TAXONOMIC CHARACTERS AND CULTURAL CONDITIONS OF A BACTERIAL STRAIN, AT-25, WHICH PRODUCES A GALACTAN SULFATE WITH ANTITHROMBIN ACTIVITY

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Strain AT-25 isolated from a soil sample collected in Japan, showed potent antithrombin activity in its culture filtrate. The strain was a strictly aerobic, gram-positive and pleomorphic rod. The properties of the strain, especially the presence of LL-diaminopimelic acid (LL-DAP), tetrahydrogenated menaquinone with eight isoprene units (MK-8(H₄)), and DNA with 72.9 mol% of guanine plus cytosine, indicate that strain AT-25 is closely related to *Arthrobacter simplex* and *Arthrobacter tumescens*, while these species do not belong to the genus *Arthrobacter* in strict meaning. The unique characteristics of strain AT-25 are requirement of SO₄²⁻ for growth and the production of a galactan sulfate with antithrombin activity. The most suitable medium for the production of galactan sulfate was a fermentation medium consisting of 0.05% Na₂SO₄, 2% glycerol, 0.5% (NH₄)₂HPO₄, 0.1% KH₂PO₄, and 0.1% yeast extract (pH 7.2).

In the course of extensive screening, a bacterium which produced a substance with potent antithrombin activity in the broth was isolated from a soil sample. The substance with high molecular weight was revealed by Inoue, et al. to be a novel galactan sulfate (6, 7).

Sulfated polysaccharides occur in nature widely distributed in higher organisms, seaweeds and sea cucumbers. However, only a few reports have described sulfated polymers from microorganisms: a dermatan sulfate-like polymers from in the capsule of *Clostridium welchii* (1), a sulfated heteroglycan in the cell wall of *Halococcus morrhuae* (13) a glycoprotein containing sulfate ester residues in halobacteria (15).

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Therefore, it is interesting to investigate the microorganism that produces galactan sulfate. In this paper, we report on the taxonomic characters of the bacterium and the cultivation medium for the production of the galactan sulfate.

MATERIALS AND METHODS

Microorganism. A bacterial strain, AT-25, isolated from a soil sample at Edogawa-ku in Tokyo, Japan, was selected while screening for antithrombin activity.

The strain was deposited at The Fermentation Research Institute, where it has been assigned accession number FERM BP-No. 1357.

Medium and cultivation for screening. A basal medium consisting of 2 g glycerol, 0.5 g $(\text{NH}_4)_2\text{HPO}_4$, 0.1 g KH_2PO_4 , 0.05 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g NaCl and 0.1 g yeast extract in 100 ml deionized water, was adjusted pH to 7.0 with NaOH and was used unless otherwise noted. The strain was cultivated in 500-ml flasks containing 100 ml of the medium on a reciprocating shaker at 30°C for 4 to 5 days.

Assay of antithrombin activity. A sample of the culture filtrate was taken out, centrifuged at $2,000 \times g$ for 10 min to remove the cells and insoluble materials, and the antithrombin activity of the supernatant was assayed as follows. The reaction mixture consisted of 280 μl of the supernatant diluted appropriately with 0.1 M NaCl-0.05 M Tris-HCl buffer, with 20 μl (200 milliunits) of topical bovine thrombin added. The formation of the clot was observed with the naked eye at intervals of 15 s to determine the clotting time. The percentage of inhibition was calculated by the following equation:

$$\text{Percentage of inhibition} = (CT_s - CT_0) \times 100 / CT_s,$$

where CT_s and CT_0 are the clotting times with and without a filtrate sample, respectively.

The antithrombin activity of the supernatant was shown as $\text{ID}_{50} \mu\text{l/ml}$ (the 50% inhibition dose).

Taxonomic examination. The morphological, cultural and physiological properties of strain AT-25 were examined according to the methods described by Cowan (4) and the Manual of Microbiological Methods (3). Other taxonomic characterization was based on Bergey's Manual of Determinative Bacteriology, 8th edition (8) and Bergey's Manual of Systematic Bacteriology vol. 2 (9). The molar G+C percentage of DNA was estimated by the thermal denaturation method of Marmur and Doty (11) using a Gilford type 250 spectrophotometer. DNA was prepared according to the method of Marmur (10). DNA from *Micrococcus flavus* IFO 3242 was used as a standard. The isoprenoid quinone was isolated by the method described by Collins et al. (2), and determined with Shimadzu gas chromatograph mass spectrometer system GCMS-QP 1000. The isomer of diaminopimelic acid in the cell wall was determined by the method of Hoare and Work (5). Cell wall was prepared by the method of Work (16).

RESULTS

Taxonomic features of bacterial strain AT-25

1. *Morphological characteristics.* The strain changed cell form after 48 h of culturing when it was grown on yeast extract-malt extract agar at 30°C. The young culture was composed of short rods with rounded ends, $0.5\text{--}0.75 \times 0.7\text{--}1.5\text{ }\mu\text{m}$ and the older culture was composed almost entirely of spherical cells. The optical microscopic photographs of the strain are shown in Fig. 1. The strain was not motile. It was gram-positive, not acid-fast and had no endospore.

