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EXPERIMENTAL ARTICLES

Soil Strain of *Bacillus subtilis* Harboring a Large Plasmid That Mediates High-Frequency Conjugal Mobilization

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Abstract—The ability of a soil strain of *Bacillus subtilis* harboring a large plasmid, p19, to mobilize a small staphylococcal plasmid, pUB110, was studied. The latter plasmid was transferred to the recipient cells of *Bacillus subtilis* 168 at a high frequency (about 10^{-2} per recipient cell) both on the filter surface and in liquid medium. Mobilization was initiated 40 min after the beginning of the contact between donor and recipient cells.

Key words: large plasmid, conjugation, mobilization.

In natural isolates of *Bacillus subtilis* and related bacilli, small cryptic plasmids of about 7–10 kb in size frequently occur, whereas large plasmids of several tens kb are rare [1–4]. Unlike small plasmids, the large ones undergo theta but not sigma replication; if the cells harboring large plasmids also contain *mob*-gene-carrying small plasmids, the latter are transmissible to recipient cells. This type of plasmid transfer is referred to as conjugal mobilization [5, 6]. It has been demonstrated in *B. natto*, a species which is closely related to *B. subtilis* and harbors a large plasmid pLS20 [7] and in a soil *B. subtilis* strain harboring a large plasmid, p1387-3 [8]. This process of plasmid transfer is still little studied. Mobilization was observed when the partner cells were grown together on a solid surface (filters, agarized medium) for a long time (16–18 h); the mobilization of plasmid p1387-3 occurred at extremely low frequency.

We have isolated a natural strain, *B. subtilis* 19, harboring a large plasmid, p19, and introduced in it a small staphylococcal plasmid, pUB110, carrying the *mob* gene.

The aim of the present work was to study the ability of plasmid p19 to mediate conjugal mobilization of plasmid pUB under various conditions.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strain *Bacillus subtilis* 19 was isolated from soil (see below). Regenerating protoplasts of these bacteria were transformed with the staphylococcal plasmid pUB110, which confers resistance to kanamycin (Kan^r) [9]. Strain *B. subtilis* (*natto*) 3334 UM4 harboring plasmid pLS20 was provided by S. Bron from Groningen University, The Netherlands. Protoplasts of this strain were also transformed with plasmid pUB110. Both strains served as donors. The recipient cells were those of strain *B. subtilis* trpC2 thr5 from the laboratory collection. Plasmid marker Cm^r (resistance to chloramphenicol) was introduced into chromosomes of the recipient cells by the method described in [10].

Media and experiments on conjugation. The experiments on conjugation in liquid medium were conducted with donor and recipient cells grown separately in liquid LB (Fluka) medium in 250-ml flasks (50 ml of medium) under aeration at 37°C for 5 h. A stationary-phase culture was used as inoculum. The growth media were supplemented with 50 μg/ml of kanamycin in the case of donor cells and 5 μg/ml of chloramphenicol in the case of recipient cells. 2- to 3-ml samples were taken from 5-h cultures of donor and recipient cells to trap the cells on membrane filters. Afterwards, the cells washed from the filters with 0.5 to 1 ml of fresh LB medium containing no antibiotics were mixed in equal volumes to obtain a thick cell suspension, which was incubated at 30°C and slow stirring for 2.5–3 h. Then, serial dilutions of the culture supplemented with kanamycin and chloramphenicol (50 and 5 μg/ml, respectively) were plated onto LB agar (Fluka) to reveal conjugates that received plasmid pUB110 and retained the Cm^r marker; the parental cells were unable to grow on this medium. In addition, the dilutions were plated on LB-agar containing either chloramphenicol or kanamycin to determine the viable cell count of donor and recipient strains, respectively.

In experiments on conjugation on filters, 2-ml samples from stationary-phase donor and recipient cultures were applied to filters, which were then put on the surface of LB agar and incubated for 16–18 h at 30°C. The cells grown were washed off with 1 ml of fresh LB medium, and the cell suspension obtained was plated onto antibiotic-containing agarized medium, as described

