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EXPERIMENTAL ARTICLES

Obtaining of Intrapopulational Dissociants of Some Bacilli and the Use of DIR-PCR for Their Identification

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Abstract—The paper is the first to suggest methods for rapid obtaining and genotypic identification of phenotypic (colonial–morphological) dissociants of bacterial cultures. For revealing the potential dissociation ability and obtaining dissociants, the use of bacterial cystlike refractile cells (CRC) is recommended. These cells are characterized by enhanced variability; upon their first passage, an abrupt increase in the dissociation index is observed as a result of the emergence of cells that form morphologically different types of colonies. The approaches elaborated were tested with *Bacillus cereus, B. subtilis*, and *B. licheniformis*, for which colonial–morphological dissociants of various types were obtained after the first passage of CRC (both of those formed in the developmental cycle of the bacteria and of those arising as a result of an artificial increase in the concentration of anabiosis autoinducers in the cultivation medium). The genomic distinctions between dissociants of *B. cereus* and *B. subtilis* were estimated using polymerase chain reaction with a primer system designed based on the analysis of nucleotide sequences of complete prokaryotic genomes available in the GenBank database (DIR-PCR). The application of the proposed method allowed distinctions to be revealed between the genomes of dissociants of *Bacillus cereus* and *B. subtilis*, which is in agreement with the hypothesis that assumes reversible intragenomic rearrangements to be the basis of bacterial dissociation into subpopulations.

Key words: bacilli, phenotypic dissociants, resting forms, genomic fingerprints, PCR, oligonucleotide primers.

Bacterial dissociation consists in the emergence in a developing microbial population of cells that produce, upon their further division, variants that differ in a number of colonial–morphological, physiological–biochemical, and genetic characteristics and are capable of mutual interconversion with a high frequency (10^{-2}–10^{-4} per cell division) [1, 2]. According to modern concepts, populational variability of bacteria is determined by the processes of intragenomic recombination of the cellular genetic material; these recombinations may be regarded as a specific mutation type [3]. Although this phenomenon has been described for many bacteria of various taxa, the endogenous causality of populational dissociation, which results in adaptive phenotype change, remain unknown. Experimental works performed over the last decade showed an increase in phenotypic heterogeneity of populations in the stationary phase, in aging cultures, and in cultures stored for a long time [2, 4]. According to current hypotheses, this is related to the proliferative resting state of cells (with the common regulon of the stationary phase switched on) [5] and to the effect of signal molecules that control the transition of cells to the resting state and expression of the stationary-phase genes [6, 7].

The interest in the intrapopulational variability of microorganisms and the possibilities for its control has considerably increased recently due to the development of new approaches to the investigation of phenotypic variability as a genotypic phenomenon underlying such phenomena as the change in the rate of microbial syntheses and transformations, virulence, persistence of pathogenic and conditionally pathogenic bacteria, efficiency of symbioses, and replacement of productive variants of industrial strains by nonproductive variants. Therefore, estimation of the potential capacity of bacterial cultures for dissociation, isolation of subpopulations, and their identification and characterization at the molecular level are pertinent tasks. However, such popular approaches as genome mapping or analysis of nucleotide sequences of 16S rRNA or the 16S–23S rRNA intergenic region rarely yield valuable results when closely related microorganisms or intraspecies structural rearrangements of genomes are studied. Recently, methods of gene typing or genome fingerprinting, which are based on integral methods of genome characterization and do not demand knowledge of its total molecular organization, have found

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wide application. These methods make it possible to reveal distinctions between closely related microorganisms that cannot be detected by other methods. There exists a wide diversity of such methods: RFLP, AP-PCR, RAPD-PCR, DAF-PCR, AFLP, rep-PCR, and ERIC-PCR [8]. They are based on amplification of "random" regions of the template DNA with the use of one or several oligonucleotide primers specific to this or that site of prokaryotic genomic DNA. As a result of these reactions, a spectrum of PCR fragments of varying length is obtained. These DNA fingerprints are unique and allow polymorphism between microorganisms to be revealed at various taxonomic levels, including the species level. However, most such approaches are not very specific at the strain level. Therefore, new systems are required that will allow phylogenetically close prokaryotic strains to be reliably distinguished.

