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EXPERIMENTAL ARTICLES

Comparative Characterization of the Conjugation Capacity and Large Plasmids in Native Strains of *Bacillus subtilis* from Different Regions of the East European Plain

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Abstract—The properties of large plasmids harbored by *Bacillus subtilis* strains isolated from soils of Moscow and Moscow oblast and from different regions of the Republic of Belarus have been studied. All large plasmids in the collection of strains from Belarus were capable of conjugative mobilization of the small plasmid pUB110 and were similar in size and other properties. Most of the tested plasmids harbored by strains isolated from Moscow soils had no mobilization ability; they were of different sizes and showed no homology with the replication region of plasmids from the Belarussian collection. The uniformity of the plasmids present in strains from Belarussian soils may be due to their active horizontal transfer under natural conditions.

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Key words: soil strains of *Bacillus subtilis*, large plasmids, conjugation.

Different native strains of *Bacillus subtilis* often harbor small cryptic plasmids [1, 2]. Large plasmids occur in these strains more rarely. We have a representative collection of native *B. subtilis* strains isolated from soils of the central part of the East European Plain (Moscow and surroundings) and its western outskirts (six regions of the Republic of Belarus) [3, 4]. In the tested portion (42 strains) of the Moscow collection, 12 strains were found to harbor large plasmids ("Moscow" plasmids). In the collection of 55 *B. subtilis* strains isolated from forest and meadow soils and other natural sources of different regions of Belarus (all of these strains are close to *B. subtilis* 168), nine strains harbored large plasmids. "Belarussian" plasmids were about 100 kb in size [4]. The structure of the *rep* regions in one of them, pBS72, differed from the structure of the corresponding regions of the already known large plasmids [5]. Later, it turned out that the *rep* regions of the other eight large plasmids resemble those of pBS72 [4]. It was assumed that this resemblance was a result of permanent conjugal transfer of these plasmids under natural conditions [4], particularly as conjugative properties had been previously revealed in one of them, p19 [6]. To verify this assumption, the present work was undertaken, with the objective of studying the capacity for conjugation in all of the "Belarussian" strains harboring large plasmids and to determine the degree of their similarity to "Moscow" plasmids by assessing the sizes and conjugative properties of the "Moscow" plasmids and performing Southern hybridization of the *rep* region of one of the "Belarussian" plasmids with "Moscow" plasmids.

Judging from the data obtained, all "Belarussian" plasmids are capable of conjugative mobilization of the small plasmid pUB110 and form quite a homogeneous group according to this and other properties. Nearly all plasmids from Moscow soil strains were incapable of conjugative mobilization; they were heterogeneous in terms of their sizes and exhibited no homology with the "Belarussian" group plasmids.

MATERIALS AND METHODS

The bacterial stains and plasmids used in this work are listed in Table 1.

Plasmid DNA for further transformation was isolated by the alkaline method of Birnboim and Doly [7]. Competent cells of *Bacillus subtilis* were obtained and transformed according to Anagnostopoulus and Spizizen [8], with slight modifications accepted at the Biological Faculty of Belarussian State University. Regenerating protoplasts were obtained and transformed according to Chang and Cohen [9]. Conjugal transfer (mobilization) of plasmid pUB110 in liquid medium was performed by the technique accepted at our laboratory (Institute of General Genetics, Russian Academy of Sciences) [10]. Cultures of donor strains (carrying a

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Table 1. Bacterial strains and plasmids used in this work

Strains and plasmids	Source
B. subtilis BS1 (pBS1)*	Collection of soil strains, Belarussian State University
B. subtilis BS15 (pBS15)*	"
" BS8 (pBS8)*	"
" BS57 (pBS57)*	"
" BSN1(pBSN1)	"
B. subtilis BS2 (pBS2)*	"
B. subtilis BS4 (pBS4)	"
" BS19 (p19)*	"
The same strains with introduced plasmid pUB110	This work, except for strain BS19 (p19 pUB110) obtained previously [6]
B. subtilis 544 (p544)	Collection of soils strains, Laboratory of Genetics of Microorganisms, Inst. Gen. Genet., Russ. Acad. Sci.
" 1337 (p1337)	"
" 1420 (p1420)	"
" 1440 (p1440)	"
" 1523 (p1523)*	"
" 1564 (p1564)*	"
" 1567 (p1567)*	"
" 1847 (p1847)*	"
" 1899 (p1899)	"
" 1420 (p1420 pUB110)	This work
" 1440 (p1440 pUB110)	"
" 1899 (p1899 pUB110)	"
" BS19 (pUB110)	Museum of strains, Laboratory of Genetics of Microorganisms, Inst. Gen. Genet., Russ. Acad. Sci.
" BS19–634 Str^R	"
" 168 (pUB110) trpC2 thr5	"
" 168 trpC2 thr5	"
E. coli JM105 (pMTL19)	"

