumulated suggesting widespread occurrence of the analogues of the sexual process (horizontal transfer of chromosomal genes and plasmids) in many bacterial species in their natural habitats. This evidence has been obtained in different ways, including direct experiments with microcosms simulating environmental conditions [1–3]. The data on horizontal gene transfer in bacilli are conflicting. An opinion has been expressed that bacilli belong to bacteria in which horizontal gene transfer plays a minor role in the variation of the gene pool as compared to spontaneous mutations. The above opinion rests on the data on intrapopulational variation in some bacilli isolated from soil in various areas of the globe [4]. However, the so-called spontaneous transformation of *B. subtilis* has been recorded in soil microcosms [5, 6]; several reports have been published on conjugal plasmid transfer in *B. thuringiensis* in soil and water microcosms, as well as in infected insects [7–9].

We recently described conjugal transfer mediated by the large plasmid p19 harbored by the soil strain *B. subtilis* 19. This plasmid promotes mobilization and transfer of the small plasmid pUB110 into cells of the recipient strain *B. subtilis* 168 at a rather high frequency (about 1% of recipient cells, both on solid and liquid nutrient medium) [10]. In this study, we aimed to determine whether conjugal transfer of plasmid pUB110 can occur in soil microcosms during coincubation of partner cells and in the digestive tract of the snail *Helix pomatia*.

MATERIALS AND METHODS

Bacterial strains and plasmids. In experiments with conjugation, the donor cells were those of *Bacillus subtilis* 19 (p19 pUB110) resistant to 50 μg/ml of kanamycin; the strain resistance was determined by the small plasmid pUB110, which was preliminary introduced into regenerating protoplasts of the donor strain [10]. The recipient cells of *B. subtilis* trpC2 thr5 were resistant to 5 μg/ml of chloramphenicol and 15 μg/ml of streptomycin.

Soil in microcosms. We used garden soil dried at 50°C to a constant weight and, if necessary, sterilized in an autoclave at 1 atm for an hour. Glucose-free Spizeisen medium (3 ml) was added to 10 g of dry sterile soil.

Inoculation of partner strains into soil, incubation conditions, and counting transconjugants. In experiments with a culture containing vegetative cells, the partner strains were first grown separately in LB liquid medium (Fluka) for 4.5 h at 37°C under aeration. These cultures (10 ml of each) were centrifuged, resuspended in 2 ml of fresh LB medium, and introduced under agitation into soil-containing 50-ml flasks. After this, the soil humidity comprised 60% of the water-holding capacity, pH 5.5. The flasks were closed with cotton plugs.

To obtain spores, the bacteria were plated on dishes with LB agar supplemented with glucose, yeast extract (Sigma), and meat extract (Difco) to final concentrations of 0.5, 0.1, and 0.3%, respectively. These dishes were incubated for four days. The grown culture was washed off the agar with phosphate buffer (pH 7.2), centrifuged, and resuspended in 3 ml of the same buffer. To disrupt vegetative cells, lysozyme was added to this suspension (1 mg/ml) and, after incubation for 30 min at 37°C, sodium dodecyl sulfate was added to a final concentration of 1%. After additional incubation for 10 min, the suspension was centrifuged (10 min at low speed), washed with sterile distilled water, centrifuged once more, and heated at 80°C for 20 min.

