TWO NEW CARBAPENEM ANTIBIOTIC-PRODUCING ACTINOMYCETES: *KITASATOSPORIA PAPULOSA* SP. NOV. AND *KITASATOSPORIA GRISEA* SP. NOV.

YOSHIHIRO NAKAMURA, EIJI ONO, TORU KOHDA and HIROSHIRO SHIBAI

Applied Research Department, Central Research Laboratories, Ajinomoto Co., Inc., 1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210, Japan

(Received for publication May 18, 1988)

Two new carbapenem antibiotic-producing actinomycetes, the cell-walls of which contain LL-diaminopimelic acid and *meso*-diaminopimelic acid, were isolated from soil. The two strains were subjected to taxonomic studies, which involved morphological, cultural, physiological and chemotaxonomical characterization, the latter including the cell-wall chemotype, whole-cell sugar composition, phospholipid composition, menaquinone system and DNA base composition. These strains were identified as new species of the genus *Kitasatosporia*. The proposed names are *Kitasatosporia papulosa* for strain AB-110 (IAM 13637, FERM 9000, JCM 7250) and *Kitasatosporia grisea* for strain AA-107 (IAM 13638, JCM 7249).

The carbapenem antibiotics exhibit potent and broad antibacterial activity, and potent β-lactamase inhibitory activity. They are powerful examples of naturally-occurring β-lactam antibiotics, such as penam (penicillins), cephem (cephalosporins), 7-methoxycephem (cephamycins), 7-formylaminocephem (cephabacin Fs), monocyclic β-lactam (nocardicins, sulfazecins and SQ-26180), clavam (clavulanic acids) and carbapenem (thienamycins, *etc.*) antibiotics.

Many carbapenem antibiotic-producing microorganisms have been isolated. However, they are limited to strains of the genera *Streptomyces*[1~9], *Erwinia*[10] and *Serratia*[10]. No microorganism in any other genus has been reported to be a carbapenem antibiotic producer. Therefore, we tried to isolate carbapenem antibiotic producers from among so-called 'rare actinomycetes', which are actinomycetes other than those of the genus *Streptomyces*.

We performed cell-wall analysis to select 'rare actinomycetes'. Cell-wall components, such as LL-diaminopimelic acid (A_2pm), *meso*-A_2pm, glycine, arabinose, galactose, xylose and madurose, have been used as key criteria in the classification of actinomycetes[11,12]. Among such cell-wall components, the isomer types of A_2pm can be used for the differentiation of 'rare actinomycetes' from the genus *Streptomyces*. The latter together with some other genera (*Streptoverticillium*, *Microellobosporia*, *Sporichthya*, *Nocardioides* and *Erytrosporangium*) contain only LL-A_2pm. However, most other actinomycete-genera contain only *meso*-A_2pm, both LL- and *meso*-A_2pm, or no A_2pm. Therefore, determination of the isomer type of A_2pm is practically useful for the selection of 'rare actinomycetes'.

A search for carbapenem antibiotic-producing 'rare actinomycetes' by our screening program led to the isolation of two new producers, AB-110 and AA-107. The cell-walls of most of the five hundreds or more of carbapenem antibiotic producers found in our screening contained only LL-A_2pm. Therefore, they were considered to belong to the genus *Streptomyces* and thus were not studied further. However, the cell-walls of strains AB-110 and AA-107 contained both LL- and *meso*-A_2pm. This type of carbapenem antibiotic-producing actinomycetes has never been reported previously. Strain AB-110 was found to produce a carbapenem antibiotic, AB-110-D[13], which showed not only potent

and broad antibacterial activity but also potent β-lactamase inhibitory activity. This antibiotic has never been reported as an actinomycete-product.

Several strains of actinomycetes which contain both the LL- and *meso*-isomers of A_2pm have been reported. KAWAMOTO *et al.*[14] found that some strains in the genus *Micromonospora* contain a small amount of LL-A_2pm together with a large amount of *meso*-A_2pm. A same finding was also reported by NELSON *et al.*[15]. ŌMURA *et al.*[16~21] isolated some strains which have the *Streptomyces* morphology but contain both LL- and *meso*-A_2pm in equal amounts. ŌMURA *et al.*[17] proposed a new genus *Kitasatosporia* for their isolates. The genus *Kitasatosporia* is characterized by the morphological resemblance to the genus *Streptomyces* and the cell-wall chemotype X[17] (both LL- and *meso*-A_2pm, glycine and galactose). Recently, TAMAMURA *et al.*[22], SHIMAZU *et al.*[23], INAOKA *et al.*[24] and IWAMI *et al.*[25] also reported some strains belonging to the genus *Kitasatosporia*. TAKAHASHI *et al.*[26] reported a strain belonging to the genus *Nocardiopsis*, which contains a small amount of LL-A_2pm together with a large amount of *meso*-A_2pm.

In this paper we describe two new species, *Kitasatosporia papulosa* sp. nov. AB-110 and *Kitasatosporia grisea* sp. nov. AA-107, and compare them with those strains containing both LL- and *meso*-A_2pm.

Materials and Methods

Isolation and Selection of Microorganisms from Soil

Strains AB-110 and AA-107 were isolated through heat treatment of soil samples[27], the standard dilution and plating technique, and selective isolation for carbapenem antibiotic-producing actinomycetes[28]. The A_2pm type was determined by the method of HASEGAWA *et al.*[29], immediately after they were identified as carbapenem antibiotic producers[28]. One colony grown on oatmeal - yeast extract - malt extract (OMYM) agar was hydrolyzed with 0.1 ml of 6 N HCl at 120°C for 15 minutes and then subjected to ascending chromatography on a thin cellulose sheet with a solvent system of methanol - 6 N HCl - pyridine - water (80 : 26 : 4 : 10) for 3 hours. Isomers of A_2pm were detected by means of the ninhydrin reaction. OMYM medium contained of oatmeal 20 g, malt extract 2 g, yeast extract 2 g, glucose 2 g, $CoSO_4 \cdot 7H_2O$ 0.006 g, $ZnSO_4 \cdot 7H_2O$ 0.003 g, $MnSO_4 \cdot 4 \sim 5H_2O$ 0.003 g and $FeSO_4 \cdot 7H_2O$ 0.003 g per 1 liter of distilled water, and was adjusted to pH 7.0.

