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EXPERIMENTAL ARTICLES

Influence of Introducing the Genetically Modified Strain *Sinorhizobium meliloti* ACH-5 on the Structure of the Soil Microbial Community

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Abstract—To study the influence of genetically modified microorganisms (GMM) on the number and structure of the soil microbial community, we introduced the genetically modified strain of *Sinorhizobium meliloti* into soil under controlled laboratory conditions. The analysis of the dynamics of soil microorganisms of all the main groups (archaea, bacteria, fungi) using the PCR with real-time detection and the analysis of the species structure of all the indicated components using T-RFLP were carried out for a month. The results of the quantitative PCR demonstrated that none of the components of the soil microbial community was appreciably influenced by the GMM introduced. The number of GMM decreased over a month more than 300-fold. The analysis of the dynamics of the eubacterial, archaeal, and fungal communities using T-RFLP did not detect fundamental changes in their structure.

Key words: genetically modified microorganism, soil microbial communities, T-RFLP.
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The assessment of the risk, caused by introduction of genetically modified microorganisms (GMM) into soil requires accurate evidence of the survival, spread, and the possibility of transfer of gene-engineered constructs to other organisms, as well as of the influence of the modified strain on the structure of the local microbial community [1, 2]. All the biosafety problems indicated are of a general character and hold not only for GMM, but also for the usual introducer strains that have not been subjected to genetic manipulations. However, the problems connected with the release of GMM strains into the environment have been given much attention over the last 10 to 15 years. Thus, it was shown that the content of genetically modified strains sharply decreased over 1 or more months after their introduction and afterwards remained at a constant level for a long time [3, 4]. For the rhizospheric and symbiotic GMM, interaction with the host plant is one of the most important factors determining the dynamics [5, 6], especially in those cases when the presence of a gene-engineered construct ensures increased competitiveness of the strain introduced [7]. Many investigations were devoted to the possibility of horizontal transfer of gene-engineered constructs, because this problem causes great concern, especially in the case of determinants of resistance to antibiotics [8, 9].

The possible influence of genetically modified microbial strains on the local microbial communities is a highly essential aspect of biological safety. This is, undoubtedly, one of the most serious problems arising when the GMM-related risk is assessed. The main difficulty is determined by the high level of genetic diversity of soil microorganisms, particularly by the fact that most of them are nonculturable. Thus, the number of microorganisms revealed on nutrient media inoculated with soil samples is usually 10^6–10^7 CFU per 1 g of soil. The results of analyzing the DNA isolated from soil indicate that the number of microorganisms is at least one or two orders of magnitude higher and may exceed 10^9 cells per 1 g of soil [10–12]. Moreover, the archaea whose number usually is about 3% [13], and in certain cases as high as 20% [14], account for a considerable part of the microbial community. Until recently, this group of microorganisms was not taken into account when the problems related to GMM release into the environment were analyzed.

Despite the difficulties described, a substantial number of investigations have been carried out in order to detect the changes in the structure of the soil microbial community upon introduction of the GMM strains [3, 7, 15–18]. It is important to point out that all these works dealt only with the bacterial part of soil communities or were even limited to its rhizospheric part. The results obtained are rather controversial and give evi-

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dence of both the absence of a visible effect and its presence. The effect of the plants on the structure of the soil microbial community seems to be much more pronounced than the effect of introducing genetically modified strains [7].

All the above determined the aims of this work. An attempt was made for the first time to carry out a complex assessment of the influence of a GMM strain on the soil microbial community as a whole, including bacteria, fungi, and archaea. For this purpose, the methods of analysis not related to the cultivation of microorganisms were used. PCR with real-time detection is among such methods; it permits an approximate quantification of the major groups of microorganisms (bacteria, archaea, and fungi) [19, 20] and of individual taxonomic groups, species, and strains [20–23]. Our attention was drawn to T-RFLP, a method for assessing the changes in the structure of microbial communities based on detecting the fluorescence-labeled terminal restriction fragments of the amplified site of the 16S rRNA gene [24]. The power of this method to differentiate between the microorganisms of different taxa is based on nucleotide polymorphism in the gene sites (for example, 16S rRNA), which are flanked by conservative primers.