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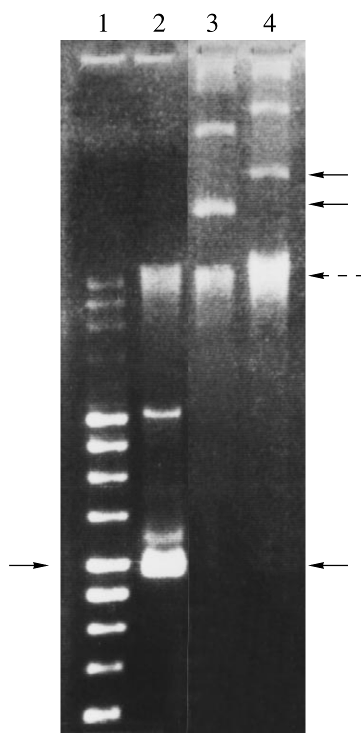

Fig. 1. Electrophoresis of plasmid DNAs: 1, standard set of supercoiled DNA fragments of different size (supercoiled DNA ladder, Gibco BR); 2, plasmid p1414 (7.949 kb); 3, plasmid pLS20 (54.6 kb); 4, plasmid p19. Arrows on the right indicate supercoiled forms of all DNAs; an arrow on the left indicates a 8.066-kb fragment on lane 1; the dotted arrow indicates the fraction of chromosomal DNA fragments.

above. Thus, the experiments with conjugation on filters were a day more time-consuming and more laborious.

DNA isolation and electrophoresis. Plasmid DNA was isolated from *B. subtilis* cells by the method of Birnboim and Doly [11]. The preparation obtained was analyzed by electrophoresis in 0.8% agarose (Sigma) in a Tris–borate buffer.

RESULTS

1. Some Properties of Strain B. subtilis 19 and of the Large Plasmid p19

Strain 19 was isolated from the forest soil in Belarus. It was sensitive to the phages AR1, AR3, AR9, SP01, and phi105. Transformation of *B. subtilis* 168 cells (in terms of the transfer of the chromosomal marker *trp*C2) by DNA of strain 19 was efficient to the same extent as the transformation by strain 168 own DNA (data not shown). Plasmid p19 seems to be somewhat larger than plasmid pLS20 judging from the data of electrophoresis (54.6 kb and about 57 kb, respectively) (Fig. 1).

2. Conjugal Mobilization of Plasmid pUB110 by Cells Harboring Plasmids p19 and pLS20 under Various Conditions

The efficiency of mobilization was studied under conditions when a mixture of donor and recipient cells was either applied onto filters to be grown for 16 to 18 h or incubated in liquid medium for 3 h. The data from one of eight experiments, which yielded similar results, are shown in the table. As it can be seen, the efficiency of plasmid pUB110 mobilization mediated by plasmid pLS20 was low on filters (10^{-6} per recipient cell), and in liquid medium, no mobilization was observed. In our previous experiments, when donor and recipient cells were coincubated on the surface of solid medium for 12–14 h, the frequency of plasmid transfer was similar, 10^{-6} [8]. The frequency of pUB110 mobilization mediated by plasmid p19 was much higher, 10^{-2} to 10^{-3}; in some experiments, as many as 10% of recipient cells received plasmid pUB110. The results were little dependent on whether the cells interacted on the filter or in liquid medium. We also added a suspension of particles of clay mineral montmorillonite to the liquid medium, which had earlier proved to be an efficient approach to increasing the efficiency of mobilization due to the coabsorption of cells and DNA on the mineral particles [12]. However, the addition of montmorillonite had no effect on conjugal mobilization (data not

Conjugal transfer of plasmid pUB110 from cells of strains harboring large plasmids, p19 or pLS20

Donor strains	Recipient strain	Number of conjugants in 1 ml of the conjugation mixture		Frequency of conjugation per recipient cell	
		in liquid medium	on filters	in liquid medium	on filters
B. subtilis 19 (p19, pUB110)	*B. subtilis* 168 trpC2 thr5	3×10^6	3.5×10^6	3.2×10^{-3}	5×10^{-3}
B. subtilis 19 (p19, pUB110)		0	0	–	–
B. subtilis (*natto*) 3335UM4 (pLS20, pUB110)	*B. subtilis* 168 trpC2 thr5	0	1.2×10^2	–	4×10^{-7}
B. subtilis (*natto*) 3335UM4 (pLS20, pUB110)		0	0	–	–

shown). Testing by the replica-plating method of 3000 conjugant colonies on minimal medium supplemented with various combinations of tryptophan and threonine showed that the cells of all colonies contained the recipient chromosome gene. No transfer of chromosome markers (at least of *trpC2* and *thr5*) was observed. We also verified the possibility that kanamycin-resistant cells may arise from spontaneous transformation by plasmid DNA released into the medium. However, the addition of DNase (Sigma, 100 μg/ml) to the mixture of donor and recipient cells in liquid medium had no effect on the frequency of mobilization.