In connection with the aforesaid, the aims of this work were (1) development of methods for rapidly revealing a wide spectrum of phenotypic (colonial–morphological) bacterial dissociants on the examples of the bacilli *B. cereus, B. subtilis*, and *B. licheniformis* and (2) investigation of the possibility of application of the DIR-PCR method for identification of intraspecies subpopulations and estimation of the variability of their genomes.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. The subjects of this study were the bacterial strains *Bacillus cereus* 504 (VKM) and *Bacillus subtilis* 720 (VKM), which were maintained on potato agar slants, and *Bacillus licheniformis* 103, maintained on nutrient agar.

To obtain resting forms of bacilli, the following media were used. The mineral base was composed of (g/l) $(NH_4)_2HPO_4$, 1.0; KCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.2; $FeCl_3 \cdot 6H_2O$, 0.001; and $MnSO_4 \cdot H_2O$, 0.0017. Medium 1, used for sporulation, consisted of the mineral base, 20% nutrient broth, and 0.2% glucose. Medium 2, used to obtain cystlike refractile cells (CRC) under conditions of C/N imbalance, consisted of the mineral base, yeast autolysate (0.2 g/l), and glucose (6–20 g/l). Medium 3, used to obtain CRC under conditions of nitrogen limitation, consisted of the mineral base (where $(NH_4)_2HPO_4$ and KCl were replaced with K_2HPO_4 (1 g/l) and urea (0.08 g/l)) and 1.6% glucose. In all media, the pH after sterilization was 7.0 ± 0.2. Five- to six-day-old endospore suspension was used as the inoculum, introduced in an amount that yielded an initial optical density of 0.2 (Specord, $\lambda = 600$ nm, $l = 10$ mm). Cultivation was performed in 250-ml flasks with 50 ml of medium at 28°C on a shaker at 140 rpm. Induction of CRC formation by d_1 factors was performed by their introduction into a suspension of cells from the growth retardation phase as described earlier [7]. Variability was evaluated by CRC plating (onto potato agar in case of *B. subtilis* and onto nutrient broth–wort (1 : 1) agar (NBWA) in case of *B. licheniformis*).

DNA isolation. DNA from the above microorganisms was isolated as described earlier [9].

PCR fingerprinting. Amplification of genomic DNA was performed on a Cetus 480 (Perkin Elmer, Sweden) thermal cycler using the primers KRPN1, 5'-TCIIAAGCTTCA-3', and KRPN2, 5'-CGCCIGGIGGAT-3', where I is inosine. Optimization of PCR conditions was performed according to the Taguchi procedure [10]. The amplification mixture (25 μl) was of the following composition: 1× buffer for BioTaq polymerase (17 mM $(NH_4)_2SO_4$; 6 mM Tris–HCl, pH 8.8; 6 mM $MgCl_2$), 5 nM dNTP, 50 ng of template DNA; 12.5 pM of each primer; and 1.25 U of BioTaq DNA polymerase (Dialat LTD, Russia). The temperature profile of the reaction was as follows: primary denaturation at 94°C for 3 min; then, 35 cycles of 94°C for 30 s, 37°C for 40 s, and 72°C for 1 min; and final extension at 72°C for 7 min. Analysis of PCR products was performed by electrophoresis at 6 V/cm in 1.5% agarose gel containing ethidium bromide. For the documentation of the electrophoretic separation of PCR products, the BioDocII system (Biometra, Germany) was used.