* These strains, in addition to a large plasmid, harbor one small cryptic plasmid.

large plasmid and the mobilized plasmid pUB110) and of the recipient strain were grown separately in LB medium (Fluka) without antibiotics at 30°C overnight and then diluted 50-fold in fresh medium and grown at 37°C to a concentration of $4–8 \times 10^8$ cells/ml. The donor and recipient cultures, 0.1 ml each, were mixed in 0.8 ml of fresh medium and incubated under weak aeration for 3 h. Then the conjugation mixture was plated onto selective media (LB agar with antibiotics). Kanamycin (Km) and streptomycin (Str) were added in concentrations of 15 μg/ml and 25 μg/ml, respectively. Kanamycin prevented the growth of recipient cells, and streptomycin prevented the growth of donor cells. Growth was observed only for recipient cells that had received upon conjugation the mobilized plasmid pBU110, which made them resistant to kanamycin. Separately grown donor and recipient cultures were plated onto the same media as controls. Cell titers of the conjugation partners were determined by plating aliquots of the conjugation mixture dilutions onto LB agar containing one of the antibiotics. The frequency of conjugal transfer was defined as the ratio of the number of transconjugants to the number of recipient cells. Typical data of one of three or more experiments are presented.

Plasmid DNA electrophoresis was performed in 1% agarose gel (Sigma) in Tris–borate buffer. The *rep*-region of p19 cloned in plasmid pMTL19 [5] was used as a probe for DNA–DNA hybridization. The DNA of pMTL19 was digested by restriction endonuclease *Eco*RI and subjected to electrophoresis in agarose gel. The required fragment was isolated from the gel, purified from agarose with a Glass Milk kit (Sileks, Russia), and labeled with ^{32}P-dATP using a Prime-a-Gene kit (Promega). DNA transfer to Haybond-N nylon filters (Amersham Biosciences) and probe hybridization were performed by standard methods [11]. Hybridization and washing of filters was carried out at 65°C.

RESULTS

Conjugative properties of the large plasmids under study. We investigated the capacity for conjugal transfer of large plasmids in eight strains of *Bacillus subtilis* from the Belarussian collection and nine strains from the Moscow collection (Table 1). Not only can conjugative plasmids be transferred themselves; they also promote the transfer (mobilization) of other plasmids (usually small ones). Since the available large plasmids were cryptic, we could judge their conjugative properties only by the ability to mobilize small plasmids carrying certain selective markers. As such a plasmid, we took the staphylococcal plasmid pUB110 carrying the gene of resistance to kanamycin (Km^R). Plasmid pUB110 was introduced into the cells of strains with large plasmids by transformation of competent cells or transformation of regenerating protoplasts. All strains from the Belarussian collection proved to be able to transform the competent cells with pUB110

Table 2. Conjugal transfer of plasmid pUB110 from *B. subtilis* strains from Belarussian and Moscow collections harboring large plasmids

Donor strain	Recipient strain	Number of conjugants per 1 ml of conjugation mixture	Conjugation frequency (per recipient cell)
Belarussian collection:			
BS1 (pBS1 pUB110)	BS19-634Str^R	2.2×10^6	1.8×10^{-3}
BS15 (pBS15 pUB110)	BS19-634Str^R	5.1×10^6	3.8×10^{-3}
BS8 (pBS8 pUB110)	BS19-634Str^R	2.3×10^4	2.2×10^{-5}
BS57 (pBS57 pUB110)	BS19-634Str^R	4.5×10^6	3.9×10^{-3}
BSN1 (pBSN1 pUB110)	BS19-634Str^R	4.2×10^5	5.7×10^{-4}
BS2 (pBS2 pUB110)	BS19-634Str^R	4.6×10^6	3.5×10^{-3}
BS4 (pBS4 pUB110)	BS19-634Str^R	4.7×10^5	4.3×10^{-4}
BS19 (p19 pUB110)	BS19-634Str^R	4.6×10^6	2.6×10^{-3}
"Moscow" strain			
1387 (p1387-3 pUB110)*	RM125Cm^R	2.7×10^3	10^{-7}

* For the "Moscow" strain, data from [12] are presented; conjugation with this strain was carried out on filters.

