Identification and Characteristics of Actinomycetes Useful for Semicontinuous Treatment of Domestic Animal Feces

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Selected strains of actinomycetes useful for practicing semicontinuous treatment of swine and poultry feces were identified as Streptomyces antibioticus S-4, S. punicusus N-50-2, S. nigrifaciens N-9-3, Thermoactinomyces vulgaris HIR-60, and Thermomonospora viridis HIR-50. These five obligately aerobic strains grew preferably on nonsterilized fresh swine feces in 24 h without any additives. They assimilated offensive volatile fatty acids in the swine and poultry feces. Cultures of these five strains were mixed and used as seed for the practical treatments of 1 ton of swine feces over the wide temperature range of 15 to 60°C. Strain HIR-50 grew most predominantly on both fresh swine and poultry feces at 50 to 55°C and decomposed uric acid. For the efficient penetration of mycelia into the feces, manures were mixed once a day so as not to break the solid mass, and the dehydration rate of feces had to be controlled in proportion to the mycelial growth rate. The actinomycete biofertilizer thus manufactured in 10 days was odorless and promotive of plant growth.

Animal wastes were traditionally treated by composting under anaerobic and thermophilic conditions for 2 to 6 months in Japan. The resulting manure created odor nuisance due to volatile fatty acids, amines, ammonia, and hydrogen sulfide. The increase in livestock industry caused a waste disposal problem. Recent interest in the use of organic matter for improvement of soil fertility urged the development of a proper treatment system for a large amount of feces before reuse as plant nutrients.

Previous studies (6, 14) reported that swine or poultry feces were deodorized in practical semicontinuous treatment by using a mixed culture of mesophilic and thermophilic actinomycetes. The semicontinuous treatment of swine feces was carried out in an open-top plastic structure (2 by 30 m) equipped with an automatic moving agitator and provided with air ventilation. One ton of freshly excreted swine feces was mixed with 10 kg of the seed culture, piled up to a depth of 20 to 30 cm on the concrete floor of the plastic house, and incubated. After 10 days of incubation, a matured biofertilizer was produced and the biochemical oxygen demand reached its minimal value. A dry product was obtained because of the increase in fermentation temperature up to 60°C and the action of sunlight and air. A portion (usually 10% and more than 30% in winter) of the finished product was used as seed for the next batch of feces to be treated. The amount of volatile fatty acids, a major source of malodor, was significantly reduced in 24 h.

The objectives of the present study were isolation and identification of coprophilous actinomycetes useful for the semicontinuous treatment of domestic animal feces and the optimization for composting on the basis of their cultural characteristics and physiological properties.

MATERIALS AND METHODS

Preparation of swine and poultry feces extract agar media.

Swine feces (100 g) or poultry feces (400 g) were added to 400 ml of tap water. The mixture was left to stand at room temperature for 10 min and filtered through two layers of gauze. After the addition of 1.5% agar, pH was adjusted to 8.3 with 2 N Na2CO3, and the mixture was sterilized at 121°C for 30 min.

Isolation and selection of actinomycetes for feces treatment. Approximately 1,000 strains of actinomycetes were isolated from the manures collected in the northern part of Kyushu, Japan, and in the suburbs of Bangkok, Thailand, by repeated plating on the swine and poultry feces extract agar medium. The isolates were tested for growth on sliced fresh swine and poultry feces. The selected strains have been applied to the practical treatment of swine or poultry feces for 7 years. Finally, five strains (S-4, N-50-2, N-9-3, HIR-60, and HIR-50) of actinomycetes were reisolated from the treated feces as the predominant strains. These strains were maintained on slants of the swine feces extract agar medium.

Taxonomic studies of actinomycetes. Cultural characteristics and physiological properties of isolates were studied by using media described by Shirling and Gottlieb (13) and Waksman (15). Isolates were grown at 30 or 50°C for 21 days, and results were recorded every 7 days. Utilization of carbon sources was investigated by the method of Pridham and Gottlieb (10). Identification was performed according to the Bergey’s Manual of Determinative Bacteriology (4) and Biseibutsu no Bunrui to Dotei (9).