DNA amplification with the use of primers specific to IS50 mobile elements [11], P150 (5'-GAGGACAACGGTTC-3') and tPtRNA (5'-CTACCAGRSSCACCA-3') (Ignatov, unpublished), was performed under the same conditions except that the $MgCl_2$ concentration in the 1× buffer for BioTaq polymerase was 2 mM instead of 6 mM.

Design of primers and optimization of conditions for DIR-PCR. Using an earlier developed algorithm [12], analysis of the 87 complete microbial genomes available at that time in GenBank was performed. As a result, a set of oligonucleotide DIR-primers was designed that allow minor distinctions to be revealed between genomes of closely related bacteria. Twenty-four primers were chosen for the present work. The testing of these primers and the elaboration of conditions optimal for DIR-PCR were performed with dissociants of *B. cereus* and *B. subtilis.*

Amplification, isolation, and sequencing of the 5'-terminal region of the 16S rRNA gene. Amplification was performed with universal primers on a Cetus 480 (Perkin Elmer, Sweden) thermal cycler using the thermostable BioTaq DNA polymerase (Dialat LTD, Russia) according to the manufacturer's recommendations. The universal primers 11–27F, 5'-GTTTGATCMTGGCTCAG-3' (forward), and 530R, 5'-GTGCCAGCMGCCGCGG-3' (reverse) [13], were used. The temperature profile of the reaction was as follows: first cycle, 94°C for 3 min, 50°C for 1 min, and 72°C for 3 min; 30 subsequent cycles, 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and final extension at 72°C for 7 min. Analysis of PCR products was performed by electrophoresis in 1% agarose gel. Isolation and purification of PCR products were performed using low-gelling-point agarose and a Wizard DNA Purification

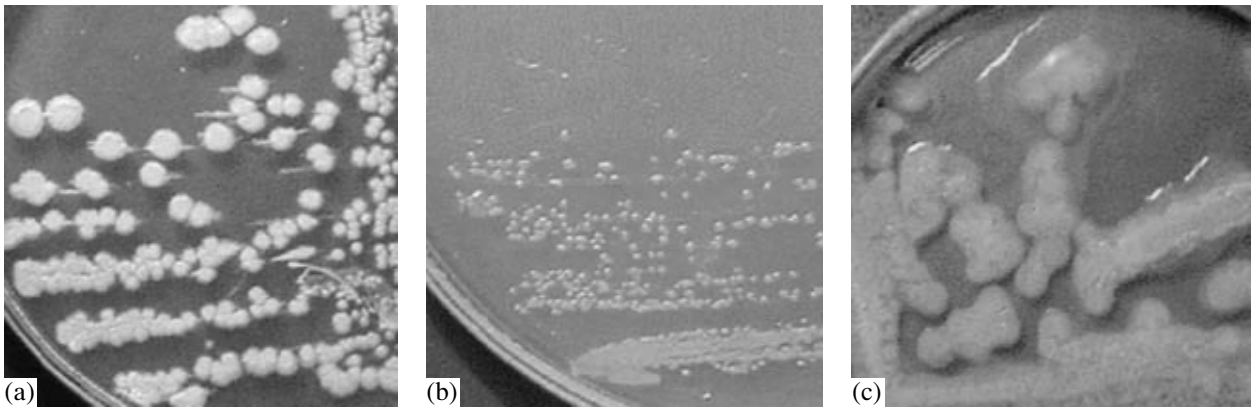

Fig. 1. Photographs of *B. subtilis* dissociants obtained after plating of cystlike refractile cells onto potato agar: (a) R type; (b) S type; (c) M type.

System kit (Promega, United States) according to the manufacturer's recommendations.

Sequencing of purified 16S rRNA gene fragments was performed using a Silver Sequencing kit (Promega, USA) according to the manufacturer's recommendations with minor modifications. Electrophoresis was run on Macrophore (Pharmacia, Sweden) and SQ3 Sequencer (Hoefer, USA) devices; the thickness of the polyacrylamide gel was 0.19 mm. For sequencing, the same primers were used as for the PCR.

