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EXPERIMENTAL ARTICLES

Some Approaches to the Selective Isolation of Actinomycetes of the Genus *Actinomadura* from Soil

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Abstract—Some approaches to the selective isolation of actinomycetes of the genus *Actinomadura* from soil are described. The approach that involves the thermal treatment of soil samples and their plating onto Gauze 1 medium with the antibiotics nystatin, nalidixic acid, and rubomycin provides for an increased amount of actinomaduras isolated from the soil actinomycete complex and for a decreased amount of streptomycetes.

Key words: actinomycetes, *Actinomadura*, isolation of actinomaduras.

Actinomadura is a rare genus of actinomycetes. The first representatives of this genus (*A. madurae* and *A. pelletieri*) were isolated from clinical sources. In the tropical and subtropical regions of Africa and America, actinomaduras are known as the causal agents of human actinomycetoma. The natural habitat of actinomaduras is superficial soil horizons, from which they can get onto human lower limbs and then into wounds [1].

The fraction of actinomaduras in the soil actinomycete complex does not exceed 5% and increases in the zonal direction from soddy podzolic soil to chernozem, light chestnut, and serozem soils [2–4]. Actinomycetes of the genus *Actinomadura* are minor components of the actinomycete complexes of forest, meadow, steppe, and semiarid biogeocenoses [5]. They are most abundant in the F layer of forest floors, in the Ad horizon of meadow biogeocenoses, and in the upper horizons of steppe and semiarid biogeocenoses. This can be explained by the specific nutritional requirements of actinomaduras. The most appropriate conditions for isolation of actinomaduras from chernozem soil take place at the early and late stages of succession initiated by soil wetting to the withering level and at the early stages of succession initiated by soil wetting to the lowest soil moisture-capacity level.

Among the genus *Actinomadura*, there are many producers of antibiotics, vitamins, enzymes, and other valuable biologically active substances [1], which calls for the development of selective approaches which would allow the detection of actinomaduras in soil and the evaluation of their abundance, as well as the isolation of soil actinomaduras for biotechnological purposes.

There are several such approaches (see table). This work was undertaken to select the approaches that limit the growth of soil bacteria and common streptomycetes and thus create appropriate conditions for the isolation of soil actinomaduras.

MATERIALS AND METHODS

Experiments were performed using soil samples taken from the A1 horizon of ordinary chernozem soil under the forb–fescue–feather grass steppe in the Kamennaya Step (Stony Steppe) reserve.

To enumerate soil actinomycetes, soil suspensions were plated onto a medium with sodium propionate [6], as well as Gauze 1 and Gauze 2 media [7]. The media were supplemented with nystatin (50 μg/ml) to inhibit the growth of fungi, nalidixic acid (1.5 and 10 μg/ml) to inhibit the growth of nonmycelial bacteria, and rubomycin (1 μg/ml) or carminomycin (1.5, 3, 5, and 10 μg/ml) to inhibit the growth of streptomycetes. Before plating, soil samples were thermally treated, and soil suspensions were filtered through sterile cotton-wool traps. The thermal treatment included the heating of air-dried soil samples at 120°C for 1 h or the heating of soil suspensions at 60°C for 10 min. The inoculated plates were incubated at 28°C for 2–3 weeks.

Actinomycetes were enumerated based on the morphological characteristics of colonies examined under an optical microscope at a magnification of 400× and isolated in pure cultures using oat agar [7]. The isolated cultures were identified using the identification criteria of *Bergey's Manual* [18]. Cultures were preliminarily assigned to the genus *Streptomyces* if we observed vegetative hyphae from 0.5 to 2.0 μm in diameter forming a highly branched, poorly septate aerial mycelium carrying chains composed of 3–50 nonmotile spores; straight or spiral sporophores were either monopodial or verticillate; and whole-cell hydrolysates contained L,L-diaminopimelic acid (DAPA) and no differentiating sugars.

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Some approaches to the selective isolation of actinomycetes of the genus *Actinomadura* from soil

Approach	Ref.
Freezing–thawing of soil samples	[8]
Differential centrifugation of soil suspensions	[9]
Heating of soil suspensions at 40°C for 20 min and treatment with 2% yeast extract, 0.2% valine, 0.2% humic acid, and SDS solution	[10]
Treatment of soil suspensions with 1% phenol for 45 min or 25% (50%) ethanol for 1 h	[11]
Treatment of soil samples at 15°C with 0.01 N NaOH for 5–10 min	[11]
Heating of soil samples at 110°C for 1 h and treatment of soil suspensions with 1% phenol	[10]
Heating of soil samples at 90–100°C for 5–45 min or 2 h	[12]
Treatment of soil samples with superhigh-frequency fields and electric pulses	[13]
Use of selective nutrient media with streptomycin, rubomycin, bruneomycin, kanamycin, and rifampicin	[14–16]

Cultures were assigned to the genus *Micromonospora* if we observed well-developed, branched substrate mycelia with a diameter of 0.5 μm; single nonmotile spores were either sessile or occurred on sporophores, often bundled; aerial mycelium was scarce, if any, and sterile; and whole-cell hydrolysates contained *meso*-DAPA, xylose, and arabinose.

