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MICROBIOLOGY

# Description of the Phylogenetic Structure of Hydrolytic Prokaryotic Complex in the Soils

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**Abstract**—With the help of the molecular-biological method of cell hybridization in situ (FISH), the abundance of a physiologically active hydrolytic prokaryotic complex in chernozem and gley-podzolic soils is determined. The total proportion of metabolically active cells, which were detected by hybridization with universal probes as representatives of the domains Bacteria and Archaea, in samples of the studied soil, was from 38% for chernozem up to 78% for gley-podzolic soil of the total number of cells. The differences in the structure of chitinolytic and pectinolytic prokaryotic soil complexes are detected. Along with the high abundance of Actinobacteria and Firmicutes in the soils with chitin, an increase in phylogenetic groups such as Alphaproteobacteria and Bacteroidetes is observed.

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Polysaccharides are natural polymers of monosaccharides joined by glycosidic bonds in linear or branched complexes. They are a part of the matrix of cell walls both of eukaryotic and prokaryotic organisms.

It is well known that pectin is the dominant uronic polysaccharide of cell walls of sphagnum mosses and yellow-green algae. Large amounts of pectin are found in fruits (apples and citrus) (Donchenko and Firsov, 2007), and chitin, in addition to what is found in the integument of water and marine species (crustaceans, for example), is an integral component of cell walls of fungi and integuments of invertebrates (Terekhov, 2003). The polysaccharides in the soil are relatively quickly utilized by microorganisms and are actively involved in soil-chemical reactions. They form complex compounds with metal ions and enter into chemical or adsorption interactions with clay minerals, which contributes to creation of soil structure. The content of polysaccharides in the soil, depending on the method of extraction and the soil type, ranges from 0.06 to 3% by weight of the soil and from 1 to 14% by weight of organic matter of the soils (Terekhov, 2003). With the growing use of biopolymers in industry, agriculture, and medicine, not only is synthesis becoming an important issue, but so is the destruction of large quantities of these compounds. Therefore, it is a relevant question to consider the activity and substrate specificity of extracellular enzymes in natural systems and, thus, their participation in the microbial remineralization of organic carbon.

The purpose of this work is to study chitinolytic and pectinolytic microbial complexes and identification of the most active groups of hydrolytics in the soils. The objectives of the study included assessment of the diversity and abundance of individual phylogenetic groups of prokaryotic microorganisms of chitinolytics and pectinolytics in the soils; segregation of dominants of chitinolytic and pectinolytic communities, and identification of the most active groups of microorganisms involved in decomposition of the studied polysaccharides.

## MATERIALS AND METHODS

The objects of study were samples of the upper (2–10 cm) humus horizons of soils of different bioclimatic zones: gley-podzolic (Yamalo-Nenets Autonomous Okrug, city of Nadym, organic carbon 0.8%, pH of water 4.1) and common chernozem (Voronezh Oblast, city of Voronezh, organic carbon 3.1%, pH of water 6.8).

In determining the structure of the hydrolytic microbial complex in the studied samples, we used the method of microcosms with initiation of microbial succession by moisture and introduction of purified polysaccharides: chitin (ICN Biomedicals, Germany) and pectin (Sigma, Germany). The mass of the introduced substrates in all the variants did not exceed tenths of a percent of the sample weight. The results were taken by 10 replications for each studied variant.

The prokaryotic microbial community of these soils was investigated by using the in situ-hybridization with rRNA-specific fluorescently labeled oligonucleotide probes (FISH-fluorescent in situ hybridization) (Amann and Ludwig, 2000; Dedysh et al., 2006; Manucharova et al., 2008). This molecular-biological method combines possibilities of identification and