Phylogenetic analysis. Genetic distances (*D*) were calculated according to Nej [14]. Dendrograms were constructed using the neighbor-joining algorithm implemented in the TREECON software package [15]. Statistical significance of the obtained trees was estimated by bootstrap analyses of 500 alternative trees.

RESULTS AND DISCUSSION

Earlier works devoted to study of intraspecies diversity of resting forms of *Bacillus cereus* showed that, under conditions of repressed sporulation, the developmental cycle culminates in the formation of CRC, which in bacilli are resting cells alternative to spores. When determining one of the main characteristics of the resting forms—the persistence of reproductive ability—we noted increased variability in the suspensions of CRC of *B. cereus*, manifested in a considerable increase, in the first passage on solid medium, in the number of colonies differing from the initial (dominant) type. The minor variants isolated were identical to the variants that constitutively emerge in the process of phenotypic dissociation of *B. cereus* cultures obtained by traditional (many times repeated) passages. In both cases, the variants exhibited stable colonial–morphological characteristics. The *B. cereus* variants—dominant, mycoid, transparent, and white—differed in a number of morphological and physiological–biochemical characteristics. In addition, a correlation was found between the morphotypes of the resting forms and the extent of dissociation exhibited upon plating of resting forms; the extent of dissociation was always higher in endospore populations than in CRC populations of *B. cereus* [7]. Proceeding from these results, at the first stage of the present work, we considered the possibility of using the above-discussed approaches (obtaining of CRC-type resting forms with unstable phenotype) for the assessment of the potential phenotypic variability of other bacilli and for obtaining of dissociants in the first passage after plating CRC onto solid media.

The relation of the populational variability to the type of the microbial forms giving rise to the microbial population was confirmed in the course of work with the cultures *B. subtilis* strain 720 and *B. licheniformis* strain 103. CRC of *B. subtilis* were obtained on C/N-imbalanced medium designed to suppress sporulation (medium 2). The initial culture was represented by cells yielding R-type colonies. After plating of 1.5-month-old CRC onto potato agar, S- and M-type populations were obtained (Fig. 1, table). The dissociants obtained were identical in the colonial–morphological characteristics to the variants described earlier as a result of studying intrapopulational variability of *B. subtilis* strain R-623 [16].

The original strain *B. licheniformis* 103 was represented by a population of cells that produced folded, rough R-type colonies on solid medium (NBWA). CRC of this bacterial variant were obtained by growing the culture on the sporulation-suppressing medium 3, which is nitrogen-deficient and glucose-excessive. After plating of 7-day-old CRC onto NBWA, the initial R-type underwent dissociation (Fig. 2, table). The population grown was represented by R-type (Fig. 2d), S-type (Fig. 2b), and P-type colonies (Fig. 2c). In addition, colonies of R(–) (Fig. 2d) and D (Fig. 2f) types occurred in minor amounts. After many repeated passages, the obtained dissociants of *B. subtilis* and *B. licheniformis* retained their colonial–morphological properties, producing up to 75–90% colonies with characteristics peculiar to the given variant. Characteristics