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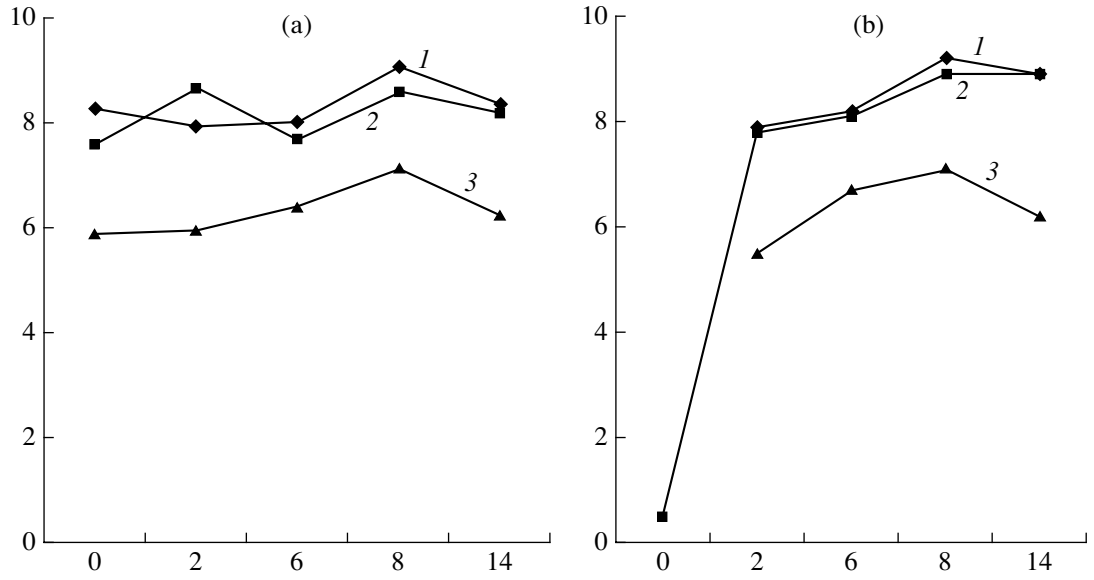

Fig. 1. Conjugation in sterile soil. (a) Soil was inoculated with partner bacteria from a growing liquid culture. (b) Soil was inoculated with spores of partner bacteria. *Y* axis, log of the number of viable bacterial cells in 1 g of soil; *X* axis, duration of the experiment (days). *1*, The number of recipient cells; *2*, the number of donor cells; *3*, the number of transconjugants.

This spore preparation was stored in the cold. Before inoculation into soil it was heated again at 80°C for 20 min. Similar amounts of spores and vegetative cells were introduced into the soil. The cells or spores of the two partner strains were coincubated at 30°C or at room temperature (about 22–23°C) for 10–18 days.

For plating samples from soil microcosms, 9 ml of physiological saline was added to 1 g of soil and the suspension was agitated for 20 min on a shaker and then for 1 min on a vortex. After precipitation of large soil particles, the suspension was plated at various dilutions. Viable recipient cells were counted after plating the suspension onto dishes with LB agar containing chloramphenicol and streptomycin (5 and 15 μg/ml), whereas viable donor cells were counted on medium containing kanamycin (50 μg/ml). Transconjugants were counted on medium containing all three antibiotics. In experiments with nonsterile soil, the growth of mycelial fungi was inhibited by the addition of nystatin (100 μg/ml).

Experiments with feeding donor and recipient cells to snails. After two days of starvation, the mollusks were fed with lettuce leaves covered with a suspension of donor and recipient cells and placed into individual clear vessels. The snails were kept at room temperature (their body temperature, measured with a thermometer introduced under the shell, was 1–2°C higher than the outside ambient temperature). About 100 mg of feces, whose excretion began 10–12 h after snail feeding with lattuce, was suspended in 1 ml of physiological saline and plated at various dilutions onto media of three types (as when plating soil, see above).

RESULTS

Conjugation in sterile soil. Figure 1a shows the results of one of several experiments that involved introduction of the partner cultures into sterile soil and incubation for 14 days at 30°C. The number of viable cells that formed colonies after plating onto solid medium remained nearly unchanged from the moment of soil inoculation to the end of the experiment. The number of transconjugants slightly increased over eight days and then decreased. After incubation for two days, about 943000 transconjugants was determined to be present in 1 g of soil (about 1.03% of the number of recipient cells).