RNA-specific oligonucleotide probes

| Probe | Target group of organisms | Target area of rRNA 16S | Nucleotide sequence of the probe (5'–3') | Formamide, %[1] | NaCl, mM[2] | Link |
|---|---|---|---|---|---|---|
| EUB338 I<br>EUB338 II<br>EUB338 III | Bacteria<br>Bacteria (Planctomycetales)<br>Bacteria (Verrucomicrobiales) | 338–355 | GCT GCC TCC CGT AGG AGT GCA GCC ACC<br>CGT AGG TGT GCT GCC ACC CGT AGG TGT | 20 | 225 | Amann et al., 1990 |
| ARCH915<br>ARC344 | Archaea | 915–934<br>344–363 | GTG CTC CCC CGC CAA TTC CT TCG CGC CTG<br>CTG CTC CCC GT | 30 | 112 | Stahl and Amann, 1991 |
| ALFlb<br>ALF968 | Alphaproteobacteria | 19–35<br>968–986 | CGT TCG YTC TGA GCC AG[3] GGT AAG GTT<br>CTG CGC GTT | 20 | 225 | Manz et al., 1992;<br>Dedysh et al., 2001 |
| BET42a | Betaproteobacteria | 1027–1043[4] | GCC TTC CCA CTT CGT TT | 35 | 80 | Manz et al., 1992 |
| GAM42a | Gammaproteobacteria | 1027–1043[4] | GCC TTC CCA CAT CGT TT | 35 | 80 | Manz et al., 1992 |
| SRB385Db | Deltaproteobacteria | 385–402 | CGG CGT TGC TGC GTC AGG | 20 | 225 | Rabus et al., 1999 |
| CF319a<br>CFB560 | Cytophaga-Flavobacterium | 319–336<br>560–575 | TGG TCC GTG TCT CAG TAC<br>WCC CTT TAA ACC CAR T[3] | 35<br>30 | 80<br>112 | Manz et al., 1996 |
| HGC69a | Actinobacteria | 1901–1918[4] | TAT AGT TAC CAC CGC CGT[5] | 25 | 159 | Roller et al., 1994 |
| LGC354A,<br>LGC354B,<br>LGC354C[6] | Firmicutes | 354–371 | TGG GAA GAT TCC CTA CTG C, CGG GAA GAT<br>TCC CTA CTG C, CCG GAA GAT TCC CTA CTG C | 35 | 80 | Meier et al., 1999 |
| HoAc1402 | Acidobacteria | 1401–1420 | CTT TCG TGA TGT GAC GGG[7] | 10 | 450 | Juretschko et al., 2002 |

Notes: [1], concentration of formamide in hybridization buffer; [2], concentration of NaCl in the washing buffer; [3], Y = C or T, W = A or T, R = A or G; [4], target molecule 23S of rRNA; [5], probe used in conjunction with unlabeled oligonucleotide 5'-TATAGTTACGGCCGCCGT-3'; [6], equimolar mixture of three labeled oligonucleotides; [7], probe is used in conjunction with unlabeled oligonucleotide 5'-CTTTCGTGACGGG-3'.

determination of the abundance of individual phylogenetic groups of microorganisms in different natural substrates and is widely used in the practice of contemporary research for the study of microbial communities in marine and freshwater ecosystems, turfs and rhizosphere of plants, and many other natural and anthropogenic environments (Rabus et al., 1999; Amann and Ludwig, 2000; Dedysh et al., 2001; Pankratov et al., 2005). It is based on identification of microorganisms by the nucleotide sequence of the rRNA *16S* gene and provides an opportunity to explore microbial diversity directly in situ, bypassing the stage of sowing on nutrient media, and allows considering living, metabolically active cells, including those in soil samples of different natures.

This study was carried out with use of the spectrum of probes specific for representatives of Archaea and Bacteria domains, as well as of individual phylogenetic groups of bacteria. The use of FISH analysis allowed considering living, metabolically active cells in soil samples with chitin and pectin and in the control.

The soil suspension (1 : 10) was sonicated (2 min, current intensity 0.4 A, frequency 22 kHz). The cells of microorganisms were separated from the larger soil particles by three-fold centrifugation at 1000 rpm for 10 min. The suspension obtained during three cycles of treatment was centrifuged at 10000 rpm for 10 min. The resulting precipitate was filled with sterile distilled water up to a volume of 2 mL.

Fixation of the samples was carried out using formaldehyde as a fixing agent (Amann et al., 1995; Pernthaler et al., 2002; Sekar et al., 2003). Two mL of the suspension was centrifuged at 10 000 prm for 5 min. The resulting precipitate was resuspended in 0.5 mL of phosphate buffer (8 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4$, 0.2 g of $NaH_2PO_4$, 1 L of $H_2O$, pH 7), and 1.5 mL of 4% formaldehyde solution was added in the phosphate buffer and incubated at room temperature on a shaker for 1.5 h. The fixed material was collected by centrifugation (8000 rpm for 2 min), washed twice with phosphate buffer, resuspended in a mixture of ethanol and phosphate buffer (1 : 1), and stored until analysis at –20°C.