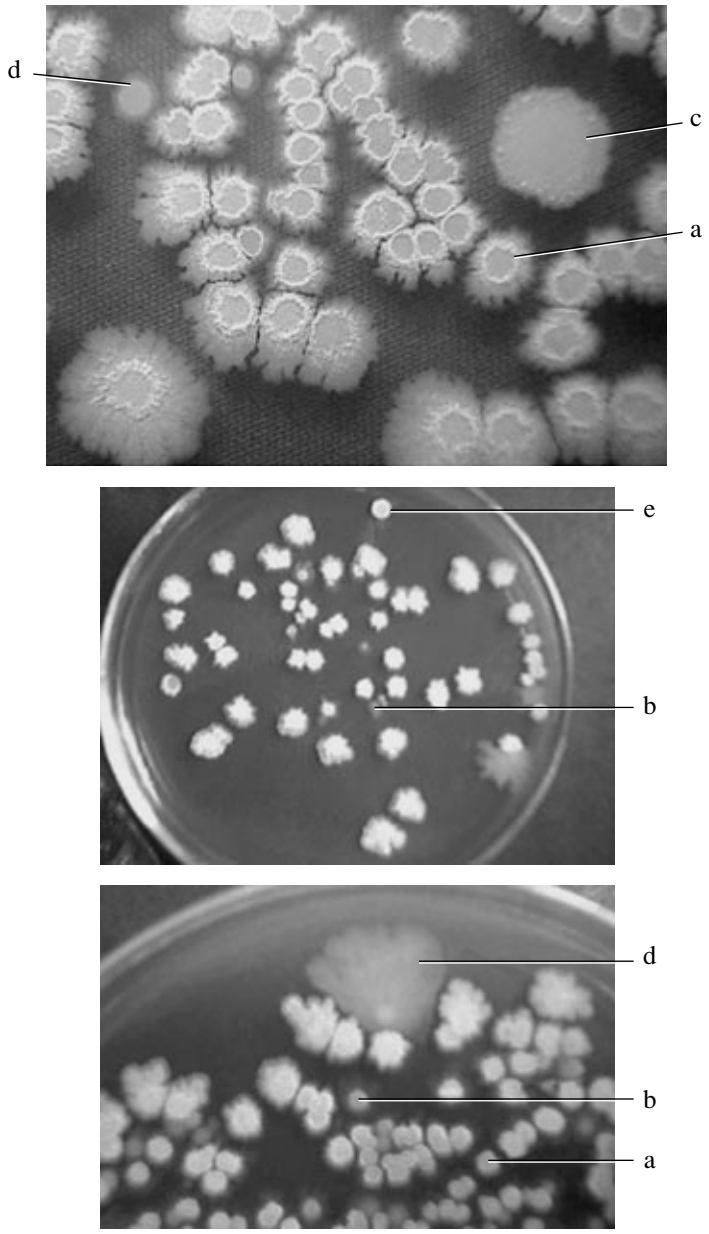

Fig. 2. Photographs of *B. licheniformis* dissociants obtained after plating of cystlike refractile cells onto NBWA: (a) R type; (b) S type; (c) P type; (d) R(–) type; (e) D type.

of the colonies of *B. subtilis* and *B. licheniformis* dissociants are given in the table.

For cultures of *B. subtilis* and *B. licheniformis*, CRC were also obtained in another way: upon an artificial increase in the content of the d_1 autoregulatory factors (anabiosis autoinducers [7]) in cultures undergoing the growth retardation phase. After plating of such CRC, a sharp increase in the dissociation index was observed in the grown populations of both species, and the following colonial types emerged: R, S, and M types for *B. subtilis* and R, R(–), S, and P types for *B. licheniformis* (the characteristics of these types were presented above).

Enhanced populational variability controlled by the level of d_1 factors was also shown in other bacteria: *Salmonella typhimurium* and *Staphylococcus aureus.* In works devoted to stress resistance of *S. aureus*, resting forms of staphylococci were obtained upon introduction of exogenous 4-*n*-hexylresorcinol (a chemical analogue of the d_1 factor) [17]. After their plating onto solid media, inversion of the initial hemolytic variant into a variant producing small G-type colonies without hemolysis zones occurred. Bacteria from the G-type colonies were characterized by slow growth and a lower number of generations (evidenced by the size of the colonies) and possessed some other distinctive