DNA. The frequency of transformation of the "Belarussian" strains under study varied. In strains BSN1 and BS4, the frequency was up to $1.1–1.78 \times 10^{-5}$ per recipient cell, which was comparable with the frequency of transformation of *B. subtilis* 168 (3.2×10^{-5} per recipient cell). In strains BS1, BS15, BS57, and BS2, the frequency of transformation was about 10 times lower ($1.2–3.3 \times 10^{-6}$ per recipient cell). In strains BS8 and BS19, the frequency of transformation was very low (1×10^{-7} per recipient cell). In the work with the Moscow collection, we succeeded in introducing pUB110 into competent cells of only three strains: *B. subtilis* 1420, 1440, and 1899. The frequency of transformation of these strains was much lower than in *B. subtilis* 168: 1.5×10^{-6} per recipient cell in strain 1420, 1×10^{-7} in strain 1440, and 5×10^{-8} in strain 1899. Thus, in contrast to the "Belarussian" strains, most of the "Moscow" strains (*B. subtilis* 544, 1337, 1387, 1523, 1564, 1567, and 1847) were not transformed by plasmid DNA. Previously, plasmid pUB110 was introduced into regenerating protoplasts of strain 1387 [12]. We failed to introduce pUB110 by any of the methods into the rest of the six strains with large plasmids, so they were not used in further experiments on mobilization.

It should be noted that the frequency of transformation by plasmid DNA, low as it was, depended also on the strain from which pUB110 DNA had been isolated. Thus, in the strains from the Belarussian collection, transformants appeared only if plasmid DNA had been isolated from the derivative "Belarussian" strain BS19 (pUB110) devoid of the large plasmid. The pUB110 DNA isolated from the laboratory strain *B. subtilis* 168 did not transform the "Belarussian" strains in our experiments. On the contrary, all three "Moscow" strains could be transformed only by a plasmid DNA preparation from *B. subtilis* 168. Probably, these distinctions were determined by distinctions in the restriction–modification systems.

The obtained plasmid transformants were tested by electrophoresis for the presence of the large plasmid inherent in this strain and plasmid pUB110. Transformants carrying both plasmids were used as donors in further experiments on pUB110 mobilization.

Summarized data on the mobilization capacity of large plasmids are presented in Table 2 (including the data of experiments with plasmid p1387-3 from strain 1387 from our previous work [12]). The frequency of mobilization in liquid medium in five "Belarussian" strains—BS1 (pBS1 pUB110), BS15(pBS15 pUB110), BS57(pBS57 pUB110), BS2(pBS2 pUB110), and BS19 (pBS19 pUB110)—reached the level of 10^{-3} per recipient cell; similar values were obtained in our previous works with strain BS19 (pBS19 pUB110) [10, 12]. In three strains (BS8 (pBS8 pUB110), BSN1 (pBSN1 pUB110), and BS4 (pBS4 pUB110)), the frequency of mobilization was 1–2 orders of magnitude lower. It should be noted, however, that the observed decrease in the absolute number of "mobilizants" and the frequency of pUB110 transfer in strain BS4 (pBS4 pUB110) was probably due to a drastic decrease in the number of donor cells in the conjugation mixture (previously, we observed such an effect, associated with mutual bacteriostatic or bactericidal action, during hybridization of bacilli from different genera [13]). Another situation was observed for the strains of the Moscow collection with introduced pUB110. Only plasmid p1387-3 from strain

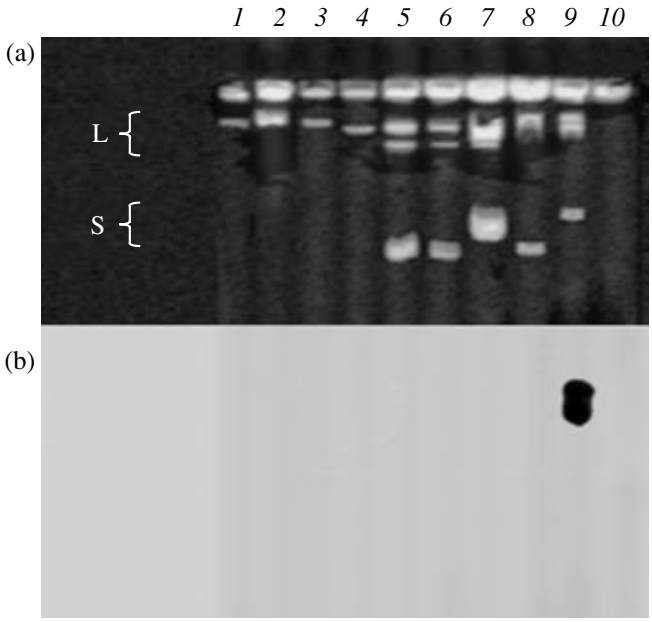

Fig. 1. Electrophoregram of the DNAs of plasmids from the Moscow collection (a) and results of their hybridization with the *rep* region of the "Belarussian" plasmid p19 (b). L, large plasmids, S, small plasmids. Lanes *1–8* correspond to the "Moscow" strains: *1*, 1899; *2*, 1337; *3*, 1420; *4*, 1440; *5*, 1523; *6*, 1564; *7*, 1567; *8*, 1847. Lane *9* corresponds to DNA of "Belarussian" strain BS19; lane *10*, to DNA of the laboratory plasmidless strain *B. subtilis* 168 trpC2 thr5. The large plasmids p1523, p1564, p1567, and p19 were represented by two subforms (lanes *5, 6, 7, 9*). Strains *B. subtilis* 1523, 1564, 1567, 1847, and BS19 harbored, in addition to large plasmids, small cryptic plasmids (lanes *5, 6, 7, 8, 9*).