The genetically modified strain *Sinorhizobium meliloti* ACH-5 carrying the GFP cassette was used as the inoculant strain. The bacteria of this species are capable of both saprophytic existence in soil and symbiotic nitrogen fixation with the plants related to the alfalfa cross-inoculation group (alfalfa, sweet clover, trigonella). The experiment is essentially a model and to some degree a technical one; therefore, we conducted it in the absence of the host plant to exclude its influence on the soil microbial community. This allowed us to study the influence of the inoculant strain as such upon the soil microbial community.

MATERIALS AND METHODS

GMM. The genetically modified strain ACH-5 obtained by site-directed mutagenesis is a derivative of strain *S. meliloti* 1021 (NC_003078) capable of both saprophytic existence in soil and formation of a nitrogen-fixing symbiosis with alfalfa (*Medicago*). Strain ACH-5 contains the GFP cassette integrated into the region encoding the SMb20333 gene. This gene encoding a hypothetical protein whose function is not known is localized on megaplasmid 2. The GFP cassette is a unique and highly specific marker that allows for exact identification and quantitative determination of this strain in soil. The stability of this construct was confirmed experimentally. The influence of genetic modification on the strain viability and its symbiotic activity was not revealed.

Experimental setup. The model experiment was carried out in 2-kg vessels; the humidity value was maintained at 60% of the total moisture capacity of the soil. The soddy podzolic forest soil sampled in the settlement of Belogorka, Leningrad oblast, was used for the experiment. The soil was sifted through a 3-mm sieve and ground to a homogeneous state. Two sterile vessels were used in the experiment, the control and experimental ones, each containing 2 kg of soil. The GMM strain was grown in a liquid TY medium (peptone, 10 g/l; yeast extract, 1 g/l; $CaCl_2$, 0.4g/l; pH 7.0), centrifuged, washed with the physiological saline to remove the nutrient medium, resuspended in 100 ml of the saline, and introduced into the experimental vessel; the soil was then thoroughly mixed. In parallel, 100 ml of the saline was introduced into the control vessel in a similar way. The vessels were covered with craft paper and incubated indoors at 20°C. The soil was watered once a week to 60% of the total moisture capacity. The day after watering, samples were taken from the surface layer (at a depth of 2–3 cm), at five to seven sites; the samples were mixed thoroughly and stored at –20°C. The samples were taken at days 1, 3, 7, 14, 21, and 28. On the first experimental day, the experimental vessel was sampled twice, before and immediately after introducing the GMM. The GMM titer determined by plating diluted aliquots on agarized TY medium was 7.5×10^7 CFU per 1 g of soil.

DNA isolation. DNA was isolated from the weighed portion of soil (0.5 g), which was collected into a screw-capped plastic test tube (2 ml). Buffer I (750 μl; STAB 2%; Tris-HCl, 0.1 M; EDTA, 20mM; NaCl, 1.4 M; pH 8.5) and 0.5 g of glass beads (0.35 mm) were added to each sample. The samples were heated for 30 min at 65°C. Disintegration was carried out in a FastPrep 24 homogenizer (M.P. Biomedicals) for 1 min at the maximum shaking rate. Heating was then repeated. The sample was extracted twice with chloroform; total DNA was precipitated with isopropanol, dissolved in 100 μl of the TE buffer (Tris-HCl, 10 mM; EDTA, 1 mM), and mixed with 100 μl of melted low-melt 2% agarose (Sigma). After solidification, the agarose blocks were washed several times in 2 ml of the TE buffer (three times for 3 h each). The agarose was then melted at 65°C; sodium acetate was added to the final concentration of 0.3 M; the mixture was extracted twice with phenol, once with phenol-chloroform (1 : 1), and once with chloroform; then, 350 μl of the aqueous phase was collected. The DNA was precipitated with isopropanol, washed with 70% ethanol, dried, and dissolved in 50 μl of water. The DNA yield was very significant, at least 2 μg DNA per g of soil.

Quantitative PCR. The number of the main groups of microorganisms was determined using PCR with real-time detection. Genomic DNA of *Escherichia coli* (Sigma), *Halobacterium salinarum* FG-07 (unpublished data, G. Jurgens, Helsinki University); and *Saccharomyces cerevisiae* Meyen 1B-D1606 were used as controls for bacteria, archaea, and fungi, respectively. The following primers were used: Eub338/Eub518, for

bacteria [25]; arc915f/arc1059r, for archaea [26]; and ITS1f/5.8s, for fungi [25].