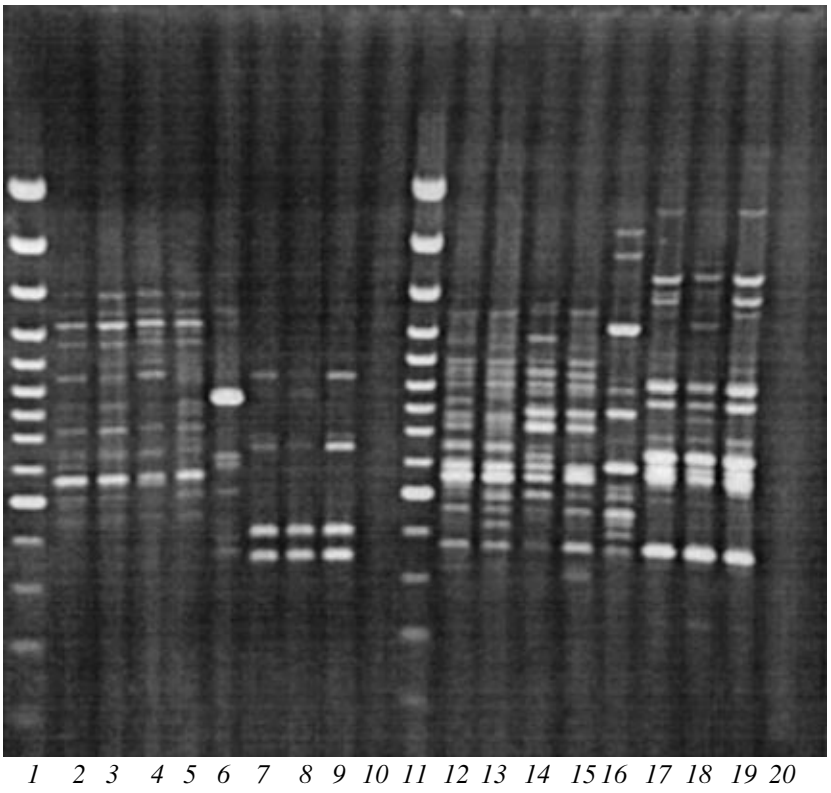

Fig. 3. Electrophoretic analysis in the presence of (*1, 11*) a 100-bp DNA molecular weight marker (Fermentas) of the PCR products obtained with primer KRPN1 (lanes *2–10*) and primer KRPN2 (lanes *12–20*) on template DNA from (*2, 12*) *B. cereus* strain 504, dominant type; (*3, 13*) *B. cereus* strain 504, mycoid type; (*4, 14*) *B. cereus* strain 504, transparent type; (*5, 15*) *B. cereus* strain 504, transparent–mycoid type; (*6, 16*) *B. cereus* strain 504, white type; (*7, 17*) *B. subtilis* strain 720, R type; (*8, 18*) *B. subtilis* strain 720, M type; (*9, 19*) *B. subtilis* strain 720, S type. (*10, 20*) Control without template DNA.