2. *Cultural characteristics.* Cultural observations were carried out on various media with the following results:

(1) Colonies on nutrient agar plate (for 3 days at 30°C): Circular, convex, entire type with smooth surface.

(2) Nutrient agar slant (for 3 days at 30°C): Moderate growth, filiform, glistening, pale brownish yellow. No soluble pigment.

(3) Nutrient broth (for 7 days at 30°C): Moderate growth, fluid turbid, sedimentation occurred. No soluble pigment.

(4) Yeast extract-malt extract agar slant (for 3 days at 30°C): Abundant growth, filiform, glistening, brownish yellow. No soluble pigment.

(5) Glucose-bouillon agar stab (for 3 days at 30°C): Abundant growth on the surface only.

(6) Gelatin stab (for 20 days at 22°C): Moderate growth, liquefaction in napiform. No soluble pigment.

(7) Litmus milk (for 14 days at 30°C): Moderate growth, coagulation with a slightly acid reaction to litmus. Reduction.

3. *Physiological and biochemical characteristics.* The physiological, biochemical and chemotaxonomic properties of the strain are summarized in Tables 1–3.

Fig. 1. Photomicrographs of *Arthrobacter* sp. AT-25 grown on yeast extract-malt extract agar slant at 30°C for cultivation times as follows (optical microscopy, stained with crystal violet): A, 8 h; B, 24 h; C, 3 days. Bars indicate 5 μm .

Table 1. Taxonomic characteristics of strain AT-25.

1. Physiological and biochemical characteristics	
OF test	no change
Liquefaction of gelatin	+
Hydrolysis of starch	—
Nitrite formation from nitrate	—
Denitrification	—
Acid formation in litmus milk medium	+
Hydrogen sulfate formation	—
Indole formation	—
Voges-Proskauer test	—
Methyl red test	—
Catalase activity	+
Oxidase test	—
Urease activity	—
Phosphatase activity	—
Assimilation of sodium citrate	—
Tolerance to NaCl	below 7.5%
Temperature range for growth	10°C–37°C
Optimum temperature for growth	25°C–30°C
pH range for growth	6–8.5
Optimum pH for growth	7–7.5
Aerobic	+
2. Chemotaxonomic characteristics	
G + C content of DNA	72.9%
Cell wall contains	
LL-DAP	+
Lysine	—
Glycine	+
Aspartic acid	—
Type of Menaquinone	MK-8 (H ₄)

+, positive; —, negative.

Cultural conditions of the bacterial strain AT-25

The essential components in the medium for the growth and the production of the galactan sulfate was examined.

The results of omission tests of the medium components are shown in Fig. 2. The strain did not grow and produce antithrombin activity in the absence of MgSO₄, and it was found that SO₄²⁻ was essential to produce antithrombin activity, and Mg²⁺ was not, as shown in Table 4. Na₂SO₄ (0.05%) was enough for the strain to grow and produce the galactan sulfate with antithrombin activity.

Strain AT-25 used various ammonium salts or nitrate as sole nitrogen sources in the medium with glycerol as the carbon source, but it did not use ammonium oxalate, as shown in Fig. 3.

Table 2. Cleavage of carbohydrate by strain AT-25.

Carbon source	Acid formation	Gas formation
Arabinose	—	—
Xylose	—	—
Glucose	—	—
Mannose	—	—
Galactose	—	—
Fructose	—	—
Maltose	—	—
Sucrose	—	—
Lactose	—	—
Trehalose	—	—
Sorbitol	—	—
Mannitol	—	—
Inositol	—	—
Glycerol	—	—
Starch	—	—
Dextrin	—	—

+, positive; —, negative.

Table 3. Utilization of carbon source by strain AT-25.

Carbon source	Utilization
Glycerol	+++
Glucose	+++
Galactose	+++
Mannose	+
Fructose	+++
Sucrose	+++
Lactose	++
Sorbitol	—
Mannitol	+++
Starch	—

+++ , good growth; ++ , moderate growth; + , poor growth; — , no growth.

Basal medium: 2% carbon source, 0.1% yeast extract, 0.5% (NH₄)₂HPO₄, 0.1% KH₂PO₄, 0.05% Na₂SO₄, pH 7.2. Cultivation time: 5 days.

DISCUSSION

Strain AT-25 had a rod-coccus growth cycle. The strain was gram-positive, not acid-fast and used glucose in respiration but did not produce acid from glucose and other sugars in the peptone medium, as shown in Table 2. The strain did not form endospore. These results suggest that the strain belongs to the genus *Arthrobacter* based on Bergey's Manual of Determinative Bacteriology, 8th edition (8).

Moreover, the strain contained LL-diaminopimelic acid and glycine but not

Table 4. Effect of Mg^{2+} or SO_4^{2-} on growth and the production of antithrombin activity by strain AT-25.