However, in these experiments, the soil might be inoculated not only with vegetative cells but also with spores contained in the cultures; during incubation, sporulation and spore germination might occur. To follow only spore behavior in this experiment, soil samples were heated before plating (80°C for 20 min). In the heated samples, no vegetative cells but only spores were present. The results obtained with soil samples preheated prior to plating are shown in Fig. 1b. When introduced into soil, almost all bacteria of both donor and recipient strains were represented by vegetative cells sensitive to heating; afterwards, their sporulation was initiated. By the eighth day of incubation in soil, only spores were observed, the number of which was equal to the number of cells (colony-forming units) in the corresponding points of the curves in Fig. 1a. The transconjugant spores were detectable after two days of coincubation in soil; the number of these spores increased with time and then somewhat decreased. By the 14th day, the number of transconjugant spores made up about 0.2% of the number of recipient spores.

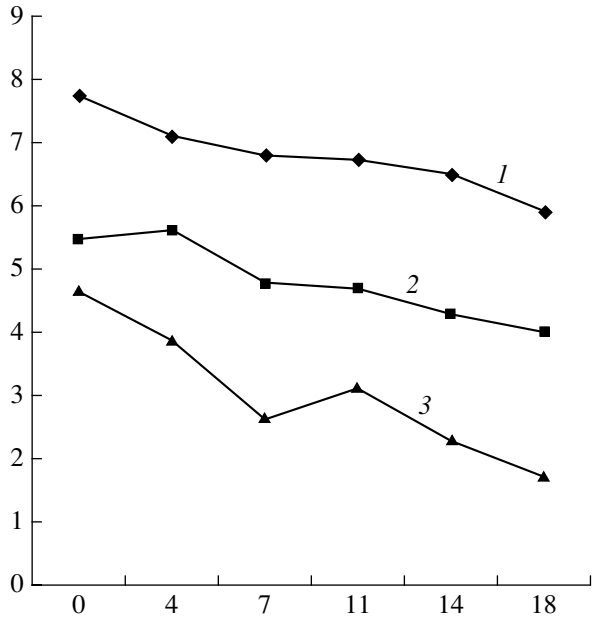

Fig. 2. Conjugation in nonsterile soil. *Y* axis, log of the number of viable cells in 1g of soil; *X* axis, duration of the experiment (days). *1*, The number of recipient cells; *2*, the number of donor cells; *3*, the number of transconjugants.

Similar experiments were conducted with the specially obtained spores (see Materials and Methods) that were introduced into soil instead of cultures of growing bacteria. From the moment of inoculation and until the 11th day of incubation, the number of viable spores of the donor strain remained unchanged; the number of spores of the recipient strain decreased nine- to tenfold (among the reasons may be germination of a portion of spores). A low number of transconjugants was determined as soon as in the first hours of spore coincubation; by the fourth day of coincubation the number of transconjugant spores made up 0.16% of the number of the recipient strain spores and later remained unchanged.

In the experiments that involved incubation at 23°C, similar results were obtained, both after inoculation of spores and vegetative cells (data not shown).

Conjugation in nonsterile soil. In the first experiments with nonsterile garden soil, we used the same strains as in the experiments described above. However, the number of viable partner bacteria decreased during the first four to six days of incubation at 30°C. The cell count of the recipient (laboratory strain 168) decreased most dramatically (by four orders of magnitude), although transconjugant formation did occur under these conditions. Therefore the soil strain *B. subtilis* 19 lacking the conjugative plasmid p19 [11] and resistant to streptomycin was used as the recipient strain in further experiments. With this strain, the decrease in the number of partner cells was not so great (about two orders of magnitude by the end of the experiment, after 18 days) (Fig. 2). Transconjugants were detectable during the entire period of the experiment; on the 11th day, they made up 0.023% of the number of recipient cells.

If soil was inoculated with spore preparations instead of vegetative cells, transconjugants were detectable only by the 14th day of incubation (data not shown).