One μL of the suspension of the fixed sample was applied to glass slides for hybridization with windows separated by a Teflon coating. The fixed cell preparations applied to glasses were treated with a solution of lysozyme (10 mg in 1 mL of 0.05 M of EDTA and 0.1 M of *tris*-HCl, 1 : 1, pH 8) to increase the permeability of cell walls of bacteria. The resulting preparations were kept for 12–24 h at room temperature or for 1 h at 37°C and then treated consecutively in a series of ethanol solutions (50, 80, and 100%). For hybridization, we used a set of rRNA-specific oligonucleotide probes designed previously to detect representatives of Bacteria and Archaea domains, as well as of individual phylogenetic groups within Bacteria (table). The synthesis of probes labeled with fluorescent dye Cy3 was carried out by Synthol (Moscow, Russia). The hybridization of products with fluorescently labeled probes was carried out in accordance with the methodology of Amann et al. (Amann et al., 1995) at a temperature of 46°C. Hybridization conditions used for the various probes, concentration of formamide in the hybridization buffer, and concentration of NaCl in the buffer for washing are shown in the table.

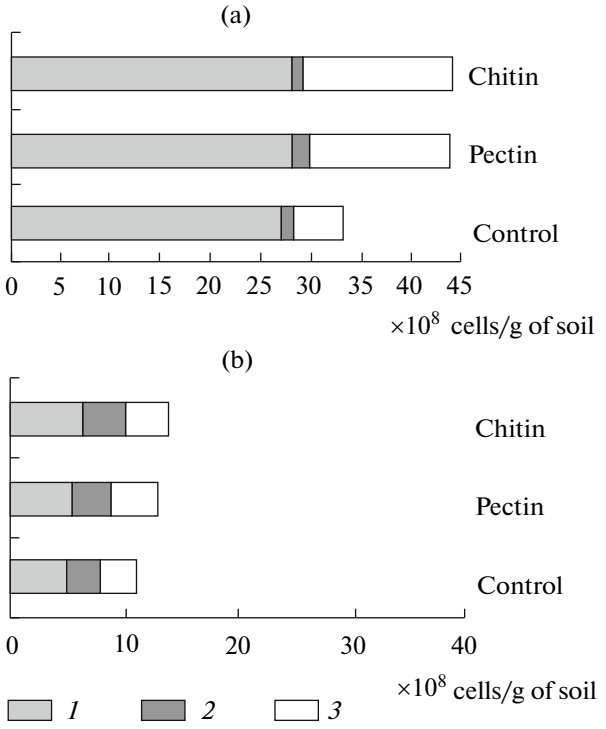


**Fig. 1.** Ratio of abundances of eubacteria (*1*), archaea (*2*), and unidentified cells (*3*) of prokaryotic microorganisms in soil samples under study on the 10th day of the experiment. (a) Chernozem; (b) gley-podzolic soil.

Upon completion of the hybridization, the products were stained with an aqueous solution of orange acridine (dilution of 1 : 10000; 2–4 min). The excess of fluorochrome was removed during washing, for which the glass was immersed in cuvettes with distilled water for 10 min. The preparations were analyzed using a Zeiss Microscope Axioskop 2 plus fluorescence microscope (Germany) with light filters, Filter set 15 for the probes and Filter set 09 for acridine orange staining. The abundance of target groups of microorganisms in the samples was determined by taking into account the number of cells hybridized with the probes in 50 fields of view of the microscope in a single cell, with subsequent calculation of the number of populations per 1 g of soil.

