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EXPERIMENTAL ARTICLES

Population Heterogeneity of Plasmid-Bearing and Plasmid-Free *Bacillus subtilis* Strains under Different Environmental Conditions

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Abstract—The population heterogeneity of recombinant and plasmid-free *Bacillus subtilis* strains introduced into aquatic microcosms was studied. After introduction, the population of the plasmid-free strain *B. subtilis* 2335 in microcosms has long been represented by both vegetative cells and spores, whereas, already ten days after introduction, the population of the recombinant strain *B. subtilis* 2335/105 (Km^rInf^+) was represented only by spores. The number of plasmid copies in the spore isolates of the recombinant strain was the same as before introduction, but the plasmid abundance in the vegetative isolates of this strain decreased. The isolates of *B. subtilis* 2335/105 obtained from microcosms and the variants of this strain obtained by ten successive subcultures on M9 and 0.1× M9 media with and without kanamycin (Km) differed in the number of plasmid copies, Km resistance, and maximum biomass yield during batch cultivation. Irrespective of the presence of Km, more than 50% of the variants subcultured on M9 medium showed reduced plasmid abundance. At the same time, about 70% of the variants subcultured on 0.1 × M9 medium with Km and 90% of the variants subcultured on the same medium without Km retained the initial number of plasmid copies. The variants subcultured on media with Km retained the initial biomass level. In more than 70% of the variants isolated from media without Km, the biomass yield increased.

Key words: *Bacillus subtilis*, recombinant plasmid, microcosm, introduction, heterogeneity

The recombinant strain *Bacillus subtilis* 2335/105 is a component of the probiotic called subalin [5]. The presence of the plasmid responsible for the synthesis of human interferon-α2 and for kanamycin resistance enhances the biosynthetic activity of cells. During the production of the probiotic, the recombinant strain was found to be highly heterogeneous with respect to the expression of the cloned genes [6]. The reasons for such heterogeneity are poorly understood.

The use of commercial preparations containing genetically modified microorganisms (GMMs) is associated with the risk of their release into the environment, where some GMMs may efficiently compete with natural microflora [7, 8].

Earlier, we showed that plasmid-free cells can rapidly accumulate in the population of the recombinant strain *B. subtilis* 2335/105 [3]. When introduced into microcosms, the recombinant strain survives predominantly in the form of spores. It is known that cells of GMMs produced from spores may differ from original cells in plasmid abundance and growth rate. Simulating the conditions of natural ecosystems in acute experiments and laboratory microcosms makes it possible to demonstrate the action of natural selection and the dynamics of recombinant plasmids [3, 7–9].

The aim of the present work was to study the population heterogeneity of the recombinant strain *B. subtilis* 2335/105 in model aquatic ecosystems with different lengths of trophic chain and in successive subcultures on different solid media.

MATERIALS AND METHODS

Microorganisms used in this work were two bacillar strains, the plasmid-free strain *Bacillus subtilis* 2335 and the recombinant strain *B. subtilis* 2335/105 containing a 5.6-kb plasmid ($Km^r Inf^+$). The latter was obtained from the Research Center "Vector," Berdsk, Belarus [4]. The isolates of the recombinant strain were obtained from model aquatic ecosystems (MAEs). The variants of this strain were obtained by the successive subculturing of the strain for about 300 cell generations on solid media prepared using complete or 10-fold diluted M9 medium [10] supplemented with 5 g/l peptone. The media either contained kanamycin or not.

Microcosms. MAEs [1] used in this study differed in the length of their trophic chains [3]. IA, IB, and IC MAEs contained only primary consumers (protozoa), while IIA, IIB, and IIC MAEs contained primary and

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secondary (daphnia) consumers. Prior to bacterial introduction, MAEs were allowed to function for three months, so that their composition could be stabilized both qualitatively and quantitatively, due to succession. Plasmid-free and plasmid-bearing strains were introduced into IA and IIA MAEs and into IB and IIB MAEs, respectively, to give the same cell concentration 2×10^6 cells/ml. IC and IIC served as control MAEs. All the MAEs were incubated at the same illuminance and temperature values.

