ISSN 0096-3925, Moscow University Biological Sciences Bulletin, 2020, Vol. 75, No. 4, pp. 206–211. © Allerton Press, Inc., 2020.
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RESEARCH ARTICLE

New Bacterial Strains of *Pseudomonas laurentiana*: Promising Agents for Agrobiotechnology

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Received February 27, 2020; revised August 17, 2020; accepted September 15, 2020

Abstract—Bacterial strains ANT 17 and ANT 56, antagonistic to plant pathogenic fungi *Bipolaris sorokiniana*, were isolated from activated sludge. Physiological, biochemical, and culture morphological properties and analysis of the 16S rRNA gene sequence and composition of fatty acids of cell walls of strains ANT 17 and ANT 56 supported its classification as the species *Pseudomonas laurentiana*. It was shown that strains *P. laurentiana* ANT 17 and *P. laurentiana* ANT 56 possess a set of properties characteristic of PGP (plant growth-promoting) microorganisms: they exhibit antifungal activity against phytopathogenic micromycetes and are capable of decomposing phosphates and synthesizing phytohormonal substances. Inoculation of cucumber, tomato, and cabbage seeds had a beneficial effect on their germination. Presowing treatment of wheat seeds under conditions of a natural infectious background with an inoculum of the isolated bacterial strains contributed to a decrease in the spread of fungi that cause root rot. The possibility of using strains *P. laurentiana* ANT 17 and *P. laurentiana* ANT 56 in biotechnology in order to increase the productivity of agroecosystems is suggested. The ability to stimulate the growth and development of plants for *P. laurentiana* strains is shown for the first time.

Keywords: Pseudomonas laurentiana, PGP microorganisms, 16S rRNA gene, antifungal activity, indolylacetic acid, cytokinins

DOI: 10.3103/S0096392520040082

The *Pseudomonas* genus is by far the most numerous group of gram-negative bacteria and it comprises more than 191 species. Representatives of this genus have wide metabolic capabilities and inhabit various environments and sources, such as water, soil, plants, and animals. They perform functions related to the decomposition of organic substances, stimulation of plant growth, etc. and can also have pathogenic effects [1].

Pseudomonas includes a large number of strains belonging to the PGPR group (plant growth-promoting rhizobacteria). PGPR can enhance plant growth using a variety of mechanisms: biological nitrogen fixation, phosphate solubilization, synthesis of phytohormones, siderophores, antibiotic substances, 1-aminocyclopropane-1-carboxylate deaminase, volatile organic compounds, induction of systemic resistance, etc. [2]. In soil, PGPRs can effectively compete with the native microbiota, survive in the rhizosphere of plants, and then function under the special conditions of this ecosystem [3].

This work describes the taxonomic position and a set of properties useful for plants in two new PGP strains of the genus *Pseudomonas*, which were identified as representatives of the species *P. laurentiana* in the framework of this study. This species was discovered relatively recently [4], and its type strain is described as a microorganism capable of oxidizing Mn(III). The ability of *P. laurentiana* strains to stimulate plant growth and development is shown for the first time.

MATERIALS AND METHODS

In this study, we employed two bacterial strains: ANT 17, which was isolated from samples of activated sludge from biological treatment facilities of an oil refinery located in the city of Orsk (Orenburg oblast), and ANT 56, which was isolated from samples of activated sludge from biological treatment facilities of an enterprise that is a large producer of soda ash, baking soda, and caustic soda in Russia (Sterlitamak, Republic of Bashkortostan).

Bacteria of the genus *Pseudomonas* were isolated on liquid Koser medium with 0.1% pyruvic acid salt as a carbon source [5].

Cultural-morphological and physiological-biochemical properties of microorganisms were determined according to generally accepted methods [6] when grown on meat peptone agar (MPA) and King A and King B selective media [5]. The primary identifi-

cation of bacteria was carried out according to Bergey's Manual [7].

The 16S rRNA gene was amplified using universal primers [8]. Sequencing of the obtained PCR fragments was carried out using the ABI PRISM BigDye Terminator v. 3.1 reagent kit (Applied Biosystems, United States) with subsequent analysis of reaction products using the ABI PRIZM 3730 automatic sequencer (Applied Biosystems, United States). The search for nucleotide sequences of 16S rRNA genes, homologous to the corresponding sequences of the studied strain, and the calculation of their pairwise similarity was carried out using the EzBioCloud server (http://ezbiocloud.net) [9]. Dendrograms of phylogenetic similarity were constructed in the MEGA X program [10] by the neighbor-joining method [11] using the Kimura two-parameter model [12].