Addition		Microbial growth (O.D. at 660 nm)	Antithrombin activity ID ₅₀ (μl/ml)
None		0.37	85
MgCl ₂ ·6H ₂ O	0.01%	0.72	90
	0.05%	0.65	75
	0.1%	0.74	75
	0.5%	1.18	85
MgSO ₄ ·7H ₂ O	0.01%	2.29	17
	0.05%	4.60	8.5
	0.1%	5.20	9.0
	0.5%	5.80	12
Na ₂ SO ₄	0.01%	1.76	7.0
	0.05%	3.88	2.6
	0.1%	3.92	2.6
	0.5%	3.84	2.9

Basal medium: 2% glycerol, 0.5% (NH₄)₂HPO₄, 0.1% KH₂PO₄, 0.1% yeast extract and additions (pH 7.2).

Cultivation time: 3 days.

Fig. 2. Omission test of the component of the medium containing 2% glycerol, 0.5% (NH₄)₂HPO₄, 0.1% yeast extract, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O and 0.3% NaCl (pH 7.2).

Microbial growth curves are on the left and changes of the production of antithrombin activity are on the right.

○: control, ●: -(NH₄)₂HPO₄, △: -Yeast extract, ▲: -KH₂PO₄, □: -MgSO₄·7H₂O, ■: -NaCl

Fig. 3. Effect of various nitrogen sources on growth and the production of antithrombin activity by strain AT-25 in medium containing 2% glycerol, 0.1% yeast extract, 0.1% KH₂PO₄, 0.05% Na₂SO₄ and 0.5% nitrogen sources (pH 7.2). Cultivation time: 5 days.

lysine in the cell wall peptidoglycan. The mol% G+C of DNA of the strain was 72.9%. The type of isoprenoid quinone of the cell was mainly tetrahydrogenated menaquinone with eight isoprene units. According to these results, the strain resembles *Arthrobacter simplex* and *Arthrobacter tumescens* among known species of the genus *Arthrobacter* described in Bergey's Manual of Systematic Bacteriology (9). But this does not indicate that strain AT-25 is a typical species of the genus *Arthrobacter* because those species that strain AT-25 resembles show no resemblance to *A. globiformis* which is the type species of the genus *Arthrobacter*. At present, in Bergey's Manual of Systematic Bacteriology, vol. 2 (9), *A. simplex* and *A. tumescens* are included in species incertae sedis of the genus *Arthrobacter* and their taxonomic position remains unresolved. The comparison of strain AT-25 with these species is summarized in Table 5.

O'Donnell et al. proposed (12) that *A. simplex* but not *A. tumescens*, is transferred to the genus *Nocardioides* based on the study of fatty acid, isoprenoid quinone and polar lipid composition of representative strains of the genus *Nocardioides* with *A. simplex* and *A. tumescens*. On the other hand, in 1983 Suzuki and Komagata proposed the new genus *Pimelobacter* to accommodate all the LL-diaminopimelic acid-containing coryneform species (14) and that *A. simplex* and *A. tumescens* are reclassified to *Pimelobacter simplex* and *Pimelobacter tumescens*.

Moreover, they commented (14) that the strains of the genus *Pimelobacter* and the genus *Nocardioides* should be distinguished from each other by the clear difference of the morphological characteristics although these strains had the common components of the peptidoglycan in the cell wall and the type of the major

Table 5. Comparison of strain AT-25 with other related species.

	<i>A. globiformis</i>	<i>A. simplex</i>	<i>A. tumescens</i>	Strain AT-25
Motility	negative, occasionally positive	positive and negative	negative, occasionally positive	negative
Color of colony on nutrient agar	gray-white, occasionally lemon yellow	gray-white	gray-white	pale brownish yellow
Cell wall contains				
LL-DAP	negative	positive	positive	positive
Lysine	positive	negative	negative	negative
Glycine	negative	positive	positive	positive
Menaquinone	MK-9 (H ₂)	MK-8 (H ₄)	MK-8 (H ₄)	MK-8 (H ₄)
G + C content of DNA	62.0–65.5%	71.7%	69.8%	72.9%
Growth at 37°C	negative	positive	occasionally positive	weakly positive
Production of antithrombin activity	weakly positive	negative	weakly positive	positive

menaquinone LL-DAP glycine and MK-(H₄). So they recommended that *A. simplex*, which is a pleomorphic rod and produce no aerial mycelium, should be classified to the genus *Pimelobacter*.

Strain AT-25 is a pleomorphic rod and does not produce aerial mycelium. So strain AT-25 satisfies the concept of the genus *Pimelobacter* rather than the genus *Nocardioidea*. Accordingly, it has been tentatively placed in the genus *Arthrobacter*.

Strain AT-25 is different from the known *Arthrobacter* species in some of its cultural and physiological characteristics. Especially two important characteristic of the strain are as follows: the strain produces insoluble pale brownish yellow pigment and it has the unique properties that the strain requires SO₄²⁻ for growth and produces a galactan sulfate with potent antithrombin activity in the broth.

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