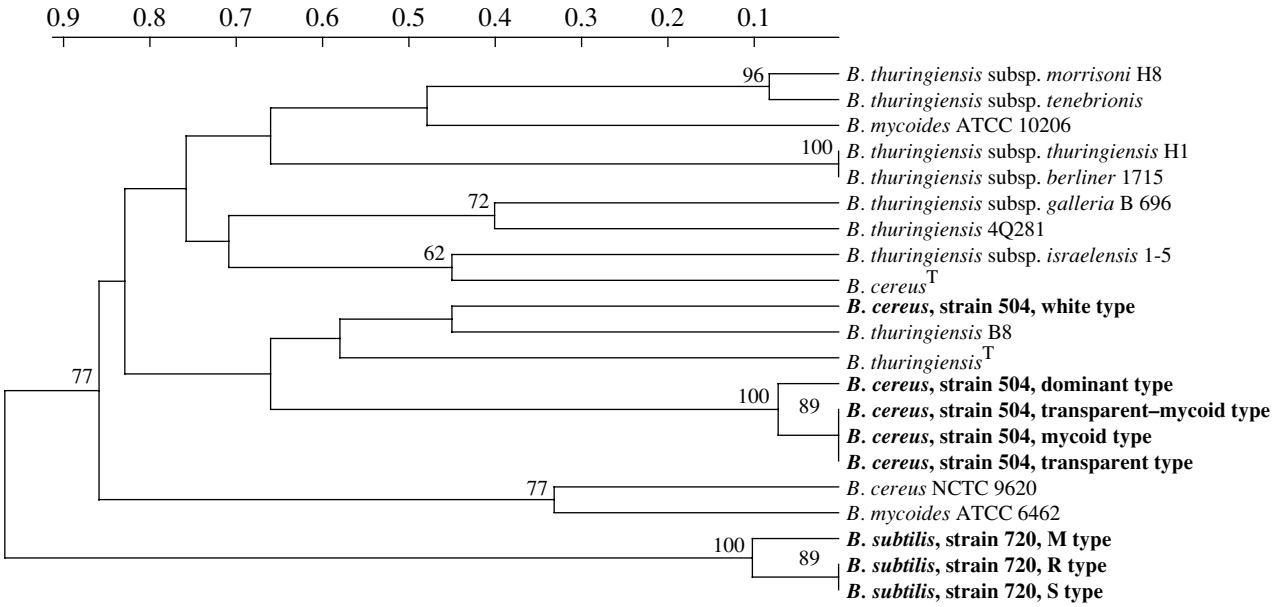

Fig. 4. Dendrogram of genetic relatedness of the bacterial groups studied, constructed based on the data from DIR-PCR for 50 polymorphous loci. Scale bar shows the fraction of distinctions.

physiological and biochemical features (e.g., those related to synthesis of extracellular proteinases); in this respect, they were similar to *B. cereus* dissociants [7]. In salmonellas, phenotypic instability was manifested in the transition of the initial virulent S type into the avirulent R type. Another approach was used in the experiments: agarized medium was supplemented with the chemical analogue of d_1 factors, 4-*n*-hexylresorcinol. Increasing concentrations of hexylresorcinol induced the transition of cells of the initial phenotype of *S. typhimurium*, which yields smooth, slimy, semitransparent, S-type colonies on solid media, into R forms, which produce dull colonies of dry consistence [18].

Thus, for the estimation of potential populational variability of bacteria and fast (in the first passage) isolation of dissociants, the following procedures can be used: (1) plating onto solid media of CRC obtained either in the natural developmental cycle or under the action of the d_1 factor and (2) plating of bacterial suspensions on solid media with a gradually increasing concentration of d_1 factors.

The next part of our work was concerned with testing the possibility of genotyping of intrapopulational variants of bacilli differing in stable phenotypic characteristics (in our case, colonial–morphological properties). To this end, we obtained PCR fingerprints of *B. cereus* and *B. subtilis* DNA with the use of either single primers or primer pairs (Fig. 3). Virtually all fingerprints exhibited unique patterns, differing from each other in the numbers, positions, and intensities of bands. In dissociants of *B. subtilis*, these distinctions were minor, being restricted to one or two bands, but in dissociants of *B. cereus* the distinctions were considerable. In the fingerprints of dissociants of the dominant, mycoid, transparent, and transitional transparent–mycoid type, the distinctions from the white type were insignificant. To make sure that these distinctions were not due to the heterogeneity of the initial cultures, nucleotide sequences were determined for the variable 5' region of the 16S rRNA genes. This analysis did not reveal distinctions in the dissociants studied, which confirmed their cultural purity (data not shown).