The composition of fatty acids in cell walls of the studied strains was determined using the Sherlock 6.1 identification system (MIDI; Microbial ID) in accordance with the technical instructions of this system [13].

The ability to synthesize indoleacetic acid (IAA) and cytokinin-like substances was detected using enzyme immunoassay as described previously [14].

The antagonistic properties of the isolated bacterial strains against phytopathogenic micromycetes were assessed in vitro by the method of cocultivation in Petri dishes [15]. The seeded dishes were incubated for 72 h at 28°C. *Fusarium avenaceum* VKM 132, *F. culmorum* VKM 844, *F. gibbosum* VKM 848, *F. nivale* VKM 3106, *F. oxysporum* VKM 137, *F. semitectum* VKM 1938, *F. solani* VKM 142, and *Bipolaris sorokiniana* IB G-12 were used as test organisms characterized by phytopathogenic activity. The latter culture is a local isolate and is stored in the Collection of Microorganisms of Ufa Institute of Biology, Ufa Federal Research Center (UFRC), Russian Academy of Sciences.

To study the effect of iron on the antifungal properties of bacterial strains, we compared the antagonistic activity of bacteria on King B medium in the absence of $FeCl_3$ and when adding 100 μg/mL $FeCl_3$.

The ability to hydrolyze phosphates was studied on Pikovskaya medium (composition: 5.0 g/L $Ca_3(PO_4)_2$, 20.0 g/L glucose, 0.2 g/L NaCl, 0.1 g/L $MgSO_4$, traces of $MnSO_4$, traces of $FeSO_4$, and 20.0 g/L agar-agar) and Muromtsev medium (composition: 10 g/L glucose, 1 g/L asparagines, 0.2 g/L K_2SO_4, 0.02 g/L corn extract, 20 g/L agar-agar, and 1.5 g/L freshly precipitated $Ca_3(PO_4)_2$).

To determine the effect of bacterization by these strains on the germination of seeds of agricultural crops, seeds of tomato, cucumber, and cabbage plants were used. For treatment, we used a diluted bacterial culture (titer ~10^5 CFU/mL) grown on King B nutrient medium. Seeds soaked in distilled water served as control. Seed germination rates were determined on the second and fourth days.

The effectiveness of antagonist strains against the complex of root rot pathogens was studied in laboratory conditions on seeds of soft spring wheat (germination rate 90.7%) with natural levels of infection of the seeding material by spores of phytopathogenic fungi *F. oxysporum* and *B. sorokiniana*. The biological preparation Rizoplan (0.5 L/t), which is based on the bacterial strain *Pseudomonas fluorescens* AR-33, was used as standard. After 7 days, the distribution and development of root rot in seedlings, as well as the spread of alternaria blight on seeds, were estimated. The data were statistically processed using MS Excel. The data in the tables are presented as mean ± standard error. The significance of differences was estimated by Student's *t*-test.

RESULTS AND DISCUSSION

Screening. As a result of screening, we isolated strains that are capable of growing on a *Pseudomonas*-selective medium and forming fluorescent pigments. The basic criterion for further selection of bacteria was the ability of the strains to suppress the growth of the phytopathogenic fungus *B. sorokiniana*, which causes helminthosporium root rot, dark brown leaf blight, glume mould, and sooty blotch on ears of cereal crops. As a result, two strains with the highest degree of antagonistic activity were selected, ANT 17 and ANT 56.