Uric acid-decomposing activity. Actinomycete strains were grown in uric acid medium (pH 7.0: glucose [5 g], peptone [10 g], yeast extract [10 g], NaCl [0.5 g], uric acid [5 g], tap water [1,000 ml]). Portions of the medium (5 ml) were dispensed into test tubes, autoclaved at 121°C for 20 min, and inoculated with a loopful of spores from the slant cultures. The culture was incubated on a reciprocating shaker at 30 or 50°C for 5 days. The culture was diluted to 100 ml with 1 N NaOH, and the solutions were diluted 10 times with 0.1 M borate buffer (pH 9.5). A293 of the solutions was determined spectrophotometrically (2).

Volatile fatty acid-utilizing activity. The mesophilic strains were grown at 30°C in liquid Waksman medium (glucose [10 g], beef extract [5 g], peptone [5 g], NaCl [5 g], tap water [1,000 ml]) on a rotary shaker for 24 h. The thermophilic strains were grown at 50°C in liquid medium A composed of

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yeast extract (5 g), peptone (1.5 g), NZ amine type A (Sheffield Chemical Co.) (3.5 g), and tap water (1,000 ml) for 36 h. To determine the volatile fatty acid-assimilating activity, oxygen uptake [Q(O$_2$); micromoles of O$_2$ per 100 mg (wet weight) of cells per hour] was measured at 30°C for mesophiles and 50°C for thermophiles with a Warburg manometer (12). Cells were harvested by centrifugation and washed twice with 0.05 M phosphate buffer (pH 8.0). A 1-ml portion of actinomycetes suspension (100 mg of cells per ml) in 0.05 M phosphate buffer (pH 8.0) was added to the main compartment of the dry, clean Warburg flasks equipped with a center well. A 0.1-ml portion of 20% KOH was added to the center well, which contained a filter paper strip. To the side arm, 0.8 ml of 0.05 M phosphate buffer (pH 8.0) and 0.1 ml of 0.1 M substrate solution were added. Substrates used as carbon sources were glucose, acetic acid, propionic acid, butyric acid, valeric acid, caproic acid, and caprylic acid (all substrates were purchased from Ishizu Pharmaceutical Co. Ltd., Japan).

Volatile fatty acids in fresh swine feces, nontreated swine feces (mixture of fresh swine feces and seed culture at the beginning of treatment), and treated swine feces were analyzed by gas-liquid chromatography. A 10-g sample of feces was acidified to pH 1 with 1.0 N HCl, and free volatile fatty acids were extracted with 2.5 ml of CH$_2$Cl$_2$ for 3 h. The extract was dehydrated with anhydrous Na$_2$SO$_4$ and concentrated to 1 ml. Sample solution (2 µl) was analyzed with a Hitachi model 163 gas chromatograph equipped with a flame ionization detector. A glass column (2 m by 3 mm) was packed with 2% diethylene glycol succinate (DEGS) plus 0.5% H$_3$PO$_4$ on Chromosorb W AW. The column was maintained at 80°C with N$_2$ as a carrier gas at a rate of 30 ml/min. Individual fatty acids were identified by comparing their retention times with those of standard fatty acids.

**Determination of treatment conditions.** The batchwise treatment of swine feces was carried out in a plastic house.

(i) **Preparation of seed.** The selected five strains were initially cultivated separately on 50 g of solid wheat bran medium [wheat bran, 50 g; Ca(OH)$_2$, 1 g; swine feces, 10 g; tap water, 40 ml] in a 500-ml Erlenmeyer flask at 30°C for mesophiles or 50°C for thermophiles for 10 days. Wheat bran cultures rich in spores of these actinomycetes were mixed and used as a starter. For preparation of a larger amount of seed culture, a nonsterilized mixture of fresh swine feces (5 kg), dried swine feces (1 kg), and Ca(OH)$_2$ (90 g) was inoculated with 5% (wt/wt) of the wheat bran culture described above (1 x 10$^{12}$ CFU/g) in an aluminum tray (900 by 360 by 60 mm) with a lid and cultivated at 30°C for 10 days.