Attempts to reveal conjugation in the digestive tract of edible snails. After plating the feces suspension obtained from snails fed with salad leaves covered with donor or recipient cells, or with both partner cells, a significant number of viable cells (colony-forming units) were detected (about 1.5×10^6 per 1 ml of suspension containing 100 mg of snail feces). Thus, the bacilli did not perish in the digestive tract of the mollusk; however, no transconjugants were detected among them.

DISCUSSION

As mentioned above, conjugal plasmid transfer has been reported to occur between bacilli, including *B. thuringiensis*, in various microcosms. In this study we demonstrated transfer of the small plasmid pUB110, mediated by the conjugative plasmid p19, between *B. subtilis* strains in microcosms with garden soil. Conjugation occurred after inoculation into soil of either the vegetative cells or spores. Plasmid transfer seems to have taken place during the first two to three days of the partner coincubation in soil, because afterwards sporulation of vegetative cells was observed. However, a portion of spores most probably germinated. In particular, that was evidently the case when spore preparations were inoculated into soil, since the rather large number of transconjugants could appear only after interaction of cells germinated from the inoculated spores. The number of transconjugants detected relative to the number of recipient cells was comparable with the data obtained in our previous experiments, when the frequency of conjugation was determined 60 min after the beginning of partner coincubation in liquid medium (conjugation of about 0.3 to 1% of the recipient cells was observed). Note, however, that conjugation conditions are different in liquid medium and soil. The number of transconjugants occurring in soil by the end of many-day-long experiments depended on several processes (adsorption of bacteria on soil particles, transconjugant propagation and dying etc.). We have shown previously that in liquid medium, conjugation occurs within a wide temperature range (from 37 to 20–22°C [10]). In experiments with soil microcosms, the partner cells were coincubated at 23°C (usual day temperature of sun light–exposed soil plots in summer near Moscow at a depth of 3–5 cm) and at 30°C (temperature of the same soil on hot days). The soil temperature was verified beforehand in summer 2001 near Moscow. In this study, we have confirmed the possibility of conjugation within the above temperature range under conditions of soil microcosms.

When incubated in microcosms with nonsterile soil, *B. subtilis* 168 cells showed sharply decreased survival over the first several days. Therefore, a strain recently isolated from soil and retaining an increased resistance to the conditions of nonsterile soil was further used in these experiments instead of the laboratory strain *B. subtilis* 168. Conjugation proved to occur under these conditions as well, although the number of transconjugants detected was an order of magnitude lower than in sterile soil. Low survival of various bacteria inoculated into nonsterile soil (due to the antagonistic activity of soil microflora) and low frequency of horizontal gene transfer under these conditions have been reported previously [1–3].

Conjugal plasmid transfer was shown to occur between *B. thuringiensis* cells in the organism of an infected insect [7–9]. In our experiments, snails were used as the host organism, as these mollusks permanently come into contact with soil and consequently with soil microflora, as well as because they are simple in handling (they have a relatively large size and can be easily maintained under laboratory conditions). We failed to reveal conjugation between *B. subtilis* strains in the snail digestive tract, although the partner bacteria did not perish under these conditions and the snail body temperature is adequate for conjugation. The digestive enzymes seem to somehow affect the bacterial surface layer to prevent conjugal contact of the bacterial cells.

Thus, our experiments with soil microcosms showed the possibility of conjugal plasmid transfer between *B. subtilis* strains in their natural habitat. The fact that the *mob* genes responsible for mobilization of small plasmids are present in a number of plasmids harbored by soil *B. subtilis* strains testifies indirectly that such plasmid transfer is a rather ordinary event [12]. In further experiments, we aim to study conjugal transfer between *B. subtilis* strains introduced into soil of different natural landscapes of the near-Moscow region.

ACKNOWLEDGMENT

This work was supported by the Russian Foundation for Basic Research (project no. 01-04-49 497).

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