Identification of new PGP strains. Cells of the studied strains ANT 17 and ANT 56 are gram-negative motile nonsporulating rods. On dense nutrient medium MPA, colonies are round with smooth edges, smooth surface, slightly convex, opaque, grayish-white, 5–7 mm in diameter. On MPA and King B medium, colonies form a yellow-green fluorescent pigment. The formation of blue pigment on King A diagnostic medium was not observed. On beveled meat peptone agar, the stroke is solid, with smooth edges. On liquid medium (meat peptone broth), the ANT 17 strain forms an abundant, dense, disk-like precipitate; strain ANT 56 forms an abundant, flocculent sediment and a thin, continuous, loose film. Colonies are catalase- and oxidase-positive, their metabolism is respiratory. The optimum growth temperature is 28°C, they grow at 4°C and in the presence of 3% NaCl. Colonies do not need additional organic growth factors. They synthesize arginine dehydrolase and do not reduce nitrate to nitrites. They do not hydrolyze gelatin, starch, or lecithin. They are not capable of forming levan from sucrose or Tween-80 lipolysis. Both the strains utilize the following carbohydrates, with the formation of acid: glucose, xylose, arabinose, and galactose. Their colonies employ a wide range of substrates: sucrose, maltose, fructose, glycerin, ethanol, propanol, butanol, hexanol, propionic, succinic, and α-ketoglutaric acids, acetate, pyruvate, lactate,

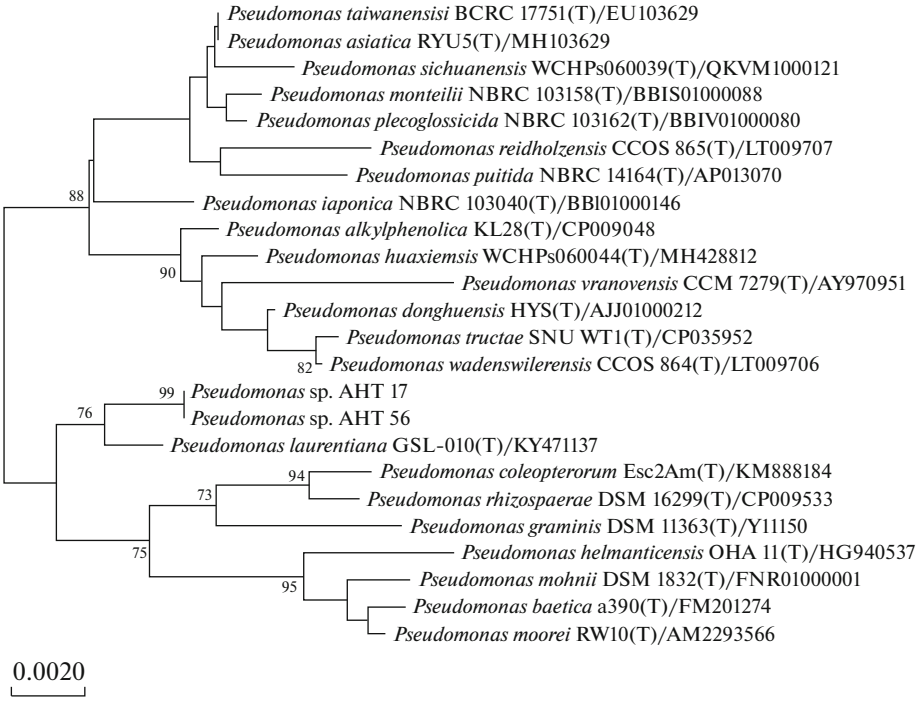

Fig. 1. Phylogenetic position of strains *Pseudomonas* sp. ANT 17 and *Pseudomonas* sp. ANT 56 based on analysis of nucleotide sequences of the 16S rRNA gene. The scale represents the evolutionary distance corresponding to two nucleotide substitutions per 1000 nucleotides. Numbers show the statistical significance of the branching order determined using the bootstrap analysis (bootstrap values above 70% are given).

citrate, DL-leucine, L-arginine, DL-α-alanine, DL-valine, L-proline, DL-lysine, L-tyrosine, L-asparagine, and phenylalanine. The strains are capable of growing on Ashby nitrogen-free medium.

In addition, the ANT 56 strain forms acid from raffinose, lactose, and rhamnose. It uses isobutyric acid, does not use mannitol, sorbitol, L-inositol, maleic, adipic, and anthranilic acids, oxalate, DL-threonine, DL-serine, DL-methionine, DL-cysteine, DL-tryptophan, D-asparagine, tetradecane, octadecane, acetamide, formaldehyde, and phenol. It produces exopolysaccharide when grown in liquid culture on potato-glucose medium and Fedorov's medium with molasses as a carbon source.

When living cells of the studied bacterial strains were applied to potato sections, no destruction of plant cells was observed, which indicates that these strains have no phytopathogenic activity.

