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EXPERIMENTAL ARTICLES

The Application of Succession Analysis in Combination with EHF Irradiation to the Selective Isolation of Actinomycetes from Soil

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Abstract—A new method employing succession analysis and extremely high frequency (EHF) irradiation is proposed for the selective isolation of actinomycetes from soil. Total actinomycetes were efficiently isolated from soil suspensions irradiated in the wavelength band 4.6–5.8 mm on the 14th and 45th days of succession initiated by soil wetting and from soil suspensions irradiated in the wavelength band 8–11.5 mm on the 7th day of succession. The rare actinomycete genera *Actinomadura, Micromonospora, Nonomuraea, Microbispora, Amycolatopsis, Pseudonocardia, Saccharothrix, Streptosporangium, Actinosynnema, Nocardioides,* and *Saccharopolyspora* were isolated by either of the two approaches (succession analysis and EHF irradiation); however, the range of isolated rare actinomycetes was considerably wider when a combination of the two approaches was used. For instance, actinomycetes of the rare genera *Actinocorallia, Promicromonospora, Actinoplanes,* and *Kibdelosporangium* were isolated only when EHF irradiation was employed at the early stages of succession.

Key words: actinomycetes, succession, EHF radiation, millimeter electromagnetic waves.

The isolation of unknown rare genera of actinomycetes may lead to the obtaining of new potent antibiotics, since the actinomycetes of rare genera produce a substantial amount of naturally occurring antibiotics. This calls for the development of new selective isolation approaches.

Earlier, we investigated the possibility of employing extremely high-frequency (EHF) radiation for the selective isolation of rare actinomycete genera from soil and showed the efficiency of EHF radiation of certain wavelengths in this respect [1, 2]. It should be noted that EHF radiation has already been used in microbiology for some purposes [3–5], but we were the first to employ it for the selective isolation of actinomycetes from natural sources.

At the same time, the succession analysis of the soil actinomycete complex makes it possible to determine the dynamics of particular actinomycete genera (many of which cannot be detected without succession) in soil samples [6]. This implies that the combination of succession analysis and EHF irradiation may essentially widen the range of actinomycetes isolated from soil.

The aim of this work was to evaluate the potential of such a combined approach to the selective isolation of actinomycetes from soil samples.

MATERIALS AND METHODS

Experimental design. Soil suspensions placed in petri dishes were irradiated from the bottom with EHF electromagnetic radiation of two wavelength bands (4.6–5.8 and 8–11.5 mm) generated using G4-141, G4-142, R2-65, and R2-69 industrial generators (Russia). Electromagnetic energy was transmitted with the aid of a waveguide duct containing an attenuator, a phase-shifting circuit, a control power meter, and plug-in horn radiators with the necessary radiation patterns. The radiation had a nonthermal intensity and was amplitude-modulated at a frequency of 1 kHz.

Samples of soil (ordinary chernozem) were collected in the Krasnodar region. Succession in soil was initiated by wetting it to a level corresponding to 30% of the total field moisture capacity. Then the moisture content of the soil was maintained at this level throughout the experiment. Soil samples were plated onto agar media on the 0th, 7th, 14th, 30th, and 45th days of succession.

Soil samples were ground in a mortar, sieved, and thoroughly mixed. A 100-mg portion of soil thus prepared was suspended in 10 ml of water by shaking for 10 min. The suspension was diluted 10- and 100-fold, and aliquots of each dilution were either irradiated (experimental soil suspension) or not (control soil suspension) with EHF electromagnetic radiation.