The *gfp*-specific primers GFP-F (5'-AGAAGAACGGCATCAAGG-3') and GFP-R (5'-GCTCAGGTAGTGGTTGTC-3') flanking the 137-bp *gfp* gene site were constructed for this work. These primers were used for the quantitative determination of the GMM strain with the following temperature profile: 94°C, 10 s; 63°C, 10 s; 72°C, 30 s; fluorescence was detected at 72°C. In all the cases, the Helicon *Taq* polymerase was used (2 units for the reaction); Amresco SYBR-green was added to the final concentration of 0.3× and isothiocyanate fluorescein to the final concentration of 10 nm). PCR with real-time detection was performed in the BioRad iCycler amplifier according to the recommendations of the authors mentioned above. When the GMM strain was detected, the total soil's DNA was diluted 50-fold and added to the reaction mixture in an amount of 1 μl (0.2–2 ng); the volume of the reaction mixture was 25 μl. Each sample was determined in three replicates. The results of the quantitative PCR were processed using the iCycler bundled software. Conversion of the DNA content to the microorganism titer was carried out in the usual way, taking the standard genome size of 4.2×10^6 for *E. coli* (bacteria); 2.6×10^6 for *H. salinarum* (archaea); and 1.2×10^7 for *S. cerevisiae* (fungi); and the calculations were performed assuming that one microorganism contained one genome. The control DNA for determining the GMM titer was isolated from the liquid culture of ACH-5 with the exactly known titer (CFU) determined by plating a series of dilutions. Thus, for a given DNA preparation, the number of microorganisms (CFU) corresponding to each of the dilutions used for the calibration was known exactly. In all the cases, we could not avoid formation of primer dimers. However, their appearance was invariably detected two or three cycles later than the product formation at the highest dilution of the control DNA template (1 pg for the reaction). The absence of primer dimers at all the dilutions of the control template was demonstrated by analysis of the melting curves of the amplificate. Since it is impossible to use the molecular probes for the group quantitative PCR, in which the final amplificate is heterogeneous and the detected amount of the soil DNA always fit in at approximately the middle of the calibration curve, we deemed it possible, with all the precautions taken, to choose the approach used.

T-RFLP. The following primers were used for T-RFLP: 63f/1494r for bacteria [27], Ar3f/Ar927r for archaea [11], and ITS1/ITS4 for fungi [28]; the primers 36f, Ar3f, ITS1 were fluorescence-labeled (the fluorophore D4, infrared, for the Sigma CEQ8000 Beckman Coulter). The PCR was performed using the Helicon *Taq* polymerase with the standard buffer supplemented with BSA to the final concentration of 100 μg/ml according to the recommendations of the aforementioned authors. The total soil's DNA was diluted tenfold and used for the PCR in an amount of 1 μl (approximately 1–10 ng). The amplified fragment was isolated from agarose after electrophoresis according to the standard technique [29]. The product (100–200 ng) was treated with endonuclease *Hae*III, reprecipitated with ethanol, dissolved in the Beckman Coulter SLS reagent supplemented with a 600 Beckman Coulter marker of the molecular weight, and separated using the Beckman Coulter automatic CEQ8000 sequencer under the conditions of capillary electrophoresis with fluorescent detection. The calculation of the peak sizes and peak areas was carried out using the Beckman Coulter Fragment Analysis program block. In order to calculate the peak size formed by the terminal fragment characteristic of strain ACH-5, T-RFLP analysis was carried out using the pure genomic DNA of this strain as a PCR template.