1387 was capable of mobilization. The frequency of pUB110 mobilization in this strain was much lower than in the strains from the Belarussian collection, 10^{-7} per recipient cell. In strain 1387, mobilization occurred only on the filter surface but not in liquid medium and only if the recipient was strain *B. subtilis* RM125 Cm^R with an impaired restriction–modification system [12]. Strains 1420, 1440, and 1899 showed no capacity for conjugal transfer of pUB110.

Hybridization of plasmids from the Moscow collection with the *rep* region of the "Belarussian" plasmids and the sizes of "Moscow" plasmids. For further comparative study of plasmids from both collections, we undertook a hybridization study to verify whether there is a homology between the *rep* regions of the "Belarussian" and "Moscow" plasmids. As mentioned above, the *rep* regions of all large plasmids from strains isolated in Belarus were similar in their structure. Therefore, it was enough to take the *rep* region of one of these plasmids (in our case, p19) as a probe for Southern hybridization.

Hybridization results are presented in Fig. 1. As evident from this figure, there was no hybridization between the *rep* regions of p19 and "Moscow" plasmids. At the same time, the p19 fragment with the *rep* region showed distinct hybridization with DNA preparation of plasmid p19.

One more characteristic of large plasmids is their size. The size of plasmid p19 from the Belarussian collection, judging from the total size of its *Cla*I fragments, was 96.7 kb [6]. The sizes of all other plasmids from the same collection, based on their DNA runs on the electrophoregram, were the same as that of p19 [4]. Among the large plasmids from the Moscow collection, the situation was quite different. Plasmid p1387-3 was 35.5 kb in size (by the total of *Eco*RI fragments) [12], plasmid p544 was 65 kb (the total of *Kpn*I fragments), and plasmid p1899 was 60 kb in size (the total of *Eco*RI fragments) (our unpublished data). The sizes of other plasmids from the Moscow collection were not determined from the total of their restriction fragments. However, the electrophoregram of native DNA of the large "Moscow" plasmids (Fig. 1) shows that they are different in size. Three of them (p1523, p1564, and p1567) are noticeably smaller than p19.

Thus, the group of large plasmids harbored by *B. subtilis* strains from Moscow soils proved to be rather heterogeneous.

DISCUSSION

According to the results of this and previous [4] works, large plasmids from *B. subtilis* strains isolated from different regions of Belarus (six geographical sites) proved to form a homogeneous group, being very similar if not identical; at the same time, they differed from the plasmids of the Moscow collection in a number of characteristics (size, capacity for cross-hybridization, and capacity for pUB110 mobilization). Their uniformity, as was supposed earlier [4], may be due to their continuous migration between cells of bacilli under natural conditions, since all of them probably exhibit a high frequency of conjugal self-transmission (judging from their capacity for mobilizing the small plasmid pUB110). Previously, we showed that pUB110 can be mobilized by conjugative plasmid p19 both in laboratory and soil microcosms [14] and in soils of some near-Moscow landscapes [15]. Certainly, it may be assumed that the bacilli harboring plasmids migrate (e.g., due to spore transfer by wind). However, the possible role of migration of plasmids themselves by conjugation or other kinds of horizontal transfer is supported by the fact that some strains harboring quite similar plasmids were quite different from each other (e.g., in antagonistic properties and in pUB110 mobilization frequency). The structure of small cryptic plasmids in the strains from Belarussian collection, according to our data, was also more uniform than the structure of small plasmids harbored by the strains from Moscow soils [16].

On the other hand, it is difficult to explain the diversity of the large plasmids in the Moscow collection. Probably, it is due to their low (or even completely

absent) conjugation activity and, as a consequence, the absence of horizontal conjugal transfer. In addition, the conditions for horizontal transfer in the soil of a huge industrial city are probably worse than in the natural landscapes of Belarus. In any case, the difference in plasmid versatility is hardly associated with geographical differences, since the climatic conditions and relief on the outskirts of the East European Plain (Belarus) and near its center (Moscow) are very similar.

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