The number of bacterial cells contained in 1 g of soil was calculated by the formula

$$N = S_1 an / v S_2 c,$$

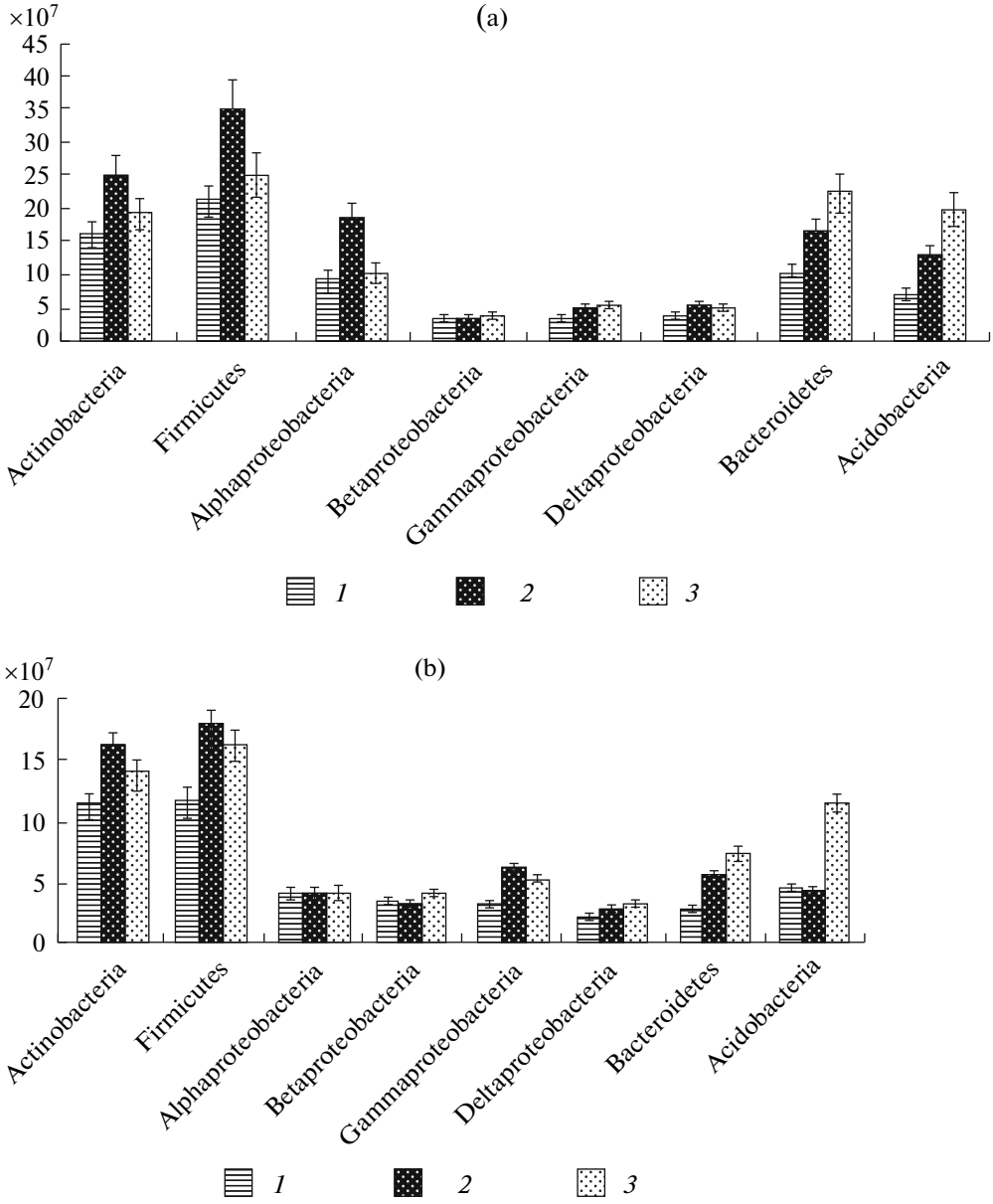


**Fig. 2.** Abundance of individual phylogenetic groups of Bacteria domain of chitinolytic and pectinolytic microbial complexes of chernozem (a) and gley-podzolic soil (b). *1*, control; *2*, chitin; *3*, pectin.

where $N$ is the number of cells (the mycelium length, μm) per 1 g of soil; $S_1$ is the square of the preparation (μm$^2$); $a$ is the number of cells (the mycelium length, μm) in one field of view (averaging over all the preparations); $n$ is the index of dilution of the soil suspension (mL); $v$ is the volume of the drop applied to the glass (mL); $S_2$ is the square of the microscope field of view (μm$^2$); $c$ is the shot of soil (g). The specific mass of microorganisms is taken equal to 1 g/cm$^3$, and the water content in the cells is 80%. The indicator of the dry biomass for a single bacterial cell of the volume of 0.1 μm$^3$ is $2 \times 10^{-14}$ g, and that for 1 m of the mycelium of actinomycetes of a diameter of 0.5 μm is $3.9 \times 10^{-8}$ g (Kozhevin, 1989).