Analyses of the microbial population and cultivation conditions were described earlier [3]. Cell concentration was expressed in colony-forming units (CFU). The maximum biomass accumulated during batch cultivations was estimated as a percentage of the control (the stationary-phase biomass of the original recombinant strain *B. subtilis* 2335/105). The presence of spores was determined as follows: 10-ml samples were heated at 80°C for 10 min and filtered through 0.1-μm-pore-size membranes (Vladipor, Russia). The filters were placed onto complete agar media to cause spore germination. Kanamycin was added at concentrations of 0.5, 5, and 50 μg/ml.

Plasmid DNA assessment. Plasmid DNA was isolated as described in the manual [2]. Plasmid abundance was semiquantitatively determined by the agarose electrophoresis of experimental samples together with the standard sample containing the known number of plasmid pBMB105 copies. The developed gel slabs were stained with ethidium bromide and photographed. The negatives were scanned to compare the intensity of plasmid bands. Samples with plasmid abundances comprising 70–100% of the control level (40–50 plasmid copies per one recombinant cell before introduction) were referred to as high-copy-number samples. Correspondingly, samples with 20–70% and less than 20% of the control plasmid abundance were referred to as medium-copy-number and low-copy-number samples. To avoid the interference of biomass with the results of the experiments, cell suspensions were normalized with respect to optical density (D_{550}). In addition, the degree of cell lysis at the stage of DNA isolation was controlled.

RESULTS AND DISCUSSION

Heterogeneity of strains introduced into microcosms. Plasmid-free and plasmid-bearing strains introduced into aquatic microcosms considerably differed with respect to the proportion of vegetative cells and spores. After the introduction of the plasmid-free strain, microcosms IA and IIA have contained mainly vegetative cells of this strain. At the same time, already ten days after the introduction of the recombinant strain *B. subtilis* 2335/105, microcosm IB contained no vegetative cells, while microcosm IIB, which has a longer

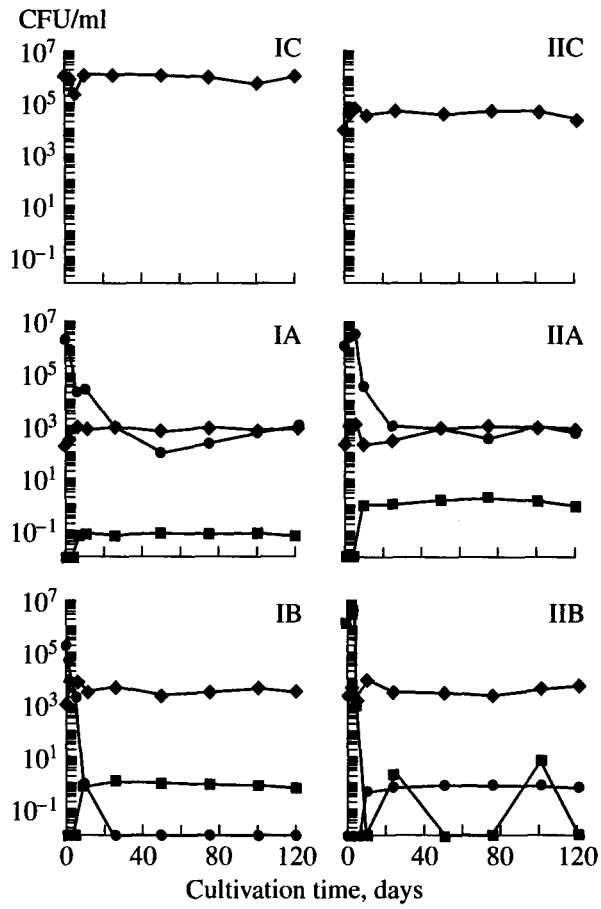

Fig. 1. Dynamics of (●) vegetative cells and (■) spores of (IA, IIA) plasmid-free and (IB, IIB) plasmid-bearing *B. subtilis* strains and (◆) indigenous microflora in microcosms with (IA, IB, IC) primary consumers and (IIA, IIB, IIC) primary and secondary consumers.

trophic chain, contained only a small number of vegetative cells (Fig. 1).