Web Links

RRNDB: http://ribosome.mmg.msu.edu/rndb/

NCBI_microbial genomes: http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi

RESULTS

Quantitative PCR. The total amount of microorganisms in the samples was determined using the PCR with real-time detection in the SYBR green variant. The approximate titer of each group of microorganisms in the sample of the control soil obtained on the first experimental day was $1.6 \times 10^9 \pm 3.7 \times 10^8$ per g of soil for bacteria; $1.2 \times 10^8 \pm 1.1 \times 10^7$, for archaea; and $2.5 \times 10^7 \pm 4.1 \times 10^6$, for fungi. The quantification was carried out for all the soil samples taken from the control and experimental vessels. In a similar way, the GMM titer was determined in the samples obtained from the experimental vessel. Interestingly, the quantitative PCR data for the sample taken immediately after introducing the strain into the soil ($7.5 \times 10^7 \pm 4.9 \times 10^6$) completely coincided with the amount of the GMM introduced (7.5×10^7). Figure 1 shows the results of the quantitative PCR. As was expected, the analysis of the melting temperature of the products obtained demonstrated the heterogeneous character of the amplificate in this respect for all microbial groups analyzed (data not shown). To assess the character of the dynamics of the number of microorganisms in the experimental and control vessels, the trend lines are shown corresponding to the linear approximation. As seen from Fig. 1, no substantial changes were observed in the dynamics of the bacterial, archaeal, and fungal components of the microbial community; at the same time, the GMM titer at day 21 of the experiment decreased more than 300-fold and was $2.3 \times 10^5 \pm 3.5 \times 10^4$.

T-RFLP. Twenty-three dominant peaks (the integral area of the peak at least 1% of the total area) were detected in the T-RFLP-gram corresponding to the bacterial community (Fig. 2a). The structure of the bacterial communit detected was highly stable and did not

differ in principle in any of the samples analyzed (six control and seven experimental samples; Fig. 1 shows the results of analysis of three samples). When comparing the sample obtained immediately after introducing the GMM to the sample obtained before its introduction, we detected the peak corresponding to the terminal fragment of the 16S rRNA gene (186.9 bp). This size is slightly different from the theoretically expected one (189 bp); however, the control T-RFLP experiment using pure DNA of the inoculant strain as a template showed that, in the series of independent experiments, this strain invariably gave a peak of precisely this size (186.7 ± 0.6 bp). Since the T-RFLP method can be used for semiquantitative assessments, the integral area of the peak corresponding to the GMM (3.2% of the total area, on the day when the GMM was introduced) was compared to the total bacterial titer in this experimental sample ($1.8 \times 10^9 \pm 2.2 \times 10^8$) determined by the quantitative PCR data; the GMM titer in the sample could therefore be assessed based on the T-RFLP data. According to the T-RFLP data, the GMM titer was 5.6×10^7, which agrees well enough with the amount of the GMM introduced (7.5×10^7). The area of this peak decreased in the course of the experiment; thus, it was discernible in the T-RFLP-grams up to day 14 of the experiment and finally disappeared in the sample obtained on day 21. The results obtained are quite consistent with the GMM dynamics revealed with the use of quantitative PCR (Fig. 1).

Figure 2b shows three T-RFLP-grams (a total of six control and seven experimental samples were analyzed) demonstrating the dynamics of the archaeal community in the course of the experiment. Unlike the stable pattern obtained for the bacterial community, here we observe certain insignificant changes. By the end of the experiment, a 136.1 bp peak appeared, and the integral area of a series of peaks 163.6–167.0 bp in size increased more than threefold. These changes were not detected in the control samples (data not shown). At the same time, the series of the dominant peaks remained unchanged. On the whole, we detected 19 dominant peaks.

Figure 2c shows three T-RFLP-grams (a total of six control and seven experimental samples were analyzed) demonstrating the dynamics of the fungal community in the course of the experiment. A total of 13 dominant peaks whose distribution was stable enough in all the samples analyzed were detected.

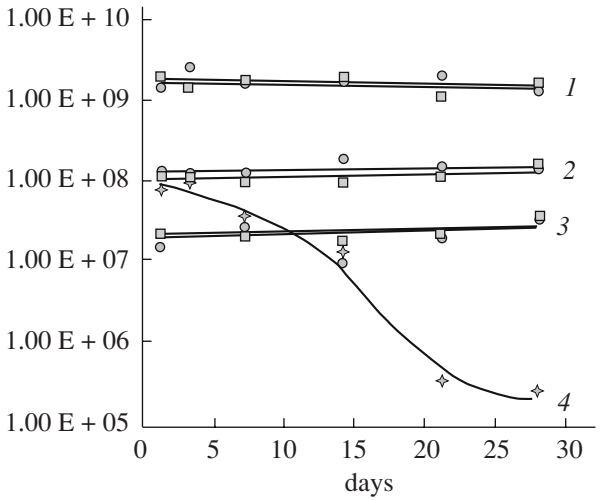

Fig. 1. Results of the quantitative determination of bacteria, archaea, and fungi in the samples by PCR with real-time detection. In all the cases, the determination errors were 9 to 23%. Bacteria (*1*); archaea (*2*); fungi (*3*); GMM (*4*). (□) Control; (◍) Experiment.

