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SHORT COMMUNICATIONS

The Morphological Characteristics and the Dynamics of Biofilms Formed by a Transgenic *Bacillus subtilis* Strain

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In nature, bacteria typically live as communities, forming microcolonies, flocs, and biofilms on various surfaces. Bacterial cells in such communities differ from free-living individual cells (occurring, for instance, in laboratory cultures) in some morphological characteristics and physiological parameters [1–3]. When occurring in aquatic environments, transgenic microorganisms may form stable associations with indigenous microbial species, which may increase their survival in nature. If transgenic microorganisms are introduced into the environment unintentionally, their high survival is unwanted. Bacteria occurring in biofilms are characterized not only by high survival rates but also by the enhanced exchange of genetic information [3].

Earlier studies showed that the transgenic *Bacillus subtilis* strain 2335(pBMB105) introduced into oligotrophic aquatic microcosms undergoes gradual lysis, so that only about 0.01% of the initial population of transgenic cells survives in the form of spores capable of germination and production of vegetative cells [4]. The aim of this work was to study the ability of *B. subtilis* 2335(pBMB105) to form biofilms on the surface of liquid media and to investigate the morphological characteristics and the cell heterogeneity of these biofilms.

The transgenic strain *B. subtilis* 2335(pBMB105) (Inf^+Km) is the principle of the antiviral probiotic preparation Subalin, which was devised for the use in veterinary and human medicine [5]. Control experiments were carried out with the wild-type strain *B. subtilis* 2335. The strains were grown in M9 medium containing (g/l) peptone, 7; $MgSO_4$, 2; $CaCl_2$, 0.05; and glucose (or glycerol), 5. In the case of *B. subtilis* 2335(pBMB105), the medium was additionally supplemented with 50 μg/ml kanamycin. The medium was inoculated either with spores or with vegetative cells. To obtain biofilms, the strains were cultivated at 37°C for 21 days without shaking. The relative number of vegetative cells and spores in the biofilms were determined using fixed and stained specimens.

There are several stages in the development of biofilms: planktonic phase, reversible attachment of cells, irreversible attachment of cells, maturation, and decline. After one day of growth, both the wild-type *B. subtilis* 2335 and the transgenic strain *B. subtilis* 2335(pBMB105) formed even, smooth biofilms, which became wrinkled in the course of further incubation. The biofilms formed by the two strains differed in the intensity of wrinkling and in the time of the wrinkling started. Irrespective of the type of inoculum used (either spores or vegetative cells), the biofilms formed by the transgenic strain had more pronounced wrinkles. The degradation of biofilms includes their smoothing, thinning, and eventually fragmentation. The biofilms formed by *B. subtilis* 2335 degraded more rapidly (on average, in 17 days) than did the *B. subtilis* 2335(pBMB105) biofilms (on average, in 21 days). Irrespective of the type of inoculum, the *B. subtilis* 2335 biofilms grown on the glucose-containing medium for 17 days represented fragments occurring on the surface of the medium. In the case of the glycerol-containing medium, such fragments were absent from the surface of the medium but occurred on the culture tube walls. In general, visual observations showed that the biofilms of the transgenic strain appeared and developed more rapidly, but degraded slower, than did the biofilms of the wild-type strain.

In the next set of experiments, we evaluated the proportion between vegetative cells and spores in biofilms as a function of the growth substrate (either glucose or glycerol), the presence of the antibiotic kanamycin (only for the transgenic strain), and inoculum type (either spores or vegetative cells). In these experiments, only intact nonlysed vegetative cells and spores were taken into account.