The isolates obtained from MAEs through vegetative cells (the so-called vegetative isolates) and spores (spore isolates) were studied with respect to biomass in the stationary growth phase, kanamycin resistance, and plasmid abundance (Fig. 2). It can be seen that the majority of the spore isolates of the recombinant strain exhibited an increased biomass in the stationary phase in comparison with the control (Fig. 2a). About 50% of vegetative isolates from microcosm IB and 12% of vegetative isolates from microcosm IIB showed a biomass level close to that of the control.

None of the vegetative or spore isolates was found to completely lose the recombinant plasmid (Fig. 2b). The vegetative isolates obtained from microcosm IB had the lowest number of plasmid copies.

For the most part, kanamycin resistance correlated with the recombinant plasmid abundance: all the spore isolates were resistant to a high concentration of kanamycin (50 μg/ml), whereas the vegetative isolates

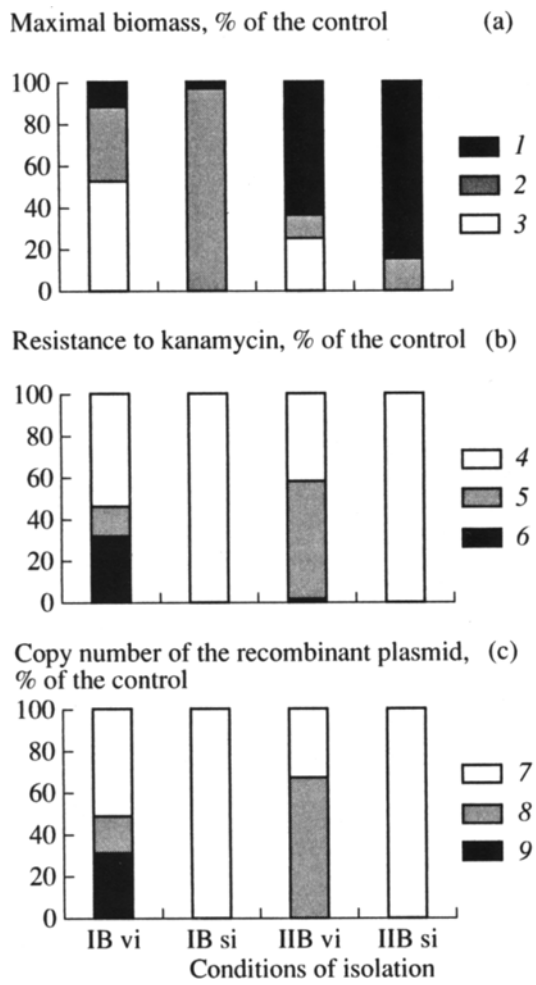

Fig. 2. Heterogeneity of the recombinant strain *B. subtilis* 2335/105 introduced into model aquatic ecosystems (microcosms): (a) maximum biomass, % of the control (*1*, 95–120; *2*, 120–170; and *3*, 170–210%); (b) Km resistance, % of the control (*4*, 0.5 μg/ml; *5*, 5 μg/ml; and *6*, 50 μg/ml); and (c) plasmid abundance, % of the control (*7*, < 20; *8*, 20–70; and *9*, 70–100). "IB vi" and "IB si" stand for, respectively, vegetative and spore isolates from microcosm IB; "IIB vi" and "IIB si" stand for, respectively, vegetative and spore isolates from microcosm IIB.