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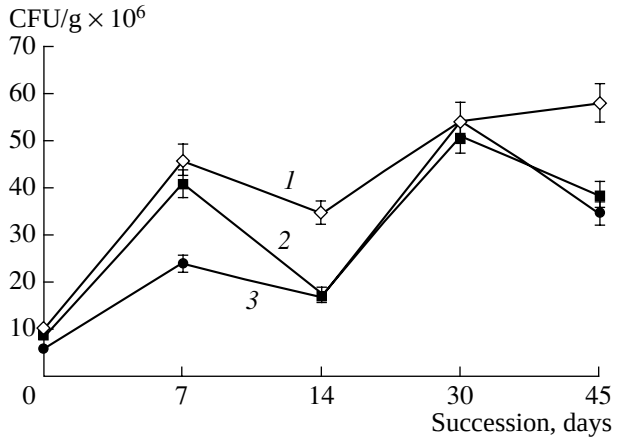

Fig. 1. The dynamics of unicellular bacteria in soil suspensions in the course of (*1*) succession without EHF irradiation, (*2*) succession combined with the exposure of soil suspensions to EHF radiation in the wavelength band 4.6–5.8 mm, and (*3*) succession combined with the exposure of soil suspensions to EHF radiation in the wavelength band 8–11.5 mm.

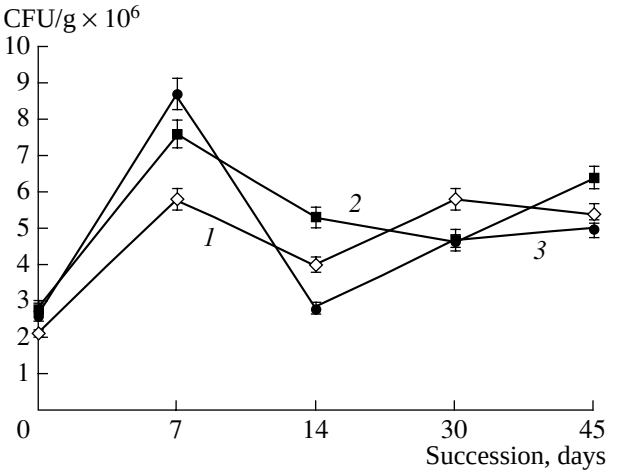

Fig. 2. The dynamics of actinomycetes in soil suspensions in the course of (*1*) succession without EHF irradiation, (*2*) succession combined with the exposure of soil suspensions to EHF radiation in the wavelength band 4.6–5.8 mm, and (*3*) succession combined with the exposure of soil suspensions to EHF radiation in the wavelength band 8–11.5 mm.

Experimental and control soil suspension dilutions were plated onto soil agar prepared as follows: Soil (200 g) was suspended in 1 l of water. The suspension was boiled for 30 min and filtered. The filtrate was brought to a volume of 1 l and used for the preparation of 2% agar medium. The pH of the medium before sterilization was 7.0. The molten soil agar cooled to 50°C was supplemented with a sterile vitamin solution (in an amount of 1 ml/l) containing 10 mg Ca pantothenate, 10 mg nicotinic acid, 1 mg thiamine chloride, and a trace amount of biotin in 20 ml of water. The agar medium was also supplemented with 10 μg/ml nalidixic acid to inhibit bacteria characterized by creeping growth and 50 μg/ml nystatin to inhibit the growth of fungi [7]. According to our earlier observations, nalidixic acid at the concentration mentioned does not inhibit the growth of actinomycetes [8]. The plates were incubated at 28°C for 2 weeks. In some experiments, the incubation was continued for 6 weeks in order to isolate slow-growing actinomycetes.

Data processing. The number of actinomycetes detected in soil was expressed as CFU per gram soil. The results were statistically processed using the methods described by Platonov [9] and Plokhinskii [10].

Taxonomic identification. The generic status of isolates was determined from their morphological properties and the chemical composition of their cell walls. To determine the structure of sporangia, isolates were grown on mineral Gauze 1 agar and oat agar [11] and examined under a Jenaval light microscope. The cell-wall diaminopimelic acid and sugars were analyzed in whole-cell hydrolysates by thin-layer chromatography on cellulose plates (Merck, Germany) as described elsewhere [11–14]. Isolates were assigned to particular actinomycete genera using the identification criteria of *Bergey's Manual* [15] and original descriptions of these genera.