DISCUSSION

This work is the first research on the influence of introducing a genetically modified bacterial strain on the number and structure of the soil microbial community on the whole (bacteria, archaea, and fungi), under the conditions of a model experiment. The main result is the demonstration of the fact that the introduction of a genetically modified strain had no significant influence on the number and structure of the soil microbial community, whereas its own number decreased significantly during the first two weeks of the experiment. This obviously results from the high resistance and complex nature of the soil microbial community, in which all the ecological niches have been already occupied and the community itself was in the state of stable equilibrium. The results obtained agree with the published data on the dynamics of the number of the introduced GMM strains [3, 4]. It is important to note that the species *S. meliloti*, including strain ACH-5, is a facultative symbiotic nitrogen fixer capable of saprophytic existence in soil in the absence of the host plant. It is obvious that the dynamics of the inoculant strain would be of a totally different character in the presence of the corresponding leguminous plant from the alfalfa's cross-inoculation group. However, the present experiment was carried out in the absence of the host plant; this approach made it possible to rule out the influence of the latter on the structure of the experimental soil microbial community, which, we will repeat again, may be much more markedly pronounced than the influence of the inoculant strain [7].

When discussing the data on the structure of the soil microbial communities and its dynamics, we would like to draw attention to the veracity of the results and to certain important technical and methodological problems. The estimates of the number of microorganisms obtained in this work (1.6×10^9 per g of soil for bacteria; 1.2×10^8 for archaea; and 2.5×10^7 for fungi) are very rough. In particular, the use of quantitative PCR for exact assessment is complicated by the fact that, in principle, it is impossible to develop the universal primers to obtain the amplificates of all the ribosomal sequences existing in a community. This results in the bias, i.e., selective preference for certain groups of