(ii) **Procedure of treatment.** Freshly excreted swine feces (100 kg) were piled up to a depth of 20 to 30 cm with 10 kg of the seed on the concrete floor of a plastic house and cultivated batchwise. Manures were mixed once a day without aeration.

**Analytical methods.** Moisture content was measured with a moisture meter (Kett Co., Japan). pH was measured with a pH meter (model HM-5B; TOA Electronics Ltd.) in the following way. A 1-g sample was suspended in 5 ml of tap water and left to stand for 10 min at room temperature, and then pH was measured. Room temperature and material temperature of manure were measured with a temperature sensor (model R 005) and recorded with an Electronic Recording Controller (model EH 200; CHINO Co., Japan). Total nitrogen was measured by the Kjeldahl methods (3). Carbon contents were measured by the procedure described in Doiyo Hiyo-gaku Ikken Note (1). Biochemical oxygen demand was determined by the Winkler method (5). The cell number of the actinomycetes was counted by plating on swine feces extract agar medium by the plate dilution method. The cell number of coliform bacteria was counted by plating on deoxycholate agar Nissui medium (Nissui Pharmaceutical Co., Japan). The cell number of anaerobic bacteria was measured with GasPak Anaerobic Systems (BBI Microbiology Systems, Div. Becton Dickinson and Co., Cockeysville, Md.).

**Pot experiments.** To assess the effectiveness of treated feces as fertilizer, pot experiments were carried out in triplicate. Humic volcanic ash soil (pH 4.7) collected at Kuroishi near Kumamoto in Japan was used for pot experiments. The soil was passed through a 16-mesh (1-mm) sieve, and the pH was adjusted to 6.0 with CaCO$_3$. A total of 600 g of soil per pot was supplemented with the treated swine feces, dried swine feces, rapeseed meal, or ammonium sulfate to give nitrogen contents of 0.1, 0.2, 0.4, 0.8, and 1.6 g per pot. Komatsuna, a kind of Chinese mustard (Brassica rapa var. preiviridis) (25 seeds), was placed on the soil surface. Water was supplied every day to keep the 60% field capacity content. The plants were grown at 25 ± 1°C in the glass house of Biotron Institute at Kyushu University. After 45 days, they were harvested and their fresh weights were measured. The pot experiments were repeated eight times by the same procedure. The fertilizer effect was expressed as the average fresh weights of the plant.

**RESULTS**

**Identification of selected actinomycete strains.** (i) **Strain S-4.** Vegetative hyphae produced a well-developed branched mycelium. Mature spore chains showed a curved shape. Sizes of the spores and mycelium were uniform. The spore surface was smooth and gray. Optimal pH and temperature for growth were pH 8.0 and 37°C, respectively. Carbon source utilization was the same as that of Streptomyces antibioticus reported by Fridham and Tresner (11). However, NaCl tolerance was 0 to 13%. On the basis of morphological and cultural characteristics, strain S-4 was considered to belong to the “Rectus Flexibilis, Smooth, Gray, C+” series described by Fridham and Tresner (11) and identified as a strain of S. antibioticus.

(ii) **Strain N-50-2.** A well-developed branched mycelium was produced, and mature spore chains were flexuous. Long spore chains were occasionally observed. Sizes of spores and mycelium were uniform. The spore surface was smooth and yellowish. Carbon source utilization was the same as that of S. puniceus (11), although NaCl tolerance (0 to 10%) was slightly stronger than that of the type strain. Strain N-50-2 was considered to belong to the series “Rectus Flexibilis, Smooth, Yellow, C–.” This actinomycete was identified as a strain of S. puniceus.

(iii) **Strain N-9-3.** Spore chains were short and flexuous. Helical chains were occasionally observed. Spore surface was smooth and gray. NaCl tolerance was 0 to 13%. Strain N-9-3 was considered to belong to the series “Rectus Flexibilis, Smooth, Gray, C–.” From the above results and the carbohydrate utilization tests, this actinomycete was identified as a strain of S. nigrifaciens (11).