As statistical data processing, variance (factor) analysis was carried out, where the introduction of chitin or pectin was considered as a factor influencing the strength of a particular phylogenetic group.

This analysis was applied to the groups of Alphaproteobacteria, Acidobacteria, and Bacteroidetes among the experimental variants. The conditions of applicability of the analysis of variance values of the studied trait are normally distributed; values are independent; variance by all gradations of the factors considered is

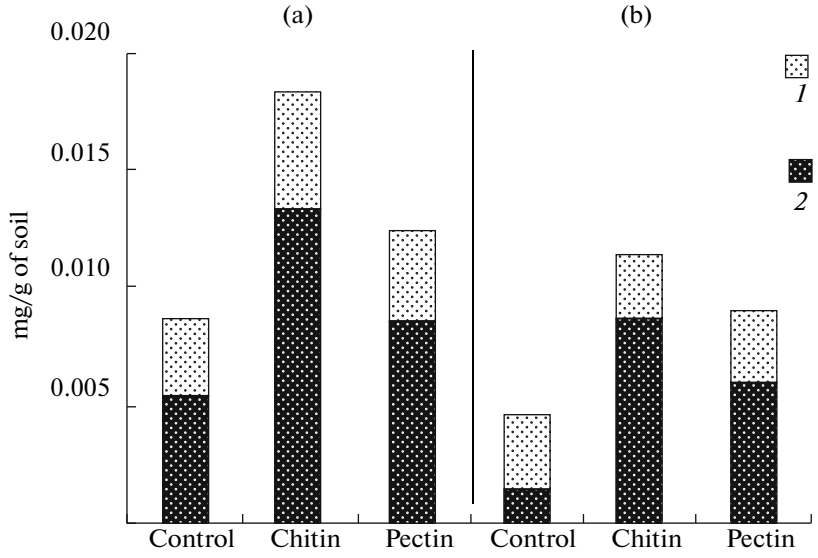


**Fig. 3.** Biomass of single-celled (*1*) and mycelial (*2*) forms of *Actinobacteria* in chernozem (a) and gley-podzolic soil (b) when adding polysaccharides and in the control on the 10th day of succession.

homogeneous (Meshalkina and Samsonova, 2008). Fisher's criterion was calculated by the LSD (least significance distance).

## RESULTS AND DISCUSSION

When introduced into the soil samples of the studied polysaccharides, the total proportion of the metabolically active cells detected by hybridization with the universal probes on the representatives of Bacteria and Archaea domains in the samples of the investigated was from 38% for chernozem up to 78% for gley-podzolic of the total number of cells isolated by staining with orange acridine (Fig. 1). These data suggest that a significant proportion of prokaryotic organisms of the chernozem occur in the form of dormant or metabolically inactive cells. Apparently, the chernozem, as a soil that is rich with organic matter and has the optimal balance of physical and chemical characteristics, is characterized by a large stock (pool) of microorganisms as compared to gley-podzolic soil.

The abundance of all identified phylogenetic groups of the Bacteria domain in the variants with both chitin and pectin was significantly higher as compared with the control and reached $4.4 \times 10^9$ cells/g of soil in samples of the chernozem with chitin (Fig. 2). Among the identified metabolically active cells in pectinolytic and chitinolytic communities of the chernozem, an increase in the abundance of bacteria belonging to the clade Actinobacteria and Firmicutes is observed. At the same time, let us note that the contribution of representatives of Acidobacteria and Bacteroidetes in the pectinolytic complex was higher compared with the chitinolytic complex. In the chitinolytic complex, along with the representatives of Actinobacteria and Firmicutes, one should note the group of Alphaproteobacteria.