Strain AB-110 was isolated from a soil sample collected at Mitsuike Park in Yokohama-city, Japan. The air-dried soil was heated at 100°C for 1 hour and then ground into a powder[27]. One ml of a 1,000-fold diluted soil suspension was plated on OMYM medium containing Augmentin (200 μg/ml). Strain AA-107 was isolated from a soil sample collected at Tosyogu-shrine in Nikko-city, Japan. The air-dried soil was heated at 120°C for 1 hour and then ground into a powder. One ml of a 1,000-fold diluted soil suspension was plated on AV medium[27] containing Augmentin (200 μg/ml). Both strains were isolated after incubation of the plate at 28°C for 3 weeks. Among about five hundred carbapenem antibiotic producers found in our laboratories, strains AB-110 and AA-107 were unique in having not only LL-A_2pm but also *meso*-A_2pm in their cell-walls. Stock cultures for the strains studied were grown on an OMYM agar slant and preserved at −85°C.

Identification Methods

Microorganisms Studied

Strains AB-110 and AA-107 were studied taxonomically together with authentic strains, *Kitasatosporia setalba* IFO 14216 (=ATCC 33774, Type strain)[17] (*Kitasatosporia setae*[18]), *Kitasatosporia griseola* IFO 14371 (Type strain)[21] and *Kitasatosporia phosalacinea* IFO 14372 (Type strain)[21], for comparison.

Each microorganism was cultivated in a 500-ml Sakaguchi flask containing 100 ml of SS medium on a reciprocal shaker at 28°C for 3 days. Then the cells were centrifuged and washed three times

with sterile saline. The washed cells were used as the inoculum for various studies. SS medium contained of starch 20 g, glucose 5 g, Soytone (Difco Lab., Michigan, U.S.A.†) 12 g, yeast extract 5 g, KH_2PO_4 1 g, $CoSO_4 \cdot 7H_2O$ 0.006 g, $ZnSO_4 \cdot 7H_2O$ 0.003 g, $MnSO_4 \cdot 4 \sim 5H_2O$ 0.003 g and $FeSO_4 \cdot 7H_2O$ 0.003 g per 1 liter of distilled water, and was adjusted to pH 7.0.

Morphological Characteristics

Morphological characteristics were examined for cultures grown at 28°C for 2~4 weeks on oatmeal agar (International Streptomyces Project (ISP) medium 3) and inorganic salts - starch agar (ISP medium 4). Examination was made with a photomicroscope (Optiphoto model with an ELWD lens; Nikon Co., Ltd., Japan.)

The spore surface was investigated under a scanning electron microscope (SEM; JSM-T20 type; Japan Electron Optics Laboratory Co., Ltd., Japan). The specimen for the SEM was prepared as follows. An agar block on which a microorganism grew was fixed with formalin gas, dehydrated through graded ethanol series, transferred into isoamyl acetate, dried by a critical point dryer (HCP-1 type; Hitachi Seisakusho Co., Ltd., Japan) and finally coated with gold by an ion coater (SC-701 type; Sanyu Densi Co., Ltd., Japan).

Cultural and Physiological Characteristics

Cultural and physiological characteristics were examined by the methods reported by SHIRLING and GOTTLIEB[30], and WAKSMAN[31] as follows.

The media used were Tryptone - yeast extract broth (ISP medium 1)[30], yeast extract - malt extract agar (ISP medium 2)[30], oatmeal agar (ISP medium 3)[30], inorganic salts - starch agar (ISP medium 4)[30], glycerol - asparagine agar (ISP medium 5)[30], peptone - yeast extract - iron agar (ISP medium 6)[30], tyrosine agar (ISP medium 7)[30], nitrate broth (ISP medium 8)[30] and Pridham and Gottlieb medium (ISP medium 9)[30] (Difco Lab.†). Sucrose - nitrate agar[31]), glucose - asparagine agar[31], nutrient agar, gelatin agar[31] and litmus milk[31] were also used. Growth ranges as to temperature and pH, and the tolerance range as to NaCl were determined in yeast extract - malt extract agar (ISP medium 2). The utilization of carbon sources was examined by growth on Pridham and Gottlieb medium (ISP medium 9) containing 1% of each carbon source.

Each medium was seeded with washed cells as mentioned above, followed by incubation at 28°C for 2~4 weeks.

Colors were determined by comparison with the color plates in the 'Methuen Handbook of Colour'[32].

Chemical Analyses of Whole-Cells and a Cell-wall Preparation

A microorganism was cultivated in a 500-ml Sakaguchi flask containing 100 ml of SS medium on a reciprocal shaker at 28°C for 3 days. The cells were collected by centrifugation, washed with sterile saline and then used as whole-cells. Cell-walls were prepared according to the method of YAMAGUCHI[33]. Amino acids and sugars in whole-cells and the cell-wall preparation were determined as described by BECKER *et al.*[34] The relative quantities of LL- and *meso*-A_2pm were determined with a TLC scanner (CS-910 model; Shimazu Seisakusho Co., Ltd., Japan) at 410 nm.

The following four kinds of cells were prepared for analyses of A_2pm according to the method of TAKAHASHI *et al.*[20]; submerged spores and filamentous mycelia in a liquid-culture of SS medium, and aerial spores and substrate mycelia in an agar-culture of inorganic salts - starch agar medium (grown at 28°C for 2 weeks.)