Cultures were assigned to the genus *Actinomadura* if we observed nonseptate substrate mycelia; scarce aerial mycelia contained short chains of spores having the form of hooks or irregular spirals with one to four coils; the diameter of spores was greater than that of hyphae; and whole-cell hydrolysates contained *meso*-DAPA, madurose, galactose, glucose, mannose, and ribose.

RESULTS AND DISCUSSION

Of the three nutrient media tested (Gauze 1 medium, Gauze 2 medium, and sodium propionate medium), Gauze 1 medium with nystatin and 1.5 μg/ml nalidixic acid turned out to greatly inhibit the growth of fungi and nonmycelial bacteria. Namely, in this case actinomycetes comprised more than 70% of all the detected bacteria.

The actinomycete complex of chernozem soil was dominated by streptomycetes (more than 80% of the total bacteria). The greatest generic diversity was observed when sodium propionate medium and Gauze 1 medium without rubomycin were used. The maximum amount of actinomaduras (8%) was detected when Gauze 2 medium with nystatin, 1.5 μg/ml nalidixic acid, and rubomycin was used (Fig. 1).

Raising the concentration of nalidixic acid to 10 μg/ml decreased the amount of nonmycelial bacteria detected on sodium propionate medium and diminished the generic diversity of actinomycetes detected on Gauze 1 and 2 media. The fraction of actinomaduras isolated in the presence of 10 μg/ml nalidixic acid was as high as 33%, being comparable with the fraction of streptomycetes detected on Gauze 1 medium with rubomycin and nystatin (Fig. 2).

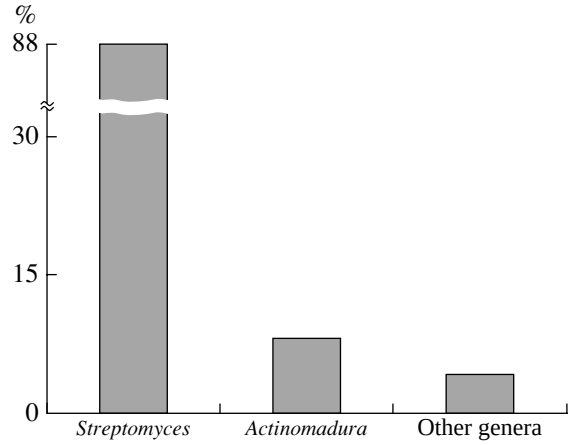

Fig. 1. Proportions between various genera detected in the soil actinomycete complex using Gauze 2 medium with 50 μg/ml nystatin, 1.5 μg/ml nalidixic acid, and 1 μg/ml rubomycin.

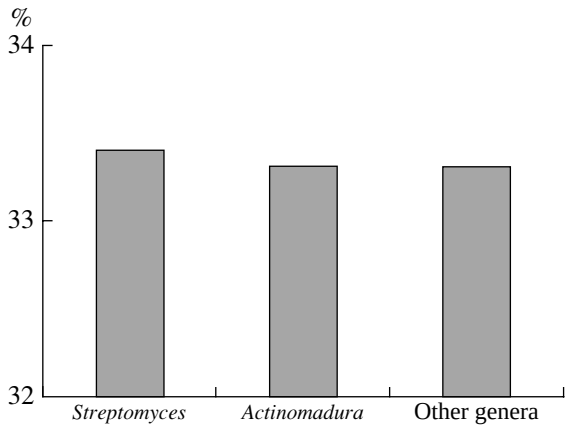

Fig. 2. Proportions between various genera detected in the soil actinomycete complex using Gauze 1 medium with 50 μg/ml nystatin, 10 μg/ml nalidixic acid, and 1 μg/ml rubomycin.

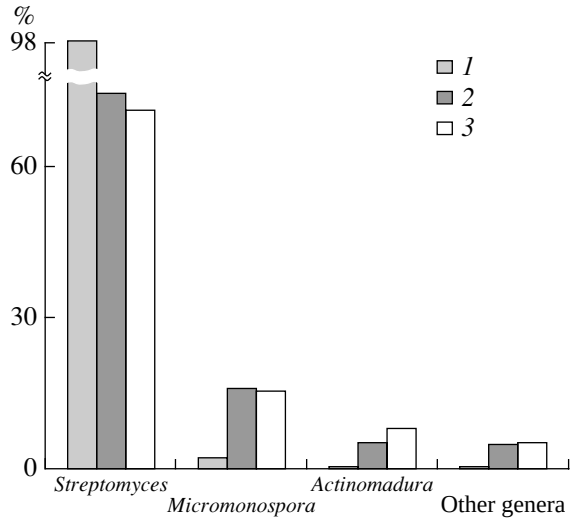

Fig. 3. Proportions between various genera detected in the soil actinomycete complex using Gauze 1 medium with 50 μg/ml nystatin; 10 μg/ml nalidixic acid; and 0, 3, and 10 μg/ml carminomycin (bars *1*, *2*, and *3*, respectively).