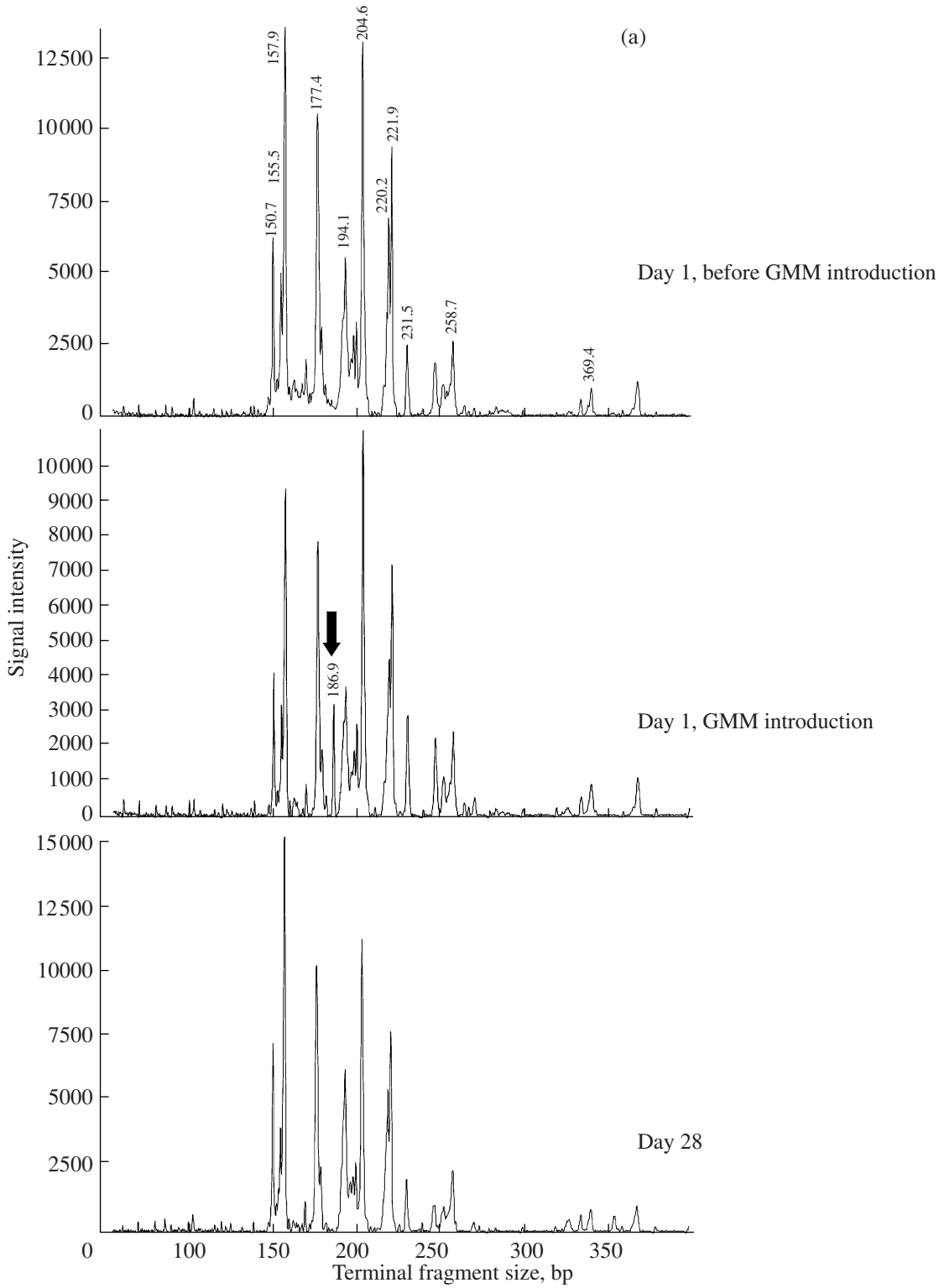

Fig. 2. T-RFLP profiling of the bacterial (a), archaeal (b), and fungal (c) communities. The sizes of the main terminal fragments are presented. In Fig. 2a, the fragment corresponding to the GMM and absent before introduction and at the end of the experiment is marked with an arrow.