Analyses of the Phospholipid and Quinone Compositions

Cells, cultivated with shaking in SS medium, were collected by centrifugation, washed with sterile saline and then freeze-dried. Lipids were extracted from the freeze-dried cells with a chloroform - methanol (2:1) mixture. Phospholipids were purified and identified by the method of LECHEVALIER *et al.*[35]. Quinones were purified and identified by the method reported by TAMAOKA *et al.*[36].

† Difco Manual 10th Ed., Difco Laboratories, Michigan, U.S.A., 1983.

Analysis of the DNA Base Composition

A microorganism was cultivated in a 500-ml Sakaguchi flask containing 100 ml of SS medium supplemented with 0.5% of glycine[37] on a reciprocal shaker at 28°C for 3 days, and then the cells were collected by centrifugation. DNA was extracted from the cells and purified by the phenol method of SAITO and MIURA[38]. The guanine plus cytosine content (G+C content) of DNA was determined by the method of TAMAOKA and KOMAGATA[39]. DNA from *Escherichia coli* K-12 was used as a reference. Its G+C content was presumed to be 51.4%.

Susceptibility to Antibiotics

The susceptibility of strains AB-110 and AA-107 to antibiotics was examined by placing susceptibility discs (Showa discs; Showa Yakuhin Kako Co., Ltd., Japan) on the agar surface of assay plates. The assay plates consisted of 30 ml of yeast extract - malt extract agar (ISP medium 2) and 1 ml of each seed culture in a plastic petri dish (75×225 mm; Eiken Kagaku Ltd., Japan). The susceptibility discs were impregnated with benzylpenicillin (20 U), cephalothin (30 μg), streptomycin (50 μg), tetracycline (200 μg), erythromycin (50 μg), fosfomycin (200 μg) or chloramphenicol (100 μg). After incubation for 2 days at 28°C, the diameters of the inhibition zones were determined.

Results and Discussion

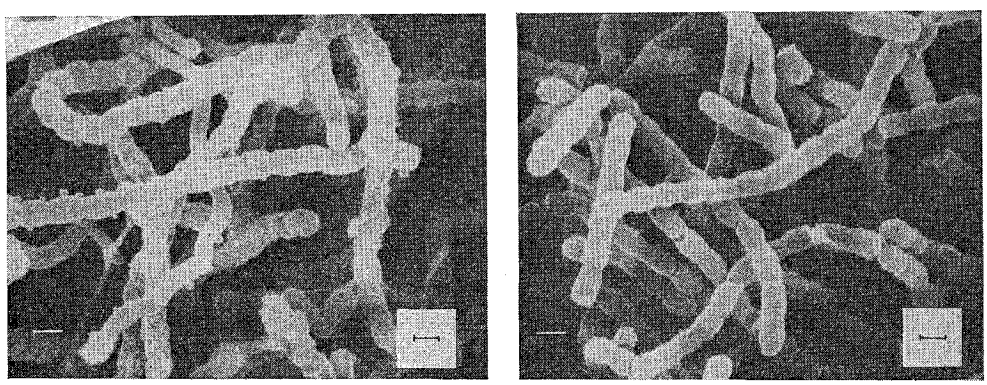
Morphological Characteristics

Strains AB-110 and AA-107 showed almost the same morphological characteristics, except for the spore surface. Long aerial mycelia developed from substrate mycelia. The structure of the aerial mycelia was of the *Rectus-Flexibilis* type. The mature spore chains comprised more than 20 spores each. The spores were cylindrical and 0.7~0.9×0.8~1.4 μm in size. The substrate mycelia in agar media were not fragmented. No zoospores, sporangia, sclerotia or any other special structures were formed. The spore surface of strain AA-107 was smooth, however, that of strain AB-110 was smooth with some warts, as shown in Fig. 1.

Cultural and Physiological Characteristics

Strain AB-110 grew abundantly on yeast extract - malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), inorganic salts - starch agar (ISP medium 4), peptone - yeast extract - iron agar (ISP medium 6), tyrosine agar (ISP medium 7) and nutrient agar. The color of the aerial mycelia that grew on oatmeal agar (ISP medium 3), inorganic salts - starch agar (ISP medium 4) and glycerol -

Fig. 1. Scanning electron micrographs of aerial spores of strains AB-110 (A) and AA-107 (B).



Cultured on inorganic salts - starch agar media for 3 weeks at 28°C.
Bars represent 1 μm.

Table 1. Cultural characteristics of strains AB-110 and AA-107.

Medium	AB-110	AA-107
Yeast extract - malt extract agar	G: Abundant	G: Abundant
(ISP medium 2)	AM: Gray	AM: Yellowish gray
	SM: Light brown	SM: Olive brown
	SP: None	SP: None
Oatmeal agar	G: Abundant	G: Abundant
(ISP medium 3)	AM: Light gray	AM: Yellowish gray
	SM: Grayish yellow	SM: Olive brown
	SP: None	SP: None
Inorganic salts - starch agar	G: Abundant	G: Abundant
(ISP medium 4)	AM: Gray	AM: Yellowish gray
	SM: Light brown	SM: Grayish green
	SP: None	SP: None
Glycerol - asparagine agar	G: Moderate	G: Poor
(ISP medium 5)	AM: Light gray	AM: Light gray
	SM: Grayish yellow	SM: Grayish green
	SP: None	SP: None
Peptone - yeast extract - iron agar	G: Abundant	G: Abundant
(ISP medium 6)	AM: Gray	AM: Yellowish gray
	SM: Light brown	SM: Grayish green
	SP: None	SP: None
Tyrosine agar	G: Abundant	G: Moderate
(ISP medium 7)	AM: Light gray	AM: Grayish yellow
	SM: Grayish yellow	SM: Olive
	SP: None	SP: None
Sucrose - nitrate agar	G: Moderate	G: Poor
	AM: White	AM: White
	SM: Pearl	SM: White yellow
	SP: None	SP: None
Glucose - asparagine agar	G: Moderate	G: Poor
	AM: Light gray	AM: Grayish yellow
	SM: Grayish yellow	SM: Olive
	SP: None	SP: None
Nutrient agar	G: Abundant	G: Abundant
	AM: None	AM: None
	SM: Dark pearl	SM: Olive
	SP: None	SP: None

G, growth; AM, aerial mycelium; SM, substrate mycelium; SP, soluble pigment.

asparagine agar (ISP medium 5) was light gray to gray. The substrate mycelia were light brown or grayish yellow. A soluble pigment was not formed (Table 1).