Considering their cultural-morphological and physiological-biochemical properties, strains ANT 17 and ANT 56 were preidentified as the genus *Pseudomonas* and deposited in the Collection of Microorganisms of Ufa Institute of Biology, Ufa Federal Research Center, Russian Academy of Sciences, under numbers IB-B 5-17 and IB-B 5-56, respectively.

For the studied microorganisms, we sequenced the 16S rRNA gene. The nucleotide sequences of strains ANT 17 (1402 bp) and ANT 56 (1344 bp) were deposited in GenBank (MN541118 and MN541119, respectively).

As a result of their comparative analysis, it was found that the ANT 17 strain shows the maximum similarity with the type strains *P. laurentiana* GSL-010^T (99.29%), *P. japonica* NBRC 103040^T (98.79%), and *P. huaxiensis* WCHPs060044^T (98.79%).

The degree of homology between the nucleotide sequences of the 16S rRNA gene of the ANT 56 strain and the strains *P. laurentiana* GSL-010^T, *P. rhizospaerae* DSM16299^T, and *P. japonica* NBRC 103040^T was 99.63, 99.18, and 99.11%, respectively.

In order to clarify the phylogenetic position, a tree was constructed based on comparative data on the nucleotide sequence of the 16S rRNA gene of the species from the *Pseudomonas* genus (Fig. 1). The dendrogram shows that the studied microorganisms form a common cluster and that their phylogenetically closest strain is the type representative of the species *P. laurentiana* (*P. laurentiana* GSL-010^T).

The fatty acid composition of the cell wall in strains ANT 17 and ANT 56 was studied as another taxonomic trait (Table 1). It was shown that hexadecanoic acid (C16:0), a combination of hexadecene and pentadecene acids (C16: 1 ω7c + C15:1 iso 2-OH), and cis-11-octadecene (C18:1 ω7c) and hexadecene (C16:1) fatty acids were predominating in profiles of both the strains. In general, the fatty acid profile of the strains

Table 1. Fatty acid content in cells of *Pseudomonas* sp. ANT 17, *Pseudomonas* sp. ANT 56, and *P. laurentiana* GSL-010^T [4] (% of total)

Fatty acids	*Pseudomonas* sp. ANT 56	*Pseudomonas* sp. ANT 56	*P. laurentiana* GSL-010^T
C10:0	0.2	–	0.1
C12:0	2.5	2.3	2.7
C14:0	1.4	1.9	1.8
C15:0	–	0.3	0.4
C16:0	37.1	33.6	32.3
C17:0	0.2	–	0.1
C18:0	0.2	0.5	0.2
C16:1	8.3	15.0	–
C18:1	1.9	0.7	–
C16:1 ω5c	–	–	0.1
C17:1 ω8c	–	–	0.1
C18:1 ω7c	11.2	10.0	8.3
C17:0 cyclo	1.0	2.8	1.1
C18:1 methyl ω7c	–	–	0.4
C10:0 3-OH	–	–	3.8
C12:0 2-OH	3.4	4.3	4.2
C12:0 3-OH	2.3	4.0	3.7
C12:1 3-OH	–	–	0.1
C16:1 ω7c + C15:1 iso 2-OH	29.2	24.6	40.4
C19:1 ω6c + C19:1 cyclo ω10c	1.1	–	0.2

"–" indicates that the given fatty acid was not detected.

corresponds to that of the type strain of the *P. laurentiana* species GSL-010.

Thus, based on the comparative analysis of the nucleotide sequence of the 16S rRNA gene and the composition of fatty acids of the cell wall, as well as cultural-morphological and physiological-biochemical characteristics, strains ANT 17 and ANT 56 were identified as representatives of the species *Pseudomonas laurentiana*.