Bacteria of other phylogenetic groups identified in the community probably have different trophic priorities or they are K-strategists that are not able to increase abundance rapidly when using relatively open-access substrates.

There is evidence in the literature that indicate the prevalence in the bacterial pectinolytic complex of sphagnum bogs of the phylogenetic groups of Proteobacteria and Bacteroidetes (Pankratov, 2007). The author explains this feature by the climatic conditions that prevail in the soils of the study area—low temperatures, pH reaction, high humidity, and lack of organic matter.

Due to the fact that one of the prevalent groups in the prokaryotic lytic microbial complex contained actinobacteria, special attention should be paid to members of this group. The mycelial forms of actinomycetes in the soil with chitin as compared to the control increase more than the single-celled actinobacteria (Fig. 3). In the soil with chitin as compared with the control sample, the mycelial forms of actinobacteria increase more than twice.

With the introduction of pectin in the soil, an increase in their abundance also occurs, but not as much. Moreover, when comparing biomasses of unicellular and mycelial actinobacteria in the samples of the studied soils, the proportion of mycelial forms is about 70% of the total biomass of actinobacteria.

Noteworthy is a noticeable increase of the Acidobacteria group in the soils with the substrates under study as compared to the control. The literature has evidence of a sufficiently high abundance of metabolically active forms of acidobacteria in sphagnum bogs.

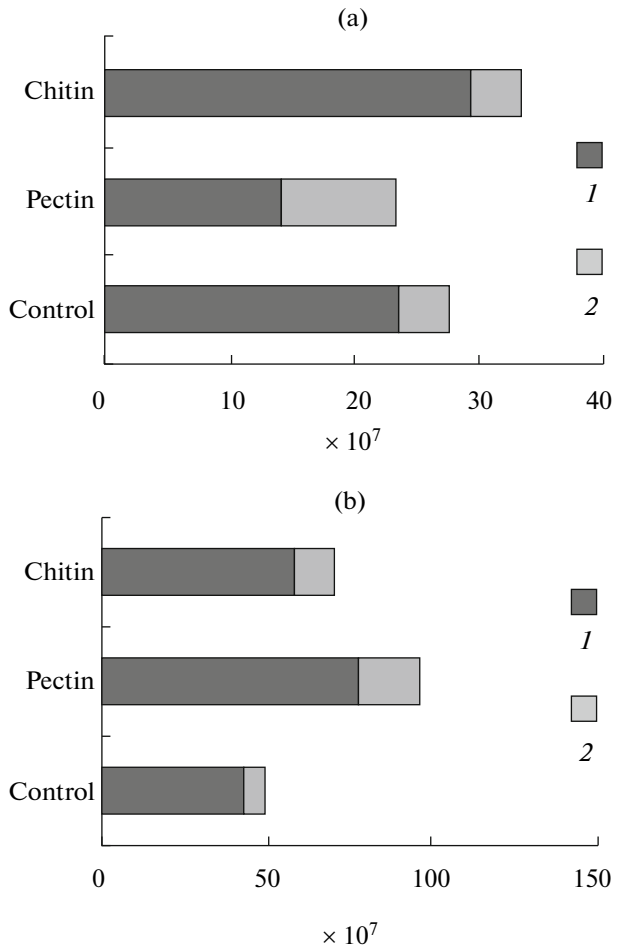


**Fig. 4.** Ratio of eubacteria (*1*) and acidobacteria (*2*) in soils under study when adding chitin or pectin on the 10th day of succession. (a) Gley-podzolic soil; (b) chernozem.