Strain AB-110 grew at 14 to 36°C, at pH 5.0 to 9.5, and in medium containing less than 3.5% of NaCl. Starch was hydrolyzed. Gelatin was liquefied. Milk was peptonized but not coagulated. Nitrate was not reduced. Melanin was not produced. Good growth occurred on D-xylose and D-glucose as single carbon sources, moderate growth on L-arabinose, D-fructose, sucrose, *i*-inositol, L-rhamnose, raffinose and D-mannitol, and no growth on cellulose.

Strain AA-107 grew abundantly on yeast extract - malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), inorganic salts - starch agar (ISP medium 4), peptone - yeast extract - iron agar (ISP medium 6) and nutrient agar. The color of the aerial mycelia that grew on oatmeal agar (ISP medium 3), inorganic salts - starch agar (ISP medium 4) and glycerol - aspargine agar (ISP medium 5) was yellowish gray or light gray. The substrate mycelia were olive brown to grayish green. A

soluble pigment was not formed (Table 1).

Strain AA-107 grew at 15 to 38°C, at pH 5.0 to 9.5, and in medium containing less than 4.5% of NaCl. Starch was hydrolyzed. Gelatin was liquefied. Milk was peptonized but not coagulated. Nitrate was not reduced. Melanin was not produced. Good growth occurred on D-xylose and D-glucose as carbon sources, moderate growth on D-fructose, L-rhamnose and D-mannitol, poor growth on L-arabinose, and no growth on sucrose, *i*-inositol, raffinose or cellulose.

Table 2. Menaquinone compositions (%) of strains AB-110 and AA-107.

	AB-110	AA-107
MK-9(H_2)	5.3	3.3
MK-9(H_4)	15.4	9.0
MK-9(H_6)	54.2	58.0
MK-9(H_8)	19.6	21.8
MK-9(H_{10})	0.9	1.0
Others	4.6	6.9

Chemotaxonomical Characteristics

The cell-walls of strains AB-110 and AA-107 in submerged-cultures contained LL- and *meso*-A_2pm, glycine and galactose, therefore, they were considered to have cell-wall chemotype X[17]. Whole-cells contained galactose. The ratio of LL- and *meso*-A_2pm in submerged mycelia was roughly estimated to be 3 : 1. Only LL-A_2pm was detected in submerged spores and aerial spores. However, both isomers seemed to be present in filamentous and substrate mycelia. This may be due to the difficulties in separating spores from mycelia. The phospholipid detected was phosphatidylethanolamine. Hence the phospholipid pattern was of type II[35]. Menaquinones were detected but not ubiquinone. The major menaquinones were MK-9 (H_6) and MK-9 (H_8), as shown in Table 2. The G+C contents of strains AB-110 and AA-107 were 72.0±0.5% and 72.0±0.3%, respectively.

Susceptibility to Antibiotics

Strains AB-110 and AA-107 were resistant to benzylpenicillin (20 U) and cephalothin (30 μg). However, they were sensitive to streptomycin (50 μg), tetracycline (200 μg), erythromycin (50 μg), fosfomycin (200 μg) and chloramphenicol (100 μg); streptomycin (50 μg) produced 49 mm and 45 mm growth inhibition zones for strains AB-110 and AA-107, respectively; tetracycline (200 μg) produced 32 mm and 35 mm zones; erythromycin (50 μg) produced 23 mm and 30 mm zones; fosfomycin (200 μg) produced 20 mm and 25 mm zones; and chloramphenicol (100 μg) produced 34 mm and 30 mm zones.

Identification of Strains AB-110 and AA-107

The morphological characteristics of strains AB-110 and AA-107 resembled those of the genus *Streptomyces*. Long aerial mycelia developed from substrate mycelia. The mature spore chains comprised more than 20 spores each. The substrate mycelia were not fragmented. Sporangia, zoospores and sclerotia were not formed. Some chemotaxonomical characteristics also resembled those of the genus *Streptomyces*. The phospholipid (type II) and major menaquinones (MK-9 (H_6) and MK-9 (H_8)) were the same as those of the genus *Streptomyces*[40~42]. The G+C contents were 72%. Those of *Streptomyces* species are generally distributed between 69 and 76%[42]. However, cell-wall and whole-cell compositions were not the same as those of the genus *Streptomyces*. The cell-walls contained LL-A_2pm, *meso*-A_2pm, glycine and galactose (cell-wall chemotype X), and whole-cells contained galactose. Therefore, these characteristics of strains AB-110 and AA-107 were considered to be those of the genus *Kitasatosporia* proposed by ŌMURA *et al.*[17]. Although strains AB-110 and AA-107 resembled some of *Micromonospora*- and *Nocardiopsis*-strains in having both LL- and *meso*-

A_2pm, the isolates were different from them chemotaxonomically or morphologically. Some strains in the genus of *Micromonospora*[14,15,42] contain LL- and *meso*-A_2pm and glycine in the cell-walls, and contain arabinose and xylose in the whole-cells. *Micromonospora* strains form spores in singly, in pairs or short chains on substrate mycelia. A strain in the genus *Nocardiopsis*[26,42,43] contains LL- and *meso*-A_2pm in the cell-walls, and contains galactose in the whole-cells. It forms fragmented substrate mycelia, spores of irregular lengths and some special structures such as sclerotia or sporangia in aerial mycelia.