Culture antagonism. The antagonistic properties of isolates were studied by cultivating them on Gauze 2 agar with the following test cultures: *Escherichia coli* K-13, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* 209P, *S. aureus* 209P/UF-2, *Micrococcus luteus* ATCC 9341, *Bacillus mycoides* R-537,

Table 1. The abundance of representatives of particular rare actinomycete genera isolated from unexposed soil suspensions in the course of succession

Genera	CFU/g × 10^4				
	0th day	7th day	14th day	30th day	45th day
Streptomyces	50	301	252	177	266
Micromonospora	70	256	80	117	73
Actinomadura, Nonomuraea	67	3	8	10	3
Amycolatopsis, Pseudonocardia	17	4	20	10	0
Saccharothrix	3	0	36	**117**	**143**
Streptosporangium	0	10	0	**70**	3
Microbispora	3	0	0	**63**	0
Actinosynnema	0	0	0	0	**17**
Nocardioides	0	0	4	0	7
Streptimonospora	0	0	0	0	**7**
Unidentified genera	3	0	4	20	27
Total actinomycetes	213	574	404	584	543
Total rare genera	163	273	152	**407**	**287**

Table 2. The abundance of representatives of particular rare actinomycete genera isolated from soil suspensions exposed to EHF radiation in the wavelength band 4.6–5.8 nm in the course of succession

Genera	CFU/g × 10^4				
	0th day	7th day	14th day	30th day	45th day
Streptomyces	40	473	396	90	247
Micromonospora	87	207	100	123	70
Actinomadura, Nonomuraea	140	40	8	70	53
Amycolatopsis, Pseudonocardia	10	13	24	3	3
Saccharothrix	0	0	0	**97**	**194**
Streptosporangium	3	0	0	3	10
Microbispora	3	0	0	**43**	0
Actinosynnema	0	0	0	0	**10**
Nocardioides	0	0	0	0	13
Actinocorallia	0	7	0	0	0
Promicromonospora	0	7	0	0	0
Unidentified genera	3	13	4	37	43
Total actinomycetes	286	760	532	466	643
Total rare genera	246	287	136	**376**	**396**

Table 3. The abundance of representatives of particular rare actinomycete genera isolated from soil suspensions exposed to EHF radiation in the wavelength band 8–11.5 nm in the course of succession

Genera	CFU/g × 10^4				
	0th day	7th day	14th day	30th day	45th day
Streptomyces	32	444	136	177	257
Micromonospora	92	164	40	77	80
Actinomadura, Nonomurae	104	192	12	23	23
Amycolatopsis, Pseudonocardia	12	32	28	16	0
Saccharothrix	0	0	52	**170**	**100**
Streptosporangium	4	4	0	0	0
Microbispora	4	**20**	0	0	0
Actinosynnema	0	0	0	0	**13**
Nocardioides	0	0	0	0	3
Actinoplanes	0	**8**	0	0	0
Saccharopolyspora	**4**	0	0	0	0
Kibdelosporangium	0	0	**4**	0	0
Unidentified genera	8	8	8	20	30
Total actinomycetes	260	872	280	483	506
Total rare genera	228	**428**	144	**306**	249

B. subtilis ATCC 6533, and *Saccharomyces cerevisiae* INA S-1.

RESULTS AND DISCUSSION

The exposure of soil suspensions to EHF radiation in wavelength bands of 4.6–5.8 and 8–11.5 mm was found to diminish the population of unicellular bacteria at certain stages of succession (Fig. 1). In the case of EHF radiation with the wavelengths 4.6–5.8 mm, the maximum decrease in the population of unicellular bacteria was observed on the 14th and 45th days of succession, whereas in the case of EHF radiation with the wavelengths 8–11.5 mm, on the 7th, 14th, and 45th days. The amount of isolated actinomycetes was maximum on the 7th, 14th, and 45th days of succession in the case of EHF radiation with the wavelengths 4.6–5.8 mm and on the 7th day of succession in the case of radiation with the wavelengths 8–11.5 mm (Fig. 2).