(iv) **Strain H1R-60.** Aerial mycelium was chalk-white. Single spores were formed directly on the mycelium or on a short sporophore. The diameters of hyphae were variable. Trunklike mycelium was thick, and branched mycelium was getting thinner at the top. Spore surface was smooth. Soluble pigment was not formed. Growth of this actinomycete occurred at 40 to 60°C and at pH 6.0 to 10.0. Optimal
temperature and pH for growth were 50°C and pH 7.5, respectively. Hydrolysis of starch and nitrate reduction were observed. These results indicated that this thermophilic actinomycete was classified as a strain of *Thermoactinomyces vulgaris* by the method of Küster (8).

(v) **Strain HIR-50.** Cultural characteristics and physiological properties of the most predominant and important strain, HIR-50, are summarized in Tables 1 and 2. Aerial mycelium was dark grayish green. Single spores and occasionally two spores were formed on aerial mycelium. The spore surface was smooth. Trunklike and branched mycelia were also found. Growth of this actinomycete occurred at 40 to 55°C and pH 6.0 to 10.0. The optimal temperature and pH for growth were 50°C and pH 7.5, respectively. Dark-green soluble pigment was produced on both nutrient agar and Waksman agar medium (15). This strain hydrolyzed starch and coagulated milk. These results indicated that this thermophilic actinomycete was a strain of *Thermomonospora viridis* according to Küster (8).

**Other physiological properties.** All five strains were obligately aerobic, because they grew abundantly under aerobic conditions but did not grow under anaerobic conditions (BBL GasPak Anaerobic Systems). The thermophilic strains HIR-50 and HIR-60 hardly grew on the ordinary culture media but grew preferably on the feces extract media. Therefore, they belong to a group of coprophilous microorganisms. All five strains assimilated volatile fatty acids (Table 3). In particular, *Thermomonospora viridis* HIR-50 assimilated n-valeric acid and *Thermoactinomyces vulgaris* HIR-60 assimilated n-butyric acid. However, caprylic acid could not be assimilated as a sole carbon source by any strain. None of these strains was able to produce antibiotics.

### TABLE 1. Cultural characteristics of strain HIR-50

<table>
<thead>
<tr>
<th>Agar medium</th>
<th>Growth of colony</th>
<th>Color of colony</th>
<th>Reverse color</th>
<th>Soluble pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose-nitrate</td>
<td>Scant</td>
<td>Opaque yellow</td>
<td>Colorless</td>
<td>None</td>
</tr>
<tr>
<td>Glucose-asparagine</td>
<td>Scant</td>
<td>Opaque yellow</td>
<td>Colorless</td>
<td>None</td>
</tr>
<tr>
<td>Glycerol-asparagine</td>
<td>Scant</td>
<td>Opaque yellow</td>
<td>Colorless</td>
<td>None</td>
</tr>
<tr>
<td>Inorganic salts-starch</td>
<td>Scant</td>
<td>Opaque yellow</td>
<td>Colorless</td>
<td>None</td>
</tr>
<tr>
<td>Oatmeal</td>
<td>Scant</td>
<td>Translucent</td>
<td>Colorless</td>
<td>None</td>
</tr>
<tr>
<td>Yeast extract-malt extract</td>
<td>Scant</td>
<td>Watery yellow</td>
<td>Colorless</td>
<td>None</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Moderate</td>
<td>Yellowish gray</td>
<td>Colorless</td>
<td>None</td>
</tr>
<tr>
<td>Nutrient</td>
<td>Abundant</td>
<td>Dark grayish green</td>
<td>Yellowish gray</td>
<td>Dark green</td>
</tr>
<tr>
<td>Peptone-yeast extract-iron</td>
<td>Scant</td>
<td>Watery yellow</td>
<td>Colorless</td>
<td>None</td>
</tr>
<tr>
<td>Czapek</td>
<td>Scant</td>
<td>Gray</td>
<td>Colorless</td>
<td>None</td>
</tr>
<tr>
<td>Waksman</td>
<td>Abundant</td>
<td>Dark grayish green</td>
<td>Yellowish brown</td>
<td>Dark green</td>
</tr>
<tr>
<td>Swine feces extract</td>
<td>Abundant</td>
<td>Dark grayish green</td>
<td>ND</td>
<td>Dark green</td>
</tr>
</tbody>
</table>

* ND, Not detected.