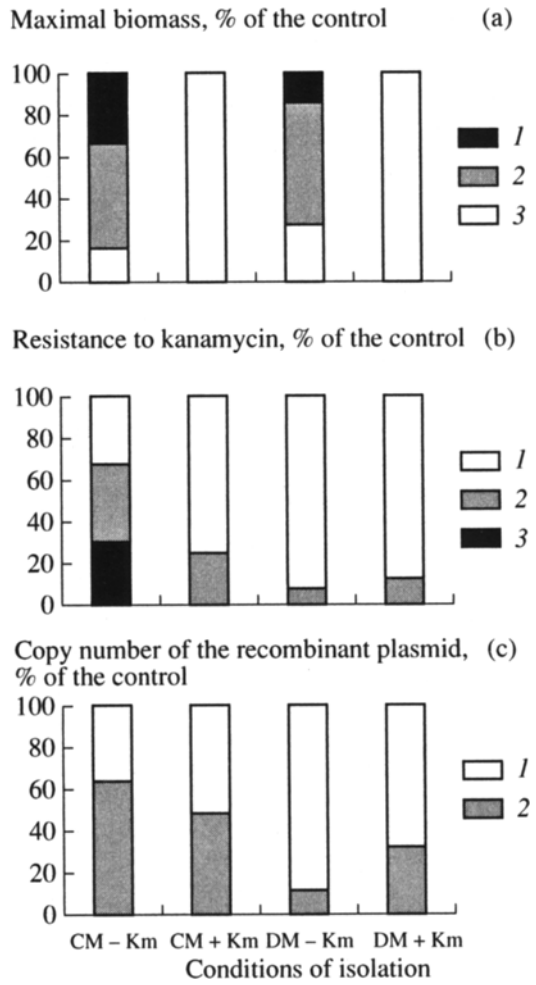

Fig. 3. Heterogeneity of the recombinant strain *B. subtilis* 2335/105 subcultured on different media: (a) maximum biomass, % of the control (*1*, 95–120; *2*, 120–170; and *3*, 170–210%); (b) Km resistance, % of the control (*1*, 0.5 μg/ml; *2*, 5 μg/ml; and *3*, 50 μg/ml); and (c) plasmid abundance, % of the control (*1*, < 20; and 2, 20–70%). "CM + Km" and "CM – Km" stand for complete medium with and without kanamycin, respectively; "DM + Km" and "DM – Km" stand for diluted medium with and without kanamycin, respectively.

obtained from microcosm IB were fairly sensitive to this antibiotic (Fig. 2c).

Heterogeneity of strains during subculturing on solid media. The different heterogeneity of the recombinant strain in the microcosms with different trophic chain lengths can be due to different concentrations of nutrients and toxic compounds [3]. The heterogeneity of the recombinant strain during subculturing on solid media was studied in relation to the key factors of natural ecosystems (complete and diluted media, the presence and the absence of kanamycin as a factor of selective pressure). To estimate strain heterogeneity, about 300 randomly taken colonies were analyzed for the same criteria of heterogeneity as in the case of the isolates obtained from microcosms (Fig. 3).

In complete medium without kanamycin, up to 65% of cells of the *B. subtilis* 2335/105 population showed an increased biomass yield in the stationary phase and fewer plasmid copies than the control cells. There was an apparent correlation between plasmid abundance and kanamycin resistance (Fig. 3). In variants 10-22 and 10-101 with high biomass in the stationary phase, the number of plasmid copies was 20% lower than in the control cells (Fig. 4 and the table). However, not all of the variants with a small number of plasmid copies showed high biomass in the stationary phase (cf. variant 10-15 in Fig. 4 and the table).

Relevant characteristics of variants of the recombinant strain *Bacillis subtilis* 2335/105 obtained through subculturing under different conditions

Variant	Medium	Maximum biomass, OD_{540} units	Maximum tolerable Km concentration, μg/ml	Plasmid abundance
2335/105	Ooriginal plasmid-bearing strain	0.7 ± 0.1	50	High-copy-number
2335	Original plasmid-free strain	1.15 ± 0.22	0.5	Plasmid-free
10-15	CM^- Km	0.65 ± 0.05	0.5	Low-copy-number
10-22	CM^- Km	1.1 ± 0.17	0.5	Low-copy-number
10-101	CM^- Km	1.07 ± 0.11	0.5	Low-copy-number
10-201	CM^+ Km	0.73 ± 0.1	50	Medium-copy-number
10-183	CM^+ Km	0.82 ± 0.09	5	Low-copy-number
10-207	DM^+ Km	0.87 ± 0.13	50	Medium-copy-number
10-48	DM^- Km	0.67 ± 0.08	50	Medium-copy-number
10-54	DM^- Km	1.21 ± 0.15	50	High-copy-number

* "CM^+ Km" and "CM^- Km" stand for complete medium with and without kanamycin, respectively; "DM^+ Km" and "DM^- Km" stand for tenfold diluted medium with and without kanamycin, respectively.

