VOL. 53 NO. 8, AUG. 2000 THE JOURNAL OF ANTIBIOTICS pp. 807 — 815

Friulimicins: Novel Lipopeptide Antibiotics with Peptidoglycan Synthesis Inhibiting Activity from *Actinoplanes friuliensis* sp. nov.

I. Taxonomic Studies of the Producing Microorganism and Fermentation

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(Received for publication November 22, 1999)

A strain that produces new lipopeptide antibiotics is a new species of the genus *Actinoplanes* for which we propose the name *Actinoplanes friuliensis* (type strain: HAG 010964). The strain is an actinoplanete actinomycete having cell wall II composition and forming sporangia. Comparisons with *Actinoplanes* spp. which have similarities with our isolate, including fatty acid analysis, showed that the isolate belongs to a new species. Taxonomic studies and fermentation are presented.

The genus *Actinoplanes* is one of the most important genera among actinomycetes in the production of secondary metabolites. Gardimycin[1] and teicoplanin[2] are two reported antibiotics from the genus *Actinoplanes*. Lipopeptides have been reported from *Actinoplanes nipponensis*[3]. The α-glucosidase inhibitor acarbose is similarly a product of *Actinoplanes* sp.[4].

In our screening program for new antibiotics active against methicillin-resistant *Staphylococcus aureus*, a strain that produced a group of new lipopeptide antibiotics (the structure elucidation will be presented in the following paper) was isolated from a soil sample collected in northern Italy in the region of Friuli. This strain contained *meso*-diaminopimelic acid in its cell wall and xylose was found as characteristic sugar in whole cell hydrolysates. Thus it is a member of the family *Micromonosporaceae* according to the taxonomic proposal of STACKEBRANDT *et al.*[5].

The colony is typically orange and forms globose sporangia, and so it belongs to the genus *Actinoplanes*. Studies of similar *Actinoplanes* species using the methods of the International Streptomycetes Project[6] and chemotaxonomic methods lead us to conclude that our strain is a member of a new species we call *Actinoplanes friuliensis* sp. nov. The strain has been deposited at the German Culture Collection (DSMZ) under number DSM 7358.

Materials and Methods

Isolation

Strain HAG 010964 was isolated from a soil sample collected at the garden entrance of a house in the Friuli Province, Italy on June 3, 1987, using the chemotactic method of PALLERONI[7] and starch-casein-sulfate agar medium recommended by VOBIS[8].

Bacterial Strains

The strains used in this study are shown in Table 1. Other strains than the type strains were *Actinoplanes utahensis* FH 2264 and our new lipopeptide producing strain HAG 010964.

For the detection of the most similar strains to the lipopetide producer the following strains were investgated by their morphological characters like colony formation and shape of sporangia on ISP media: *Actinoplanes auranticolor* ATCC 15330, *A. brasiliensis* ATCC 25844, *A. campanulatus* IMET 9244, *A. consettensis* ATCC 49799,

Table 1. Strains of the genus *Actinoplanes* used for direct comparative studies.

Species	Strain No.	Source
Actinoplanes brasiliensis Thiemann et. al. 1969	FH 2237	ATCC 25844^T
"Actinoplanes nipponensis" Routien 1977 [1]	FH 2241	ATCC 31145^T
Actinoplanes utahensis Couch 1963	FH 2264	NRRL 12052
Actinoplanes friuliensis sp. nov.	HAG 010964	own isolate

^{T} type strain of the species; [1] the species *"A. nipponensis"* has not been validly published according to Rule 27 of the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1992 [21])

A. cyaneus ATCC 21983, *A. deccanensis* ATCC 21983, *A. derwentensis* ATCC 49798, *A. digitatis* ATCC 15349, *A. durhamensis* ATCC 49800, *A. ferrugineus* ATCC 29868, *A. garbadiensis* FH 2243, *A. globisporus* ATCC 23056, *A. humidus* ATCC 49801, *A. ianthinogenes* ATCC 27366, *A. italicus* IFO 13661, *A. kinshahensis* IFO 13997, *A. liguriae* ATCC 31048, *A. lobatus* ATCC 15350, *A. missouriensis* ATCC 14538, *A. nipponensis* ATCC 31145, *A. palleroni* IFO 14961, *A. philippinensis* ATCC 12427, *A. rectilineatus* ATCC 29234, *A. regularis* ATCC 31417, *A. teichomyceticus* ATCC 31121, *A. utahensis* DSM 43147 and NRRL 12052. This date will be presented separately.

Morphology and Physiology

The morphological and physiological characteristics of the strains were observed by using agar cultures on the various media described by SHIRLING and GOTTLIEB[6]: yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract iron agar (ISP 6) and tyrosine agar (ISP 7), incubated for 10 days at 28°C. For scanning electron microscopy the strain was grown on ISP 3 agar. The small agar piece was prepared using the method of WINK *et al.*[9].

Utilization of carbohydrates was investigated on ISP 9 medium (SHIRLING and GOTTLIEB[6]) using a microtiter plate technique with twelve well plates. Sodium chloride tolerance was tested on microtiter plates (six-well) too using a technique based on the method of KUTZNER[10]. A fingerprint of enzymatic activities was obtained with the help of API 20E and API ZYM test strips[11~13].