### TABLE 2. Physiological properties of strain HIR-50

<table>
<thead>
<tr>
<th>Property</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature range for growth (°C)</td>
<td>40-55</td>
</tr>
<tr>
<td>Optimal temperature for growth (°C)</td>
<td>50</td>
</tr>
<tr>
<td>pH range for growth</td>
<td>6.0-10.0</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>7.5</td>
</tr>
<tr>
<td>Coagulation of milk</td>
<td>Positive</td>
</tr>
<tr>
<td>Gelatin liquefaction</td>
<td>Negative</td>
</tr>
<tr>
<td>Hydrolysis of starch</td>
<td>Negative</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>Negative</td>
</tr>
<tr>
<td>Formation of melanin pigment</td>
<td>Negative</td>
</tr>
<tr>
<td>NaCl tolerance (%)</td>
<td>0-7</td>
</tr>
<tr>
<td>Decomposition of uric acid</td>
<td>Positive</td>
</tr>
<tr>
<td>Growth under anaerobic conditions</td>
<td>Negative</td>
</tr>
</tbody>
</table>

### TABLE 3. Volatile fatty acid-assimilating activity of actinomycetes

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Oxygen uptake [Q(O2)]° by strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S-4</td>
</tr>
<tr>
<td>Glucose</td>
<td>14.3</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>9.8</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>8.9</td>
</tr>
<tr>
<td>n-Butyric acid</td>
<td>9.0</td>
</tr>
<tr>
<td>n-Valeric acid</td>
<td>7.5</td>
</tr>
<tr>
<td>Caprylic acid</td>
<td>3.7</td>
</tr>
<tr>
<td>Caprylic acid</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Q(O2) (microliters of O2 per 100 mg [wet weight] of cells per hour) was measured by a Warburg manometer at 30°C for mesophiles (strains S-4, N-9-3, and N-50-2) and 50°C for thermophiles (strains HIR-50 and HIR-60).

a ND. Not detected.
fatty acids in the feces were completely assimilated by actinomycetes in 24 h (Fig. 2). The white mycelia of actinomycetes penetrated vigorously into feces and matured spores were fully developed in the inner part of the feces after 3 days of incubation in the controlled plastic house. In laboratory experiments, however, mycelia grew only on the surface and were not able to penetrate into feces in an incubator because of high humidity.

Treatment of poultry feces had been very difficult because of the presence of a large amount of uric acid. However, by using these five actinomycetes, treatment of poultry feces was performed properly with respect to pH, moisture content, and cell number of coliform bacteria, as in the treatment of swine feces (Fig. 3). In addition, about 50% of the uric acid contained in poultry feces was decomposed during the treatment.

Maturity of treated swine feces was judged by (i) 40% reduction of the biochemical oxygen demand of the original organic matter, (ii) a carbon-to-nitrogen (C/N) ratio of about 10, and (iii) the smell of soil, or rather actinomycetes, in the treated feces.

**Pot experiments.** When the series of the plant cultures was fertilized with ammonium sulfate, the highest yield was obtained at a nitrogen content of 0.2 g per pot (Fig. 4). Growth of the plants was suppressed by fertilization with larger amounts of ammonium sulfate-nitrogen. In the case of rapeseed meal, maximum plant yield was obtained at a nitrogen content of 0.8 g per pot. The best yield of the plants was obtained with the treated swine feces at a highest

**FIG. 1.** Changes in pH, temperature, and moisture content during microbial treatment of swine feces. Cell numbers (CFU per gram) of thermophilic actinomycetes at 50°C (○), mesophilic actinomycetes at 30°C (△), anaerobic bacteria (▲), and coliform bacteria (□) are indicated. Other symbols: ---O---, biochemical oxygen demand; ---△---, pH; ▼, temperature of manure; ▲, room temperature; □, moisture content; ---X---, organic carbon; ---×---, total nitrogen; □, C/N ratio.