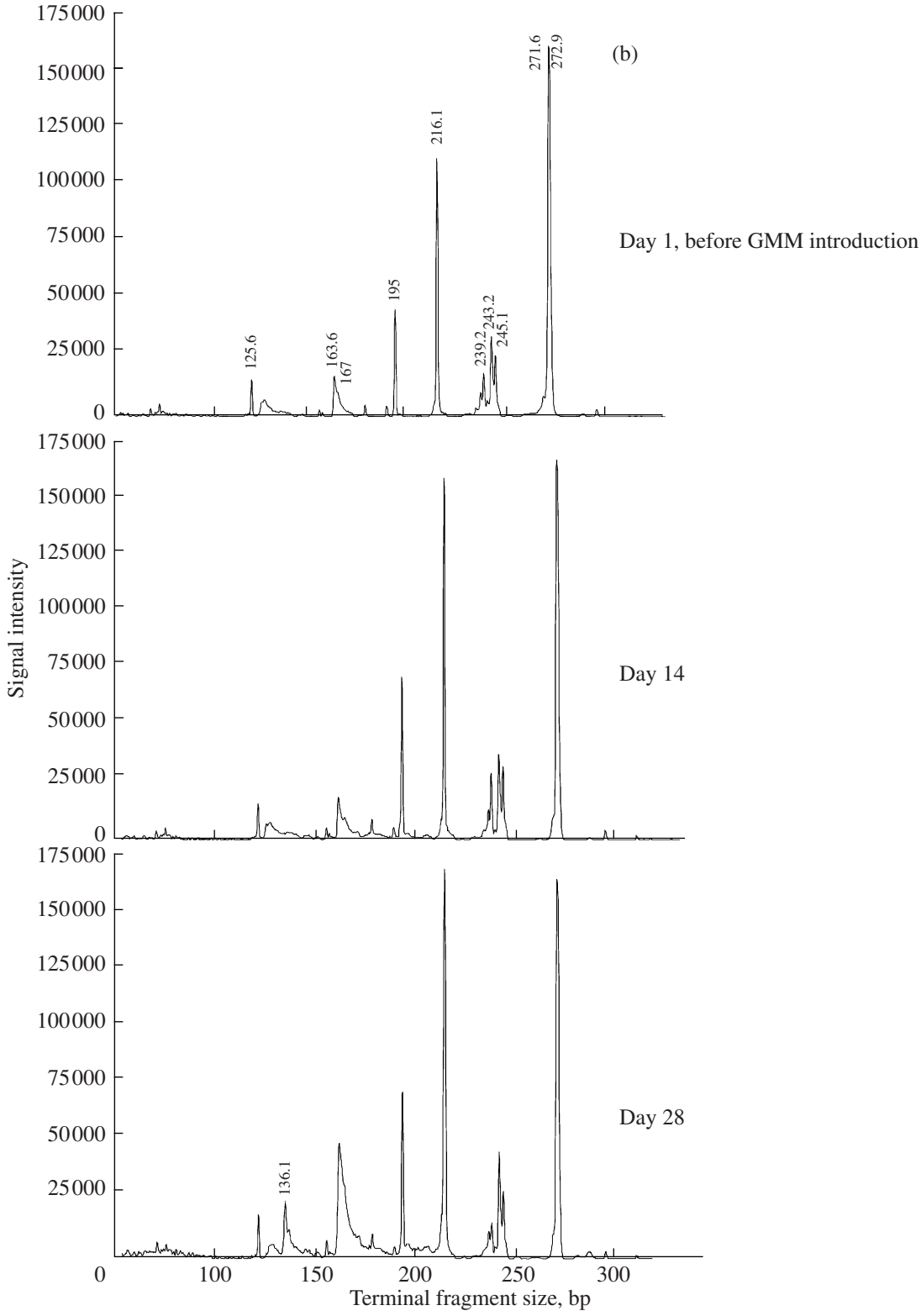

Fig. 2. Contd.

organisms. The conversion of the data on the amount of DNA in soil to the number of microorganisms carried out in this work would have been justified if they all had the same genome size and the same number of RNA operons in the genome, which is not the case. The sizes of the bacterial genome may vary between 1 and 15 Mb