Strains reported in the genus *Kitasatosporia* are *K. setae*[16,18,21], *K. griseola*[21] and *K. phosalacinea*[21]. Those have been validly published[44,45]. But other strains[23,25] in the genus *Kitasatosporia* have not yet been validly published.

The three strains, *K. setae* IFO 14216, *K. griseola* IFO 14371 and *K. phosalacinea* IFO 14372, were characterized at the species level on the basis of differences in the G+C content, mass color of aerial and substrate mycelia, color of the soluble pigment, tolerance to NaCl, gelatin liquefaction, milk coagulation, nitrate reduction, utilization of carbon sources and antibiotics produced[17,21]. *K. setae* IFO 14216 has a G+C content of 73.1%. White aerial mycelia and ivory substrate mycelia develop. A yellow maple color soluble pigment is produced. It is tolerant to less than 1.5% NaCl. Gelatin is not liquefied. Milk is peptonized and coagulated. Nitrate is not reduced. L-Arabinose, D-xylose and D-glucose are utilized, but not D-fructose, sucrose, *i*-inositol, L-rhamnose, raffinose, D-mannitol or cellulose. The antibiotic, setamycin, is produced. *K. griseola* IFO 14371 has a G+C content of 66.0%. Gray aerial mycelia and golden olive substrate mycelia develop. A pink soluble pigment is produced. It is tolerant to less than 2.0% NaCl. Gelatin is not liquefied. Milk is peptonized and coagulated. Nitrate is not reduced. L-Arabinose, D-xylose, D-glucose and raffinose are utilized, but not D-fructose, sucrose, *i*-inositol, L-rhamnose, D-mannitol or cellulose. The antibiotic, setamycin, is produced. *K. phosalacinea* IFO 14372 has a G+C content of 66.6%. White aerial mycelia and bamboo color substrate mycelia develop. A light tan soluble pigment is produced. It is tolerant to less than 2.0% NaCl. Gelatin is not liquefied. Milk is peptonized but not coagulated. Nitrate is reduced. L-Arabinose, D-xylose, D-glucose, D-fructose, sucrose, L-rhamnose and raffinose are utilized, but not *i*-inositol, D-mannitol or cellulose. A different antibiotic, phosalacine, is produced.

The results of taxonomic comparison of strains AB-110 and AA-107 with three species of *Kitasatosporia* are summarized in Table 3.

Strains AB-110 and AA-107 have very similar G+C contents to that of *K. setae*, but not to that of *K. griseola* or *K. phosalacinea*. Therefore, the isolates were considered to be related to *K. setae*.

However, strains AB-110 and AA-107, and *K. setae* were found to differ on taxonomic comparison. Strains AB-110 and AA-107 were different from *K. setae* in the ratio of LL- and *meso*-A_2pm in the cell-walls, mass color of aerial and substrate mycelia, soluble pigment produced, tolerance to NaCl, gelatin liquefaction, milk coagulation, utilization of carbon sources and antibiotics produced. According to our results, *K. setae* showed the same characteristics as those described by ŌMURA[16,17,21], except for the ratio of LL- and *meso*-A_2pm contents. The ratio of LL- and *meso*-A_2pm contents in strains AB-110 and AA-107, and *K. setae* were 3 : 1, 3 : 1 and 1 : 3, respectively, although that of *K. setae* was reported to be almost 1 : 1[16,17]. Strains AB-110 and AA-107 developed gray aerial mycelia and brown substrate mycelia. A soluble pigment was not produced. Strains AB-110 and AA-107 were tolerant to less than 3.5% and 4.5% NaCl, respectively: *K. setae* is tolerant to 1.5% NaCl. Gelatin was liquefied.

Table 3. Comparison of *Kitasatosporia papulosa* AB-110 and *Kitasatosporia grisea* AA-107 with *Kitasatosporia setae* IFO 14216, *Kitasatosporia griseola* IFO 14371 and *Kitasatosporia phosalacinea* IFO 14372.

	K. papulosa AB-110	*K. grisea* AA-107	*K. setae*[17,21]	*K. griseola*[21],[a]	*K. phosalacinea*[21],[a]
Aerial mycelium	Long spore chain	Long spore chain	Long spore chain	Long spore chain	Long spore chain
Fragmentation of substrate mycelium	None	None	None	None[21]	None[21]
Zoospores and sporangia	None	None	None	None	None
Spore chain	≧20	≧20	≧20	≧20	≧20
Spore shape	Cylindrical	Cylindrical	Cylindrical	Cylindrical	Cylindrical
Spore surface	Smooth with some warts	Smooth	Smooth	Smooth	Smooth
Aerial mycelium	Gray	Gray	White	Gray	White
Substrate mycelium	Brown	Brown	Ivory	Golden olive	Bamboo
Soluble pigment	None	None	Yellow maple	Pink	Light tan
Temp for growth (°C)	14~36	15~38	15~37	15~37	15~42
pH for growth	5.0~9.5	5.0~9.5	5.5~9.0	5.5~9.0	5.5~9.0
NaCl tolerance (%)	3.5>	4.5>	1.5>	2.0>	2.0>
Starch hydrolysis	+	+	+	+	+
Gelatin liquefaction	+	+	−	−	−
Milk peptonization	+	+	+	+	+
Milk coagulation	−	−	+	+	−
Nitrate reduction	−	−	−	−	+
Melanin formation	−	−	−	−	−
Utilization of carbon sources:					
L-Arabinose	+	+	+	+	+
D-Xylose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Fructose	+	+	−	−	+
Sucrose	+	−	−	−	+
i-Inositol	+	−	−	−	−
L-Rhamnose	+	+	−	−	+
Raffinose	+	−	−	+	+
D-Mannitol	+	+	−	−	−
Cellulose	−	−	−	−	−
Cell-wall	Type X	Type X	Type X	Type X	Type X
Ratio of LL- and *meso*-A_2pm in cell-walls	3:1	3:1	1:3 (reported to be 1:1[17])	1:1	1:1
Phospholipid	Type II	Type II	Type II[21]	Type II	Type II
Quinone	MK-9 (H_6) MK-9 (H_8)	MK-9 (H_6) MK-9 (H_8)	No data	No data	No data
GC content (%)	72.0	72.0	73.1[21]	66.0	66.6
Antibiotic produced	Carbapenems	Carbapenems	Setamycin[17]	Setamycin	Phosalacine

[a] All data from ref 21.