CHARACTERIZATION OF NEW PGP STRAINS

Antagonistic activity. Investigation of the range of antagonistic activity of bacterial strains *P. laurentiana* ANT 17 and *P. laurentiana* ANT 56 showed that these microorganisms suppress the development of phytopathogenic fungi of the genera *Bipolaris* and *Fusarium* (Table 2). The studied bacterial strains showed selectivity in relation to these micromycetes: the diameters of the regions of growth inhibition of phytopathogens varied significantly. The strains most actively suppressed the growth of *B. sorokiniana* IB G-12 and

Table 2. Range of antagonistic activity of strains *P. laurentiana* ANT 17 and *P. laurentiana* ANT 56

Species of phytopathogenic fungi	Diameter of fungal growth inhibition region, mm	
	P. laurentiana ANT 17	*P. laurentiana* ANT 56
Fusarium avenaceum VKM 132	7.0 ± 1.1	7.0 ± 0.9
F. culmorum VKM 844	12.0 ± 1.6	17.0 ± 1.9
F. gibbosum VKM 848	11.0 ± 1.5	10.0 ± 1.3
F. nivale VKM 3106	10.0 ± 1.0	8.0 ± 1.2
F. oxysporum VKM 137	10.0 ± 1.4	6.0 ± 0.8
F. semitectum VKM 1938	26.0 ± 3.0	21.0 ± 2.5
F. solani VKM 142	10.0 ± 1.2	10.0 ± 1.0
Bipolaris sorokiniana IB G-12	26.0 ± 2.8	30.0 ± 3.3

F. semitectum VKM 1938. The antagonistic activity indices of the studied strains are comparable to those of known antagonist strains of the *Pseudomonas* genus [16, 17].

Nature of antifungal metabolites. It is known that the antagonism of microorganisms can be associated with the synthesis of iron-transporting agents-siderophores, which compete with the siderophores of phytopathogenic microorganisms for the iron required for the latter.

It was shown that the addition of iron to the nutrient medium does not change the antifungal activity of bacterial strains. Thus, on King B medium with addition of $FeCl_3$, the diameter of the region of growth inhibition of the phytopathogen *B. sorokiniana* for the *P. laurentiana* ANT 17 strain was 26 mm and that for the *P. laurentiana* ANT 56 strain was 32 mm.

Therefore, it was found that the antagonistic activity of strains *P. laurentiana* ANT 17 and *P. laurentiana* ANT 56 is not limited by the presence of iron in the medium; that is, the antifungal properties of cultures are determined by the synthesis not of siderophores but, probably, antibiotic substances.

Synthesis of phytohormones is one of the most important properties of bacteria classified as PGPR. Strains *P. laurentiana* ANT 17 and *P. laurentiana* ANT 56 were examined for the ability to produce phytohormones, indole-3-acetic acid (IAA) and cytokinin-like substances. IAA is known to be involved in the regulation of cell division and growth by stretching, root differentiation, and other processes of plant growth and development [18, 19], while cytokinins and cytokinin-like substances induce cell division and are involved in maintaining the shoot apical meristem [20].

A significant amount of immunoreactive substances was found in the culture fluid of the *P. laurentiana* ANT 17 strain. This microorganism was able to synthesize IAA in the amount of 804 ng/mL of culture fluid and did not produce cytokinins. The bacterial

Table 3. Efficiency of using strains *P. laurentiana* ANT 17 and *P. laurentiana* ANT 56 against wheat seed infection

Variant	Nongerminated seeds, %	Abnormally developed seedlings, %	Wheat seedlings affected by diseases, %	
			B. sorokiniana	*Alternaria* sp.
Control	11.0 ± 0.7*	18.0 ± 0.9*	48.6 ± 1.2*	12.6 ± 0.8*
Biological preparation Rizoplan	8.0 ± 0.6*	8.6 ± 1.2*	44.6 ± 1.6*	3.6 ± 0.2*
P. laurentiana ANT 17	10.0 ± 0.2*	6.6 ± 0.5*	30.0 ± 1.5*	2.0 ± 0.1*
P. laurentiana ANT 56	10.6 ± 0.3	8.0 ± 0.7*	24.0 ± 1.1*	1.6 ± 0.07*

*Differences with control are statistically significant at $p \le 0.05$.

strain *P. laurentiana* ANT 56 synthesized IAA and cytokinins in the amount of 166 and 68 ng/mL of culture fluid, respectively. The amount of IAA accumulated in the culture fluid by the studied strains was higher than that for the phytohormone producer *Pseudomonas koreensis* IB-4 (VKM B-2830D), which we described earlier [21] and for which it was 40 ng/mL of culture fluid. Only the *P. laurentiana* ANT 56 strain produced less cytokinins.