Thus, the most appropriate conditions for the selective isolation of actinomycetes from soil are on the 14th and 45th days of succession in the case of radiation with the wavelengths 4.6–5.8 mm and on the 7th day of succession in the case of radiation with the wavelengths 8–11.5 mm.

It should be noted that the effect of EHF radiation was selective with respect to the isolation of rare actinomycete genera, to which all genera of the order *Actinomycetales*, except for the genus *Streptomyces*, are arbitrarily attributed. These genera are called rare genera because their representatives are isolated from soil much more rarely than representatives of the genus *Streptomyces.*

In the case of the unexposed control soil suspension, the number of isolated representatives of rare genera considerably increased on the 7th, 30th, and 45th days of succession (Table 1). On the 7th day of succession, predominant was *Micromonospora*, one of the most frequently encountered and studied rare genera. In addition, on the 30th day of succession, predominant were the rare genera *Saccharothrix, Streptosporangium*, and *Microbispora.* On the 45th day of succession, the isolates were dominated by the rare genera *Saccharothrix, Micromonospora, Actinosynnema*, and *Streptimonospora.*

The exposure of soil suspensions to the wavelengths 4.6–5.8 mm augmented the number of representatives of rare genera isolated on the 0th day of succession by 1.5 times as compared with the control soil suspension (Table 2). In this case, the number of representatives of rare genera isolated on the 30th and 45th days of succession was 1.5 times greater as compared with the 0th day of succession. The rare genera isolated from soil suspensions exposed to EHF radiation at the late stages of succession were represented by *Saccharothrix, Microbispora, Actinosynnema, Actinocorallia* [16],

and *Promicromonospora* (the latter two genera were not isolated from the unexposed soil suspensions at all).

Likewise, the exposure of soil suspensions to the wavelengths 8–11.5 mm increased the number of representatives of rare genera isolated on the 0th day of succession by 1.5 times as compared with the control soil suspension (Table 3). The number of representatives of rare genera isolated on the 7th and 30th days of succession was, respectively, 2 and 1.5 times greater as compared with the control soil suspension. The number of isolates of the rare genus *Microbispora* was maximum on the 7th day of succession, whereas otherwise they were isolated on the 30th day of succession (Table 2). Some cultures isolated at the early stages of succession were found to belong to the rare genera *Actinoplanes*, *Saccharopolyspora*, and *Kibdelosporangium*, which were not isolated in the other experiments.

The data presented indicate that either of the two approaches (succession analysis and EHF irradiation) allows the isolation of rare actinomycete genera from soil; however, the complex method provides for a wider diversity of the isolates. In particular, this method turned out to be advantageous for the isolation of the rare genera *Actinocorallia*, *Promicromonospora*, *Actinoplanes*, and *Kibdelosporangium*. The exposure of soil suspensions to EHF radiation led to the isolation of rare actinomycete genera in earlier periods of succession than when we attempted to isolate them using succession without EHF irradiation. This finding indicates that EHF radiation may promote the germination of actinomycete propagules spontaneously selected at the early stages of succession.

It should be noted that the fraction of antibiotically active representatives of rare actinomycete genera isolated from soil decreases in the course of succession, although the total number of isolates of rare genera increases. The exposure of soil suspensions to EHF radiation, especially in the wavelength band 4.6–5.8 mm, allows the isolation of antibiotically active cultures of rare actinomycete genera throughout the succession period and raises their fraction by 10–20%.