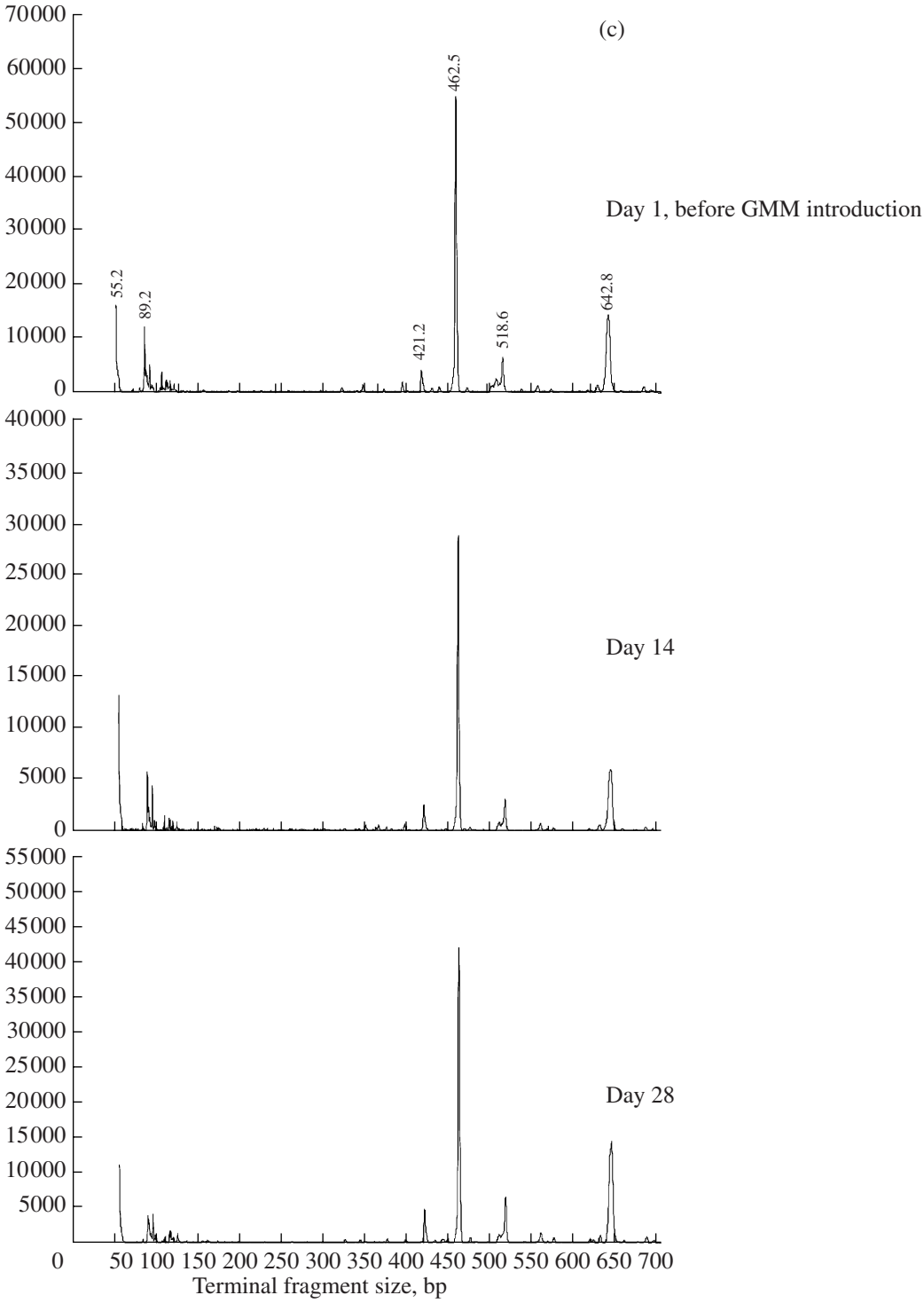

Fig. 2. Final.

(GenBank), and the number of rRNA operons in the genome may be as high as 14 in certain *Bacillus* species (RRNDB). Interestingly, the genome parameters of *E. coli* used in the present work as the control (the genome size is 4.2×10^6, and the number of rRNA operons is 4) are close to the average value for bacteria according to the GenBank and RRNDB data (3.6×10^6 and 3.0, respectively). Despite all these problems, the results obtained seem to be much closer to reality than the results of the studies relying on microbial cultures. This is confirmed, for example, by complete coincidence of the data on the number of the GMM (which

was introduced into soil in a precisely known amount) with the quantitative PCR data and, in turn, the coincidence of this number with the T-RFLP data (the share of the area of the peak corresponding to the GMM in the total peak's area calculated for the number of microorganisms according to the quantitative PCR data for bacteria). Taking into consideration all the aforesaid, interpretation of the data obtained by quantitative PCR is restricted here to the analysis of the trend lines corresponding to linear approximation; these trends indicate that the introduction of GMM in an amount of 7.5×10^7 cells per 1 gram of soil had no significant effect on the quantitative characteristics of the bacterial, archaeal, and fungal components of the soil microbial community.

This thesis is more visually confirmed by analysis of the T-RFLP data presented in this work. In fact, it is sufficient to look at the graph demonstrating a low peak corresponding to the GMM immediately after introducing the strain into soil, in the presence of the peaks corresponding to the dominant taxa, to see that the influence of GMM on the soil microbial community is more likely to be insignificant. Moreover, the T-RFLP data agree with the quantitative RCR data: the last sample, in which the peak corresponding to the GMM is still present, was taken on the 14th day of the experiment.

Interestingly, the disappearance of the peak corresponding to GMM in the T-RFLP-grams and the quantitative PCR data indicate that not only no more than 0.3% of the GMM cells introduced originally remained by the end of the 30-day experiment, but also that the DNA contained within the dead cells of this strain degraded completely.

The results of T-RFLP demonstrating the dynamics of the archaeal and fungal communities also give evidence of their high resistance and invariability, except for the small changes in the structure of the archaeal community. It is however necessary to note that T-RFLP analysis targets only the dominating taxa; a large number of less numerous species, whose variations in number are not reflected in the T-RFLP-grams, are outside its scope.

This work demonstrated the possibility of using molecular techniques in the assessment of the consequences of introducing genetically modified microorganisms into the environment, with due regard for the problems and difficulties related to the use of such methods. We believe that we should work further for optimizing and standardizing such approaches for the subsequent activity in the field of biological safety and monitoring.

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