Milk was peptonized but not coagulated. In addition to L-arabinose, D-xylose and D-glucose utilized by *K. setae*, D-fructose, sucrose, *i*-inositol, L-rhamnose, raffinose and D-mannitol were utilized by strain AB-110, and D-fructose, L-rhamnose and D-mannitol by strain AA-107. Carbapenem antibiotics were produced. Furthermore, strain AB-110 was different from strain AA-107 in spore surface, tolerance to NaCl and utilization of carbon sources. Strain AB-110 developed smooth spores with some warts.

It was tolerant to less than 3.5% NaCl, not 4.5%. In addition to carbon sources utillized by strain AA-107, sucrose, *i*-inositol and raffinose were utilized by strain AB-110.

Considering the separation of the three approved species in the genus *Kitasatosporia*[17,20], it is concluded that strains AB-110 and AA-107 are new species of *Kitasatosporia*, respectively. The proposed name for strain AB-110 is *K. papulosa* sp. nov., due to the warts on its spore surface, and that for strain AA-107 is *K. grisea* sp. nov., due to the gray color of its aerial mycelia.

Description

Descriptions of the two new species are given below.

Kitasatosporia papulosa sp. nov. (L. adj. papulosa, warty)

Morphology: Long aerial mycelia develop from substrate mycelia. The structure of aerial mycelia is of the *Rectus-Flexibilis* type. The mature spore chains comprise more than 20 spores each. The spores are cylindrical and $0.7\sim0.9\times1.0\sim1.2$ μm in size. The spore surface is smooth with some warts. The substrate mycelia in agar media are not fragmented. Zoospores and sporangia are not formed.

Color of Colonies: Aerial mycelia are gray on yeast extract - malt extract agar, oatmeal agar and inorganic salts - starch agar. Substrate mycelia are brown. A soluble pigment is not produced.

Growth Temperature: $14\sim36$°C.

Growth pH: pH 5.0~9.5.

Tolerance to NaCl: Less than 3.5%.

Physiological Characteristics: Starch is hydrolyzed. Gelatin is liquefied. Milk is peptonized but not coagulated. Nitrate is not reduced. Melanin is not formed. L-Arabinose, D-xylose, D-glucose, D-fructose, sucrose, *i*-inositol, L-rhamnose, raffinose and D-mannitol are utilized for growth, but not cellulose.

Antibiotic Susceptibility: Resistant to β-lactam antibiotics. Sensitive to streptomycin, tetracycline, erythromycin, fosfomycin and chloramphenicol.

Cell-wall Composition and Whole-cell-sugar Composition: LL-A_2pm, *meso*-A_2pm, glycine and galactose are present in the cell-walls. Galactose is present in the whole-cells.

Phospholipid and Menaquinones: Phosphatidylethanolamine is present. The major menaquinones are MK-9 (H_6) and MK-9 (H_8).

G+C Content: 72.0%.

Antagonistic Product: The type strain produces carbapenem antibiotics.

Habitat: This organism may be distributed in soil. Only a single strain is known.

Type Strain: The type strain is strain AB-110, which was isolated from soil in Yokohama-city, Japan. A culture of this strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the accession number, FERM 9000, at the Institute of Applied Microbiology, University of Tokyo, Japan, with the accession number, IAM 13637, and at the Japan Collection of Microorganisms, RIKEN, Japan, with the accession number, JCM 7250.

Kitasatosporia grisea sp. nov. (M.L. adj. grisea, gray)

Morphology: Long aerial mycelia develop from substrate mycelia. The structure of aerial mycelia is of the *Rectus-Flexibilis* type. The mature spore chains comprise more than 20 spores each. The spores are cylindrical and $0.7\sim0.9\times1.0\sim1.2$ μm in size. The spore surface is smooth. The substrate mycelia in agar media are not fragmented. Zoospores and sporangia are not formed.

Color of Colonies: Aerial mycelia are gray on yeast extract - malt extract agar, oatmeal agar and inorganic salts - starch agar. Substrate mycelia are brown. A soluble pigment is not produced.

Growth Temperature: 15~38°C.

Growth pH: pH 5.0~9.0.

Tolerance to NaCl: Less than 4.5%.

Physiological Characteristics: Starch is hydrolyzed. Gelatin is liquefied. Milk is peptonized but not coagulated. Nitrate is not reduced. Melanin is not formed. L-Arabinose, D-xylose, D-glucose, D-fructose, L-rhamnose and D-mannitol are utilized for growth, but not sucrose, *i*-inositol, raffinose or cellulose.

Antibiotic Susceptibility: Resistant to β-lactam antibiotics. Sensitive to streptomycin, tetracycline, erythromycin, fosfomycin and chloramphenicol.

Cell-wall Composition and Whole-cell-sugar Composition: LL- and *meso*-A_2pm, glycine and galactose are present in the cell-walls. Galactose is present in the whole-cells.

Phospholipid and Menaquinones: Phosphatidylethanolamine is present. The major menaquinones are MK-9 (H_6) and MK-9 (H_8).

G+C Content: 72.0%.

Antagonistic Product: The type strain produces carbapenem antibiotics.

Habitat: This organism may be distributed in soil. Only a single strain is known.