REFERENCES

1. Li, Yu.V., Terekhova, L.P., and Gapochka, M.G., Isolation of Actinomycetes from Soil Using Extremely High-Frequency Radiation, *Mikrobiologiya*, 2002, vol. 71, no. 1, pp. 119–122.
2. Li, Yu.V., Terekhova, L.P., Alferova, I.V., and Gapochka, M.G., The Use of EHF Radiation in Different Wavelength Bands for the Selective Isolation of Actinomycetes from Soil, *Biomed. Radioelektron.*, 2002, no. 5/6, pp. 20–24.
3. Isaeva, V.S., The Effect of EHF Radiation on Microorganisms, *Mezhdunar. simp. "Millimetrovye volny neteplovoi intensivnosti v meditsine"* (Int. Symp. "The Use of Nonthermal Millimeter Waves in Medicine," October 3, 1991), Moscow, 1991, part. 2, pp. 478–482.
4. Tambiev, A.Kh. and Kirikova, N.N., The Effect of EHF Radiation on the Metabolism of the Cyanobacterium *Spirulina platensis* and Other Photosynthesizing Organisms, *Biomed. Radioelektron.*, 1998, no. 3, pp. 17–24.
5. Smolyanskaya, A.Z., The Effect of Millimeter Electromagnetic Waves on Microbial Cells, *Neteplovye effekty millimetrovogo izlucheniya* (The Nonthermal Effects of Millimeter Waves), Devyatkov, N.D., Ed., Moscow: IRE AN SSSR, 1981, pp. 132–146.
6. Zvyagintsev, D.G. and Zenova, G.M., *Ekologiya aktinomitsetov* (The Ecology of Actinomycetes), Moscow: GEOS, 2001, p. 6.
7. Terekhova, L.P., Galatenko, O.A., and Alferova, I.V., The Use of Selective Media for the Isolation of Actinomycetes from Soil, in *Poisk produtsentov antibiotikov sredi aktinomitsetov redkikh rodov* (Search for Antibiotic Producers among Actinomycetes of Rare Genera), Alma-Ata: Gylym, 1990, pp. 5–12.
8. Alferova, I.V., Terekhova, L.P., and Prauzer, Kh.R., A Selective Medium with Nalidixic Medium for the Isolation of Antibiotic-producing Actinomycetes, *Antibiot. Khimioter.*, 1989, no. 5, pp. 344–348.
9. Platonov, A.E., Statistical Analysis in Medicine and Biology: Problems, Terminology, Logic, and Computer Methods, Moscow: Izd. Ross. Akad. Med. Nauk, 2000, pp. 11–16.
10. Plokhinskii, N.A., *Biometriya* (Biometry), Moscow: Mosk. Gos. Univ., 1970.
11. Gauze, G.F., Preobrazhenskaya, T.P., Sveshnikova, M.A., *et al.*, *Opredelitel' aktinomitsetov* (Manual of Actinomycetes), Moscow: Nauka, 1983.
12. Hasegawa, T., Takizawa, M., and Tanida, S., A Rapid Analysis for Chemical Grouping of Aerobic Actinomycetes, *J. Gen. Appl. Microbiol.*, 1983, vol. 29, pp. 319–322.
13. Lechevalier, M.P., Identification of Aerobic Actinomycetes of Clinical Importance, *J. Lab. Clin. Med.*, 1968, vol. 71, pp. 934–944.
14. Staneck, J.L. and Roberts, L.D., Simplified Approach to Identification of Aerobic Actinomycetes by Thin-Layer Chromatography, *Appl. Microbiol. Biotechnol.*, 1974, vol. 28, pp. 226–231.
15. *Bergey's Manual of Systematic Bacteriology*, 9th ed., Holt, J.G. *et al.*, Eds., Baltimore: Williams & Wilkins, 1994.
16. Iinuma, S., Yokota, A., Hasegawa, T., and Kanamaru, T., *Actinocorallia* gen. nov., a New Genus of the Order *Actinomycetales*, *Int. J. Syst. Bacteriol.*, 1994, vol. 44, no. 2, pp. 230–234.