The addition of carminomycin at concentrations of 3 and 10 μg/ml to Gauze 1 medium increased the fraction of actinomaduras in the soil actinomycete complex by, respectively, 2 and 6 times (as compared with the control) and decreased the fraction of isolated streptomycetes by 1.3 and 1.2 times. Likewise, the addition of carminomycin at concentrations of 1.5, 5, and 10 μg/ml to sodium propionate medium somewhat increased the fraction of actinomaduras and decreased the fraction of streptomycetes in the soil actinomycete complex (Fig. 3).

Thus, Gauze 1 medium with 50 μg/ml nystatin, 1 μg/ml rubomycin, and 10 μg/ml nalidixic acid provides for the maximum isolation of actinomaduras from chernozem soil. Gauze 1 medium with 50 μg/ml nystatin, 10 μg/ml nalidixic acid, and 10 μg/ml carminomycin is less efficient in this respect.

The maximum amount of actinomycetes (85% of the total soil bacteria) was isolated when heated and filtered soil suspensions were plated onto sodium propionate medium. The heating of soil suspensions also augmented the amount and fraction of the isolated representatives of *Actinomadura*, *Micromonospora*, and other rare actinomycete genera, especially when sodium propionate medium was used. The filtration and subsequent heating of soil suspensions raised the fraction of isolated actinomaduras to 19% (Fig. 4).

The heating of soil samples at 120°C for 1 h turned out to be still more efficient than the heating of soil suspensions. In the former case, the fraction of *Actinomadura* and other rare actinomycete genera isolated on Gauze 1 medium increased to 24 and 15%, respectively, whereas the fraction of isolated streptomycetes decreased to 61% (Fig. 5).

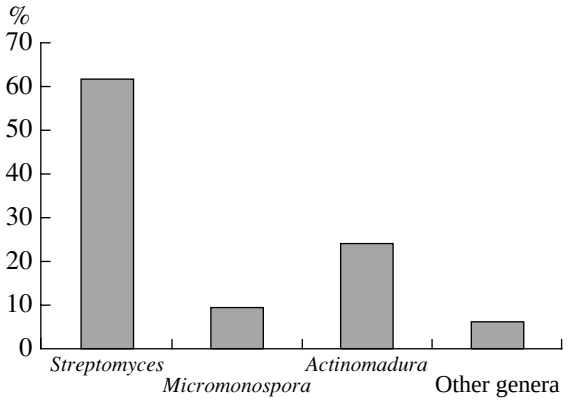

Fig. 4. Proportions between various genera detected in the soil actinomycete complex by plating filtered and heated (60°C, 10 min) soil suspensions onto sodium propionate medium with 50 μg/ml nystatin, 10 μg/ml nalidixic acid, and 1 μg/ml rubomycin.

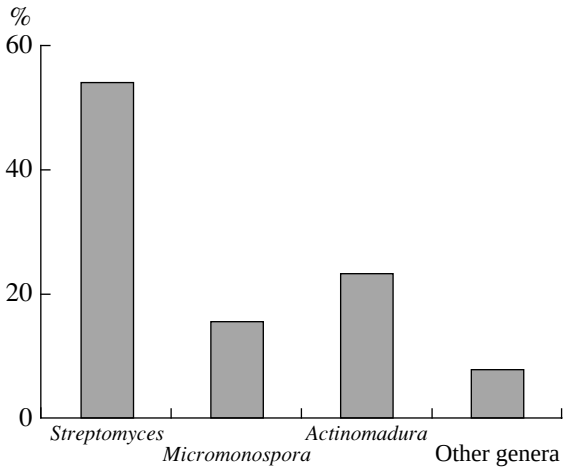

Fig. 5. Proportions between various genera detected in the soil actinomycete complex by plating heated (120°C, 1 h) soil samples onto Gauze 1 medium with 50 μg/ml nystatin and 10 μg/ml nalidixic acid.

Thus, the most appropriate conditions for the isolation of actinomaduras from soil are the heating of soil samples at 120°C for 1 h and their plating onto Gauze 1 medium with 50 μg/ml nystatin, 1 μg/ml rubomycin, and 10 μg/ml nalidixic acid. Such a selective approach raises the amount of actinomaduras isolated from soil samples from 2×10^2 to 8×10^4 CFU/g soil and diminishes the amount of isolated streptomycetes.

At the same time, the most appropriate approach to the evaluation of the fraction of actinomaduras in the soil actinomycete complex is the plating of untreated soil suspensions onto sodium propionate medium with 1 μg/ml rubomycin (or 1.5 μg/ml carminomycin), 1.5 μg/ml nalidixic acid, and 50 μg/ml nystatin. This selective approach augments the fraction of actinomaduras detected in the soil actinomycete complex and

insignificantly affects the fraction of detected soil streptomycetes.

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