Phosphate-mobilizing activity. The ability of microorganisms to dissolve organic and inorganic phosphorus compounds in the soil plays an important role in plant nutrition. Bacterial synthesis of metabolites in the form of acids and enzymes (phosphatases) affects the mobility of this organogenic element and its subsequent availability to plants. Investigation of the ability to convert phosphorus compounds in strains *P. laurentiana* ANT 17 and *P. laurentiana* ANT 56 on the nutrient media of Pikovskaya and Muromtsev showed the formation of apparent clearing zones, which indicates the mobilizing activity of these bacteria with respect to inorganic phosphates. No ability to degrade organic phosphorus compounds was detected when growing bacteria on a medium containing organic phosphorus in the form of sodium adenosine triphosphate.

Effect on plant germination. In all variants of the experiment, inoculation of seeds of agricultural crops with bacteria had a positive effect on their germination in comparison with the control, which confirms PGP properties of the studied strains. The most "responsive" to presowing treatment were cabbage and cucumber seeds: their germination on the fourth day increased by an average of 7 and 9%, respectively, compared with the control. Bacterization with strains of microorganisms accelerated germination in tomato seeds: on the second day, their germination rates was higher than the control values by 7–9%; however, the differences between the control and experimental variants were not statistically significant by the fourth day (at $p \le 0.05$).

Effectiveness against the complex of root rot pathogens. It was shown that treatment using strains *P. laurentiana* ANT 17 and *P. laurentiana* ANT 56 under conditions of natural seed infection helps reducing the spread of fungi of the *Alternaria* genus by 38.3 and 50.6%, respectively, and helminthosporium root rot caused by *B. sorokiniana* by 71.4 and 87.3% (Table 3).

The biological preparation Rizoplan used as standard showed a high degree of biological effectiveness in relation to the micromycete *B. sorokiniana* causing root rot in wheat (71.4%), but it turned out to be ineffective against alternaria blight in seedlings. This indicates that the indicators of the effectiveness against phytopathogens in the studied strains in a natural infectious background were no lower than those of known biological preparations.

When treated with the biological preparation Rizoplan and a liquid culture of the *P. laurentiana* ANT 17 strain, the number of nongerminated seeds was less than in the control (Table 3). At the same time, there were no significant differences with the control for this parameter in the variant with the *P. laurentiana* ANT 56 strain.

The indicator characterizing the proportion of abnormally developing seedlings after the bacterial treatment, including using the biological preparation Rizoplan, was 2–3 times less than in the control. This indicates a decrease in the damage to the tissues of wheat plants caused by phytopathogenic micromycetes due to the effect of antifungal metabolites of PGP bacteria.

Thus, based on the analysis of physiological and biochemical properties, the nucleotide sequence of the 16S rRNA gene, and the composition of fatty acids of the cell wall, bacterial strains ANT 17 and ANT 56 were classified as the species *Pseudomonas laurentiana*.

It was shown that strains *P. laurentiana* ANT 17 and *P. laurentiana* ANT 56 possess a number of properties useful for plant growth and development, which are characteristic of PGP microorganisms. In particular, they exhibit antifungal activity against a large number of phytopathogenic micromycetes and are capable of decomposing inorganic phosphates and synthesizing phytohormonal substances. Treatment of cucumber, tomato, and cabbage seeds with a liquid culture with a titer of ~10^5 CFU/mL had a beneficial effect on the germination of plants. Presowing treatment of wheat seeds under conditions of a natural infectious background with an inoculum of isolated bacterial strains

reduced the spread of fungi that cause root rot. In terms of their useful properties, strains *P. laurentiana* ANT 17 and *P. laurentiana* ANT 56 can be considered promising for further study in terms of their application in biotechnology in order to increase the productivity of agroecosystems.

FUNDING

This study was carried out using the equipment of the Agidel Regional Center for Shared Use in the framework of the state assignment of Ufa Federal Research Center, Russian Academy of Sciences (no. 075-00326-19-00) within the subject no. AAAA-A18-118022190100-9 at Ufa Institute of Biology (Ufa Institute of Biology, Ufa Federal Research Center, Russian Academy of Sciences).

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare no conflict of interests. The work was carried out without the use of animals and without the involvement of people as subjects.

ADDITIONAL INFORMATION

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Translated by K. Lazarev