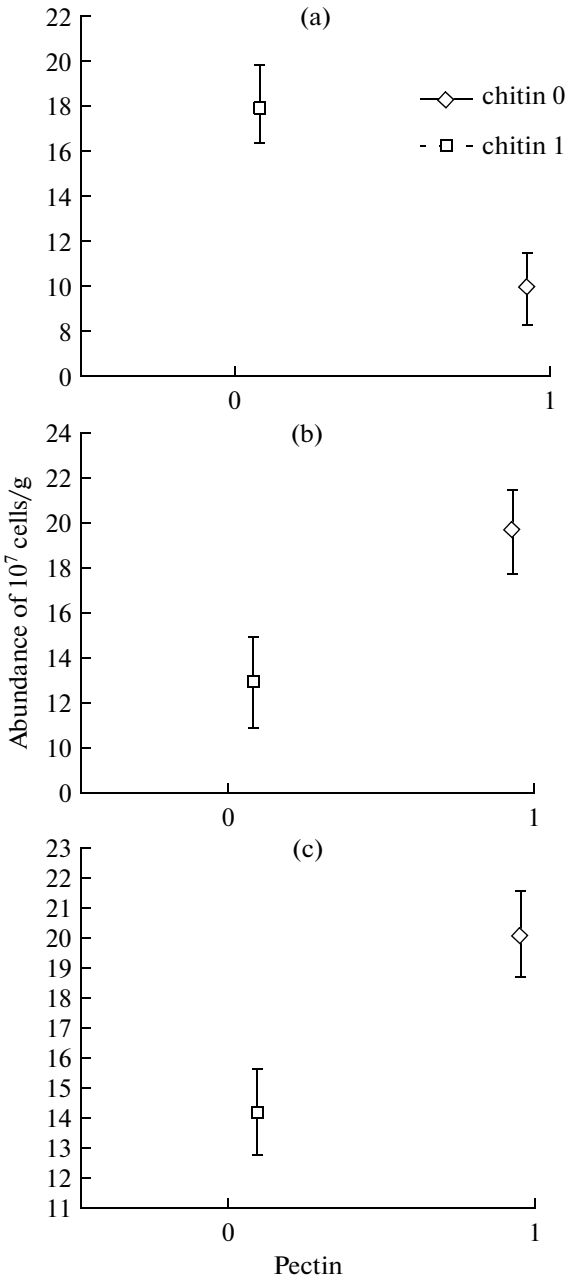


**Fig. 5.** Unweighed mean values for some phylogenetic groups of prokaryotes of chernozem: (a) *Alphaproteobacteria,* (b) *Acidobacteria*, and (c) *Bacteroidetes* when adding the polysaccharides under study. 0, polysaccharide was not introduced; 1, polysaccharide was introduced.

According to the results of Pankratov (2007), their proportion may reach 25% of the total abundance of eubacteria. However, there is no reliable data on the participation of this group in the decomposition of hard-to-reach polysaccharides. We determined the abundance of metabolically active cells of this group on the 10th day of the experiment (Fig. 4). It was shown that after the introduction of pectin, the acidobacteria abundance in the chernozem samples increased by a factor of 2 as compared to the control, and in gley-podzolic soil it increased by 3 times. Since the gley-podzolic samples were characterized by higher soil acidity (pH 5), perhaps it is connected with the sharp increase in the metabolically active acidobacteria during the decomposition of pectin.

Thus, using the molecular-biological method (FISH), differences in the structure of chitinolytic and pectinolytic complexes of the soils under study are revealed. Along with the high abundance of Actinobacteria and Firmicutes in the soils with chitin, an increase of phylogenetic groups such as Alphaproteobacteria is observed, and the increase of Acidobacteria and Bacterodetes should be noted in the pectinolytic complex.

In order to confirm the differences between the experimental variants for some phylogenetic groups (Alphaproteobacteria, Acidobacteria, and Bacteroidetes), variance (factor) analysis was carried out (Fig. 5). The numbers 0 and 1 designate the introduction of a particular substrate in the soil: 0, was not introduced; 1, was introduced. Thus, the combination of 0 for pectin and 1 for

chitin indicates that chitin was applied; 0 chitin, 1 pectin means that only pectin was introduced into the soil. The Fisher's LSD criterion was calculated (least significance distance). Using LSD, we assessed the difference between the averages. The null hypothesis is formulated for two averages and argues that these averages are equal. In all the variants investigated, the null hypothesis about equal means is rejected and the significance level is less than 0.05. The Newman–Keuls test is similar, the significance level of which is also less than 0.05 for all the phylogenetic groups under study.

Thus, on the basis of statistical processing, we can confidently assert the significant quantitative differences between the phylogenetic groups of microorganisms-destructors of the substrates under study in the chernozem. For the gley-podzolic soil, similar trends of the increase in the particular phylogenetic groups are observed.

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