Figure 2 shows that a DNA band exactly corresponding to that of plasmid pUB110 was observed after the electrophoresis of DNA from two conjugants. Similar results were observed with the eight other conjugants tested (data not shown).

3. The Time of Preparation to Mobilization in a Mixed Culture of Donor and Recipient Cells

To determine when mobilization was initiated after the beginning of contact between donor and recipient cells, the conjugation process was interrupted at certain time intervals by cell agitation. After mixing the donor and recipient cells, the culture was sampled at different times to be diluted ten times and agitated in a vortex for 30 s to disconnect the attached partner cells. Afterwards, the cells were plated to determine the viable cell count of both donor and recipient cells and the number of the recipient cells that had already received the plasmid. Figure 3 shows the results of one of five such experiments. As can be seen, mobilization occurred 40 min after the beginning of contact between cells: the number of conjugants increased over 2 h and then the curve reached a plateau. Both donor and recipient cell counts remained unchanged during this time; cell division seems to have been arrested due to the high cell concentration in the suspension (about 10^{10} cells/ml).

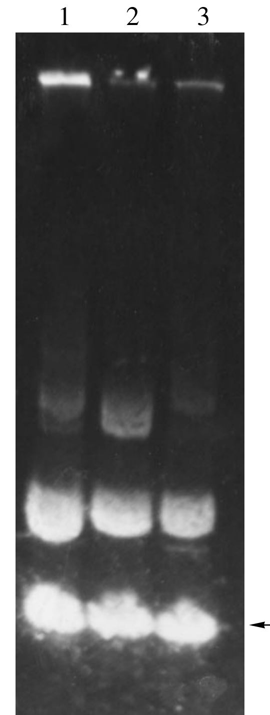

Fig. 2. Electrophoresis of (1) DNA from cell lysate of strain 19 harboring plasmid pUB110 and (2, 3) DNA from cell lysates of the two conjugants into which the plasmid was transferred. The arrow indicates supercoiled forms of plasmid DNA.

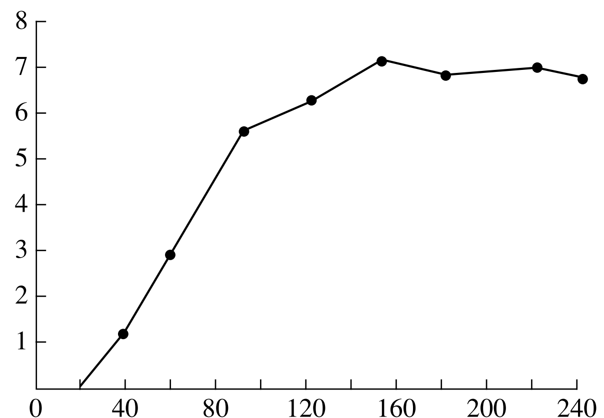

Fig. 3. Time of initiation of plasmid mobilization in a mixed culture of donor and recipient cells. Ordinate shows natural logarithm of the frequency of plasmid pUB11 transfer per recipient cell ($\times 10^{-6}$), ln; abscissa shows the time (min) elapsed after the beginning of cell contact.

CONCLUSION

In this work, the conjugative properties of the soil strain *B. subtilis* 19 harboring a large plasmid, p19, were studied. The conjugal mobilization of a small staphylococcal plasmid, pUB110, additionally introduced into this strain, proved to occur at a high frequency. The process was equally effective during the growth of donor and recipient cells on filter surface and in liquid medium.

Although the process of conjugal mobilization has been studied for a long time in certain bacilli, especially in *B. thuringiensis* [13, 14], *B. subtilis* remained little studied in this respect. We managed to isolate a soil strain of *B. subtilis* harboring a large plasmid with conjugative properties, which are superior to those of other large plasmids of *B. subtilis* described so far. This plasmid mediates mobilization of the small plasmid pUB110 at a high frequency; the experiments on mobilization can be conducted not only on a solid surface (filters) but also in liquid medium; mobilization occurred soon after the beginning of cell contact. Taken together, these properties of plasmid p19 suggest that it may be a promising model to investigate the process of small plasmid mobilization, which is still poorly studied.

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