Type Strain: The type strain is strain AA-107, which was isolated from soil in Nikko-city, Japan. A culture of this strain has been deposited at the Institute of Applied Microbiology, University of Tokyo, Japan, with the accession number, IAM 13638, and at the Japan Collection of Microorganisms, RIKEN, Japan, with the accession number, JCM 7249.

Acknowledgment

The authors wish to thank Dr. K. KOMAGATA of the Institute of Applied Microbiology, The University of Tokyo, for his kind advice and encouragement. They are also wish to thank Dr. A. HIRAISHI of the above mentioned institute for determination of the menaquinone system and the DNA base composition.

References

1) KAHAN, J. S.; F. M. KAHAN, R. GOEGELMAN, S. A. CURRIE, M. JACKSON, E. O. STAPLEY, T. W. MILLER, A. K. MILLER, D. HENDLIN, S. MOCHALES, S. HERNANDEZ, H. B. WOODRUFF & J. BIRNBAUM: Thienamycin, a new β-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. J. Antibiotics 32: 1~12, 1979
2) BOX, S. J.; J. D. HOOD & S. R. SPEAR: Four further antibiotics related to olivanic acid produced by *Streptomyces olivaceus*: Fermentation, isolation, characterization and biosynthetic studies. J. Antibiotics 32: 1239~1247, 1979
3) STAPLEY, E. O.; P. J. CASSIDY, J. TUNAC, R. L. MONAGHAN, M. JACKSON, S. HERNANDEZ, S. B. ZIMMERMAN, J. M. MATA, S. A. CURRIE, D. DAOUST & D. HENDLIN: Epithienamycins—novel β-lactams related to thienamycin. I. Production and antibacterial activity. J. Antibiotics 34: 628~636, 1981
4) OKAMURA, K.; S. HIRATA, A. KOKI, K. HORI, N. SHIBAMOTO, Y. OKUMURA, M. OKABE, R. OKAMOTO, K. KOUNO, Y. FUKAGAWA, Y. SHIMAUCHI, T. ISHIKURA & J. LEIN: PS-5, a new β-lactam antibiotic. I. Taxonomy of the producing organism, isolation and physico-chemical properties. J. Antibiotics 32: 262~271, 1979
5) IMADA, A.; Y. NOZAKI, Y. KINTAKA, K. OKONOGI, K. KITANO & S. HARADA: C-19393 S_2 and H_2, new carbapenem antibiotics. I. Taxonomy of the producing strain, fermentation and antibacterial properties. J. Antibiotics 33: 1417~1424, 1980
6) KAWAMURA, Y.; Y. YASUDA, M. MAYAMA & K. TANAKA: Asparenomycins A, B and C, new carbapenem antibiotics. I. Taxonomic studies on the producing microorganisms. J. Antibiotics 35: 10~14, 1982

7) NAKAYAMA, M.; A. IWASAKI, S. KIMURA, T. MIZOGUCHI, S. TANABE, A. MURAKAMI, I. WATANABE, M. OKUCHI, H. ITOH, Y. SAINO, F. KOBAYASHI & T. MORI: Carpetimycins A and B, new β-lactam antibiotics. J. Antibiotics 33: 1388~1390, 1980
8) TSUJI, N.; K. NAGASHIMA, M. KOBAYASHI, Y. TERUI, K. MATSUMOTO & E. KONDO: The structures of pluracidomycins, new carbapenem antibiotics. J. Antibiotics 35: 536~540, 1982
9) MIYADOH, S.; T. SHOMURA, T. ITŌ & T. NIIDA: *Streptomyces sulfonofaciens* sp. nov. Int. J. Syst. Bacteriol. 33: 321~324, 1983
10) PARKER, W. L.; M. L. RATHNUM, J. S. WELLS, Jr., W. H. TREJO, P. A. PRINCIPE & R. B. SYKES: SQ 27,860, a simple carbapenem produced by species of *Serratia* and *Erwinia*. J. Antibiotics 35: 653~660, 1982
11) LECHEVALIER, M. P. & H. LECHEVALIER: Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Bacteriol. 20: 435~443, 1970
12) GOTTLIEB, D.: Order I. Actinomycetales Buchanan 1917, 162. *In* BERGEY's Manual of Determinative Bacteriology. 8th Ed., *Eds.*, R. E. BUCHANAN & N. E. GIBBONS, pp. 657~659, Williams & Wilkins Co., Baltimore, 1974
13) NAKAMURA, Y.; K. ISHII, E. ONO, M. ISHIHARA, T. KOHDA, Y. YOKOGAWA & H. SHIBAI: A novel naturally occurring carbapenem antibiotic, AB-110-D, produced by *Kitasatosporia papulosa* novo sp. J. Antibiotics 41: 707~711, 1988
14) KAWAMOTO, I.; T. OKA & T. NARA: Cell wall composition of *Micromonospora olivoasterospora*, *Micromonospora sagamiensis*, and related organisms. J. Bacteriol. 146: 527~534, 1981
15) NELSON, R. A.; J. A. POPE, Jr., G. M. LUEDEMANN, L. E. MCDANIEL & C. P. SCHAFFNER: Crisamicin A, a new antibiotic from *Micromonospora*. I. Taxonomy of the producing strain, fermentation, isolation, physico-chemical characterization and antimicrobial properties. J. Antibiotics 39: 335~344, 1986
16) ŌMURA, S.; Y. IWAI, Y. TAKAHASHI, K. KOJIMA, K. OTOGURO & R. ŌIWA: Type of diaminopimelic acid different in aerial and vegetative mycelia of setamycin-producing actinomycete KM-6054. J. Antibiotics 34: 1633~1643, 1981
17) ŌMURA, S.; Y. TAKAHASHI, Y. IWAI & H. TANAKA: *Kitasatosporia*, a new genus of the order *Actinomycetales*. J. Antibiotics 35: 1013~1019, 1982
18) ŌMURA, S.; Y. TAKAHASHI, Y. IWAI & H. TANAKA: Revised nomenclature of *Kitasatosporia setalba*. Int. J. Syst. Bacteriol. 35: 221, 1985
19) TAKAHASHI, Y.; Y. IWAI & S. ŌMURA: Relationship between cell morphology and the types of diaminopimelic acid in *Kitasatosporia setae*. J. Gen. Appl. Microbiol. 29: 459~465, 1983
20) TAKAHASHI, Y.; T. KUWANO, Y. IWAI & S. ŌMURA: Some characteristics of aerial and submerged spores of *Kitasatosporia setae*. J. Gen. Appl. Microbiol. 30: 223~229, 1984
21) TAKAHASHI, Y.; Y. IWAI & S. ŌMURA: Two new species of the genus *Kitasatosporia*, *Kitasatosporia phosalacinea* sp. nov. and *Kitasatosporia griseola* sp. nov. J. Gen. Appl. Microbiol. 30: 377~387, 1984
22) TAMAMURA, T.; T. SAWA, K. ISSHIKI, T. MASUDA, Y. HOMMA, H. IINUMA, H. NAGANAWA, M. HAMADA, T. TAKEUCHI & H. UMEZAWA: Isolation and characterization of terpentecin, a new antitumor antibiotic. J. Antibiotics 38: 1664~1669, 1985
23) SHIMAZU, A.; K. FURIHATA, H. SETO & N. ŌTAKE: Dimorphism and phylogenic position of the genus *Kitasatosporia*. Actinomycetologica 50: 2~13, 1987
24) INAOKA, Y.; H. TAMAOKI, S. TAKAHASHI, R. ENOKITA & T. OKAZAKI: Propioxatins A and B, new enkephalinase B inhibitors. I. Taxonomy, fermentation, isolation and biological properties. J. Antibiotics 39: 1368~1377, 1986
25) IWAMI, M.; O. NAKAYAMA, H. TERANO, M. KOHSAKA, H. AOKI & H. IMANAKA: A new immunomodulator, FR-900494: Taxonomy, fermentation, isolation and physico-chemical and biological characteristics. J. Antibiotics 40: 612~622, 1987
26) TAKAHASHI, A.; Y. OKAMI & K. HOTTA: Consideration on the taxonomic position of *Nocardiopsis mutabilis*. Actinomycetologist 47: 8~14, 1985
27) NONOMURA, H. & Y. OHARA: Distribution of actinomycetes in soil. (VI) A culture method effective for both preferential isolation and enumeration of *Microbispora* and *Streptosporangium* strains in soil (part 1). J. Ferment. Technol. 47: 463~469, 1969
28) NAKAMURA, Y.; E. ONO, T. KOHDA & H. SHIBAI: Highly targeted screening system for carbapenem antibiotics. J. Antibiotics 42: 73~83, 1989
29) HASEGAWA, T.; M. TAKIZAWA & S. TANIDA: A rapid analysis for chemical grouping of aerobic actinomycetes. J. Gen. Appl. Microbiol. 29: 319~322, 1983
30) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966

31) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Vol. 2. Classification, Identification and Description of Genera and Species. Williams & Wilkins Co., Baltimore, 1961
32) KORNERUP, A. & J. H. WANSCHER (*Ed.*): Methuen Handbook of Colour. 3rd Ed. Eyre Methuen Ltd., London, 1978
33) YAMAGUCHI, T.: Comparison of the cell-wall composition of morphologically distinct actinomycetes. J. Bacteriol. 89: 444~453, 1965
34) BECKER, B.; M. P. LECHEVALIER & H. A. LECHEVALIER: Chemical composition of cell-wall preparations from strains of various form-genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~243, 1965
35) LECHEVALIER, M. P.; C. DE BIEVRE & H. A. LECHEVALIER: Chemotaxonomy of aerobic actinomycetes. Phospholipid composition. Biochem. Syst. Ecol. 5: 249~260, 1977
36) TAMAOKA, J.; Y. KATAYAMA-FUJIMURA & H. KURAISHI: Analysis of bacterial menaquinone mixtures by high performance liquid chromatography. J. Appl. Bacteriol. 54: 31~36, 1983
37) YAMADA, K. & K. KOMAGATA: Taxonomic studies on coryneform bacteria III. DNA base composition of coryneform bacteria. J. Gen. Appl. Microbiol. 16: 215~224, 1970
38) SAITO, H. & K. MIURA: Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochem. Biophys. Acta 72: 619~629, 1963
39) TAMAOKA, J. & K. KOMAGATA: Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol. Lett. 25: 125~128, 1984
40) LECHEVALIER, M. P.; A. E. STERN & H. A. LECHEVALIER: Phospholipids in the taxonomy of actinomycetes. *In* Actinomycetes. *Eds.*, K. P. SHAAL & G. PULVERER, Zbl. Bakt, Suppl. 2. pp. 111~116, Gustav Fisher Verlag, Stuttgart, New York, 1981
41) COLLINS, M. D.; M. FAULKNER & R. M. KEDDIE: Menaquinone composition of sporeforming actinomycetes. System. Appl. Microbiol. 5: 20~29, 1984
42) SEINO, A. (*Ed.*): Housenkin no Dotei Jikkensho (Identification Methods for Actinomycetes) (in Japanese). Nippon Housenkin Kenkyukai, Tokyo, 1985
43) SHEARER, M. C.; P. M. COLMAN & C. H. NASH, III: *Nocardiopsis mutabilis*, a new species of nocardioform bacteria isolated from soil. Int. J. Syst. Bacteriol. 33: 369~374, 1983
44) Validation of the publication of new names and new combinations previously effectively published outside the IJSB. List No. 11. Int. J. Syst. Bacteriol. 33: 672~674, 1983
45) Validation of the publication of new names and new combinations previously effectively published outside the IJSB. List No. 19. Int. J. Syst. Bacteriol. 35: 535, 1985