**FIG. 2.** Gas chromatogram of volatile fatty acids in swine feces. (A) Volatile fatty acids in fresh swine feces (attenuation, 16 × 10³); (B) volatile fatty acids in mixture of fresh swine feces and seed culture at the beginning of treatment (attenuation, 4 × 10³); (C) volatile fatty acids in treated swine feces after 48 h (attenuation, 4 × 10³). Peaks: 1, acetic acid; 2, propionic acid; 3, n-butyric acid; 4, isovaleric acid. Volatile fatty acids were separated by a glass column (2 m by 3 mm) of 2% DEGS plus 0.5% H₃PO₄ on Chromosorb W AW at 80°C with an injection temperature of 150°C and a carrier gas (N₂) flow rate of 30 ml/min.

**DISCUSSION**

A previous paper (14) described a novel use of actinomycetes for practical treatment of swine feces. In the present paper, the five strains of actinomycetes were selected and identified. Furthermore, the techniques for growing the actinomycetes fully inside the domestic animal feces were developed. The actinomycetes showed vigorous growth on nonsterilized fresh swine feces without any additives and assimilated the offensive volatile fatty acids as carbon sources. The feces were deodorized very rapidly at an initial moisture content of 65% of the feces at pH 8.0 to 8.5. Under these conditions, the growth of anaerobic bacteria was inhibited. Of the five strains, HIR-50 grew most predominantly on both swine and poultry feces. In the practical treatments, this thermophilic strain played the principal role in composting, because the mycelial growth phase coincided with the increase in temperature of the manure, and the dark-greenish spor set of this strain were often observed in the treated feces.

The composting process was divided into two steps, covering of feces with mycelia and penetration of mycelia into feces. The surface of the feces was initially covered with the mycelia in 30 h under the optimal conditions. Although these conditions were quite satisfactory for growth on the
surface, mycelia of these obligately aerobic actinomycetes could not penetrate into feces. Therefore, we tried a novel approach for allowing the mycelia to penetrate into feces on the basis of the principle of rice koji-making technique in sake brewing (7). During the rice koji-making process, the temperature of steamed rice rises from 35 to 42°C and the moisture content is reduced from 35 to 25% in 40 to 45 h, and then the mold mycelia penetrates into the steamed rice. In a similar way, the dehydration rate of feces was controlled so as to be proportional to the mycelial growth rate, to create the optimally moist and therefore aerobic microenvironment, mycelial growth occurred initially on the limited part of the feces surface and gradually developed on and into the feces with the gradual dehydration. The heterogeneous structure of feces as a solid mass should not be broken by excessive mechanical mixing to prevent destruction of the aerobic microenvironment.

In the pot experiment with Brassica rapa var. pereiridis, the growth of plants fertilized with treated feces was better than that of plants fertilized with air-dried feces. Since organic nitrogen derived from mycelia in treated swine feces was gradually mineralized, supplementation with such large amounts of treated swine feces as 1.6 g of N per 600 g of soil per pot did not show an inhibitory effect on plant growth. On the contrary, in the case of plants fertilized with ammonium sulfate or rapeseed meal, the highest yields were obtained in the plants cultivated at the lower nitrogen contents of 0.2 or 0.8 g of N per pot, respectively. Addition of larger amounts of ammonium sulfate or rapeseed meal inhibited the growth of plants. These data suggested that large amounts of treated feces (C/N ratio, 10) could be used as an excellent actinomycete biofertilizer and soil-improving agent. The treatment of poultry feces with actinomycetes for biofertilizer will be described in later papers.

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LITERATURE CITED