The presence of kanamycin in media (both complete and diluted) served to preserve kanamycin-resistant variants in the *B. subtilis* 2335/105 population (Fig. 3b). In spite of the small number of plasmid copies in the majority of the variants tested, their resistance to kanamycin was close to the control level (variants 10-201 and 10-207 in Fig. 4 and the table). The biomass of these variants in the stationary phase was also close to the control level (Fig. 3).

Analysis of variants isolated through subculturing on diluted media without kanamycin showed that 90% of them (Fig. 3) had a great number of plasmid copies (variants 10-54 and 10-48 in Fig. 4 and the table) and retained resistance to the high concentration of the antibiotic (50 μg/ml). In some kanamycin-resistant variants with high plasmid abundances (e.g., variant 10-54 in Fig. 4 and the table), the biomass yield in the stationary phase was close to that typical of the plasmid-free strain *B. subtilis* 2335.

Thus, the plasmid-free strain *B. subtilis* 2335 and the plasmid-bearing recombinant strain *B. subtilis* 2335/105 introduced into aquatic microcosms displayed differences in their survival under these conditions: the recombinant strain survived predominantly in the form of spores, while the plasmid-free strain, in the form of both vegetative cells and spores. These differences may be due to the increased biosynthetic activity of recombinant cells producing human interferon. Unlike the population of plasmid-free cells, the introduced population of recombinant cells becomes heterogeneous; this suggests that, trying to adapt to new environmental conditions, recombinant cells reduce the expression of heterologous genes unnecessary for survival. However, none of the vegetative or spore isolates tested was found to completely lose the recombinant plasmid. Moreover, spore isolates retained the initial number of plasmid copies. Some vegetative isolates obtained from microcosm IB exhibited fewer plasmid copies (less than 20% of the control level). Vegetative cells produced from spores in microcosms presumably also had fewer copies of the recombinant plasmid.

Analysis of *B. subtilis* 2335/105 variants obtained by successive subculturing for about 300 cell generations showed that diluted media are more appropriate for the conservation of plasmid abundance and antibiotic resistance than complete media, especially when the selective agent (kanamycin) is absent. This may be due to the lower activity of biosynthetic processes in diluted (and, hence, nutritionally deficient) media than

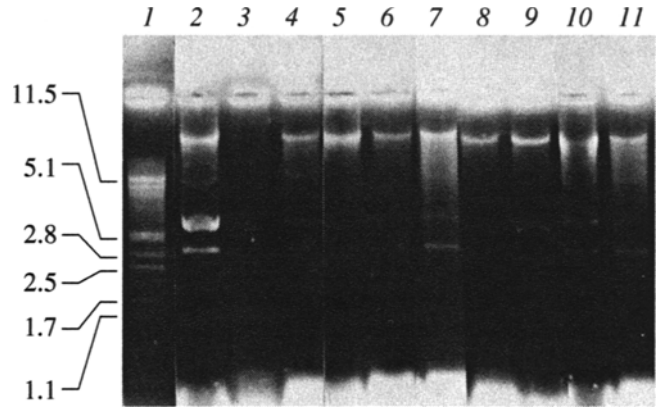

Fig. 4. Electrophoresis of cell lysates of variants of the recombinant strain *B. subtilis* 2335/105 (for their relevant characteristics, see table). Lanes: *1*, phage λ; *2*, *B. subtilis* 2335/105; *3*, *B. subtilis* 2335; *4*, variant 10-15; *5*, variant 10-22; *6*, variant 10-101; *7*, variant 10-54; *8*, variant 10-183; *9*, variant 10-207; *10*, variant 10-48; and *11*, variant 10-201.

in complete media [11]. During cultivation in deficient media without the antibiotic, some variants of the recombinant strain exhibited ample biomass in the stationary phase, high kanamycin resistance, and the initial number of plasmid copies. Such variants were not, however, revealed in microcosms four months after the introduction of the recombinant strain.

For ecological safety reasons, it should be borne in mind that the use of probiotic preparations of *B. subtilis* is associated with a risk of long-term preservation of spores containing recombinant plasmids in natural ecosystems and even of vegetative cells with a reduced level of expression of plasmid genes.

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