Colonial–morphological characteristics of dissociants of the bacteria *B. subtilis* and *B. licheniformis*

Colony type	Colony characteristics
	B. subtilis
R	Rough colonies with even edges (4–5 mm)
M	Slimy, amoeba-shaped colonies (6–7 mm)
S	Smooth, glistening, slightly convex colonies of much smaller size (1–1.5 mm) than those of other dissociants
	B. licheniformis
R	Folded, rough colonies with a craterlike center and dissected margin (5–6 mm)
R(–)	Rough transparent colonies with a dissected margin (5–13 mm)
P	Flat, rough, colonies of a larger size (13–15 mm) than those of other dissociants, having a dry solid consistence and uneven margin
S	Smooth, glistening, slightly convex colonies having a slimy consistence, a pronounced colored center, and a transparent margin; the colonies are smaller in size (3–4 mm) than those of other dissociants
D	Smooth, glistening, white, slightly convex colonies having a slimy consistence and even margins (3–4 mm)

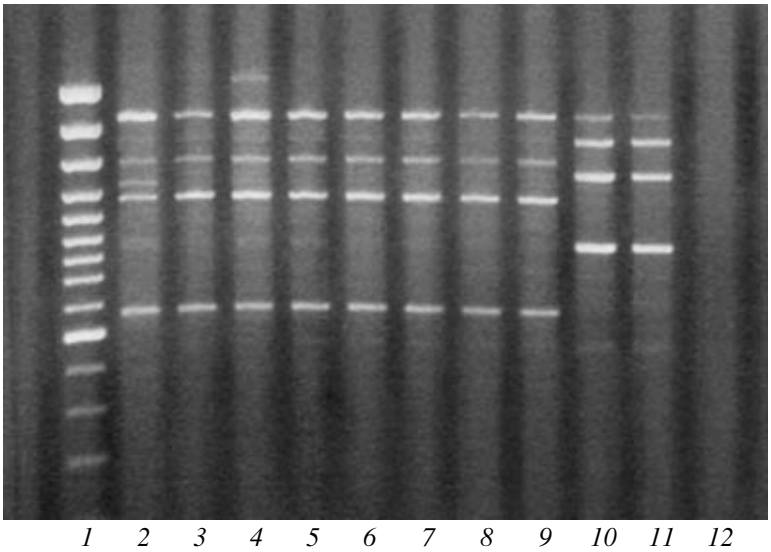

Fig. 5. Electrophoretic analysis in the presence of (*1*) 100-bp DNA molecular weight marker (Fermentas) of products obtained in DIR-PCR with primer KRPN1 on template DNA from *B. cereus* variants: (*2, 3*) *B. cereus* strain 504, dominant type; (*4, 5*) *B. cereus* strain 504, mycoid type; (*6, 7*) *B. cereus* strain 504, transparent type; (*8, 9*) *B. cereus* strain 504, transparent–mycoid type; (*10, 11*) *B. cereus* strain 504, white type. (*12*) Control without template DNA.

The dendrogram constructed based on fingerprint comparison (Fig. 4) demonstrated close relatedness of all of the dissociants: all of them, with the exception of the white variant, form a compact cluster. The white variant occurred in one group with two strains of *B. thuringiensis*, which does not contradict data in the literature. Several publications tentatively consider *B. cereus* and *B. thuringiensis* as one species [19]. It is possible that integration in the genome of a megaplasmid (or, vice versa, its excision) occurred during the formation of the white variant of *B. cereus* (megaplasmids are typical of the genomes of *B. cereus* and *B. thuringiensis*). Plasmid integration may result in considerable changes in PCR fingerprints [19]. Additional investigation of the dissociants under study with the use of primers specific to mobile elements P150 and IS50 and to tRNA genes confirmed the earlier obtained results: the analysis of the fingerprints showed the relative phylogenetic remoteness of the white variant from the dominant, mycoid, transparent, and transparent–mycoid variants (data not shown).

In addition, the stability of the fingerprints obtained was verified by running PCR on preparations of template DNA obtained from different passages of all types of dissociants (Fig. 5). The fingerprint patterns proved to be stable; the accidental factor thus does not play a role. This experiment also showed that emergence of different types of dissociants is determined by stable genome rearrangements. On the whole, the stability of the fingerprints demonstrated in this work allows DIR-PCR to be used for the identification of individual bacterial strains, at least those belonging to the species studied in the present work. This stability provides a basis for development of molecular passports of individual microorganisms.

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