When transgenic **vegetative cells** were inoculated into the glycerol-containing medium without kanamycin, the relative number of vegetative cells in the biofilm began to decrease after 10 days of incubation, while after 17 days in the case of the glucose-containing medium. In the course of further incubation, the difference between the biofilms grown on different substrates tended to decrease (figure). Similar results were obtained in experiments with the kanamycin-containing media. These experiments showed that both glycerol and kanamycin acted to decrease the content of vegetative cells in the biofilms formed by the transgenic strain. Similar results were observed for the biofilms formed by the wild-type strain, but the decline in the content of vegetative cells was observed later. It should

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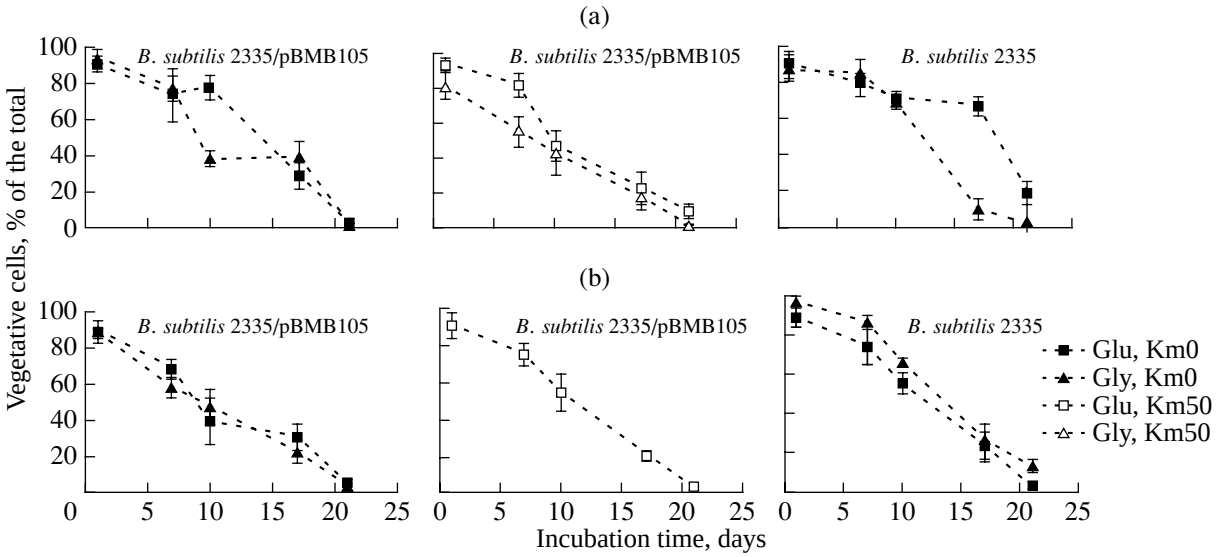

The percentage of vegetative cells in the biofilms formed on the surface of nonagitated media inoculated with (a) the vegetative cells and (b) spores of *B. subtilis* 2335 and *B. subtilis* 2335(pBMB105). Glu, Gly, Km50, and Km0 stand for the media containing glucose, glycerol, and either containing or not 50 μg/ml kanamycin, respectively.

be noted that the fragmented biofilm of the wild-type strain still contained a considerable number of vegetative cells.

In all of the variants of the biofilms developed from **spores**, the relative number of vegetative cells began to decrease already after the first days of growth, the difference between the biofilms grown on glucose and glycerol being negligible (figure).

These findings indicate that the inoculation of vegetative bacillar cells, but not spores, into the medium with glucose brought about the formation of biofilms distinguished by the catabolite repression of spore formation. Consequently, the inoculum type plays a role in the formation of *B. subtilis* films.

The inoculation of transgenic spores into the medium with glycerol and kanamycin did not lead to the formation of biofilms. This can be explained by the fact that these spores must synthesize not only proteins necessary for spore germination but also proteins necessary for the degradation of the antibiotic. In the case of glycerol, which is energetically a less favorable substrate than glucose, the load on the biosynthesizing apparatus of bacillar spores turns out to be too heavy to provide for the formation of a population of vegetative cells.

To conclude, the biofilms formed by the transgenic *B. subtilis* 2335(pBMB105) strain contain both active vegetative cells and dormant spores, which suggests that the introduced transgenic bacilli can survive in natural ecosystems for a long time. The most likely sites of their accumulation and survival in the environment are various biotic and abiotic surfaces, on which the biofilms of transgenic bacilli are formed.

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