Antimicrobial Spectrum

For the antimicrobial spectrum we used the strains described by GOODFELLOW in BERGEY's manual[14]. The bacteria were grown on Mueller Hinton agar and the fungi on Czapek Dox. For metabolite production the *Actinoplanes* strains were incubated in a medium containing soluble starch (10.0 g/liter), yeast extract (2.0 g/liter), glucose (10.0 g/liter), glycerol (10.0 g/liter), cornsteep liquor (2.5 g/liter), peptone (2.0 g/liter), NaCl (1.0 g/liter) and $CaCO_3$ (3.0 g/liter) for five days in a shaking flask culture at 28°C. After cultivation the whole culture was extracted with methanol, evaporated and dissolved in water.

Chemotaxonomic Analysis

Analysis of the whole-cell diaminopimelic acid isomers and the sugars was done by the method of HASEGAWA *et al.*[15]. The phospholipids were analyzed by the method of KUTZNER *et al.*[10].

For analysis of whole-cell fatty acid composition we developed a rapid method based on the method of MÜLLER *et al.*[16] and our own database. The strain was incubated in ISP 2 broth for 7 days at 28°C and 1 ml was transferred to the center of a sterile Sartorius filter (SM 11106) placed on an agar medium containing starch (10 g/liter), glucose (10 g/liter), glycerol (10 g/liter), cornsteep liquor (2.5 g/liter), peptone (5 g/liter), yeast extract (2 g/liter), NaCl (1 g/liter) and $CaCO_3$ (3 g/liter). Incubation was carried out for 5 days at 28°C. Two loops of cell material were transferred into 10 μl of distilled water in a microtube, 35 μl of methanolic TMSH were added and the sample was mixed. The sample was dried with a nitrogen stream (Barkey Evaporator) and extracted into 100 μl of a mixture of 9 volumes of *tert*-butyl

methyl ether and *n*-hexane (1 : 1, v/v) and one volume of methanol. The extract was used directly for the GC analysis, which was done with an HP 6890 GC (Hewlett-Packard).

Fermentation

The friulimicins were produced by fermentation in a 50-liter Braun Fermentation stainless steel stirred vessel. Frozen vegetative mycelium of *Actinoplanes friuliensis* was used at 1% to inoculate 100 ml of seed medium in a 300 ml Erlenmeyer flask. The seed medium consisted of sucrose 30 g/liter, KNO_3 2 g/liter, K_2HPO_4 1 g/liter, $MgSO_4 \cdot 7H_2O$ 0.5 g/liter, KCl 0.5 g/liter, $FeSO_4 \cdot 7H_2O$ 0.01 g/liter, yeast extract 2 g/liter and peptone 5 g/liter. The seed flasks were incubated for 120 hours at 30°C on a rotary shaker at 180 rpm. The fermenter was charged with 40 liters of a medium consisting of sucrose 11 g/liter, meat extract 6 g/liter, yeast extract 0.3 g/liter, $MgSO_4 \cdot 7H_2O$ 0.6 g/liter, K_2HPO_4 0.1 g/liter, $FeCl_3 \cdot 6H_2O$ 10 μmol and L-valine 0.6 g/liter. The medium-resin mixture was sterilized in the fermenter at 121°C for 1 hour. The fermenter was inoculated at 1% with the seed flask growth. During fermentation the temperature was controlled at 28°C. The stirring rate was 100 rpm and the air flow rate was 1 v/v/minute. Antifoam (Desmophen) was initially at 0.01%. The fermentation was terminated at 120 hours.

Fermentation Analysis

The fermentation was monitored on-line for changes in pH. The fermenter was sampled daily to evaluate growth and product formation. The formation of the compound was monitored with an HPLC system. The column used was a Nucleosil 120 RP 18 (120×4.6 mm with a 20×4.6 mm precolumn). A gradient with a potassium phosphate buffer (pH 7.0, 10 mM) and acetonitrile was used. The flow rate was between 1.5 and 2 ml/minute. With this system crude mixtures of the compounds could be detected in the culture filtrate. The retention time was between 10 and 20 minutes.

Results

Characteristics of Strain HAG 010964

Vegetative mycelium developed well on the ISP media tested (Table 2). Aerial mycelium was not formed and a red soluble pigment was produced on medium ISP 7. After 10 days on ISP 3, sporangia were formed which show an irregular shape with a smooth surface in the scanning electron micrograph. The surface of the spores was smooth.

The vegetative mycelium was orange on all the ISP media used. All carbohydrates which were tested could be utilized by the strain HAG 010964 (Table 3). The enzymatic activities are shown in Table 4. Apart from lipase (C14) and α-fucosidase, all reactions with API ZYM were positive, and with API 20E no activities were detected for H_2S production, tryptophan deaminase and indole production. Antibacterial activity (Table 5) was detectable only with the starch medium, which is also described as a production medium.

Comparison of strain HAG 010964 with *Actinoplanes brasiliensis* FH 2237 (ATCC 25844), "*A. nipponensis*" FH 2241 (ATCC 31145) and *A. utahensis* FH 2264 (NRRL 12052). After the characterization of all the *Actinoplanes* species which are listed under the point materials and methods the strains *Actinoplanes brasiliensis* FH 2237, "*A. nipponensis*" FH 2241 and *A. utahensis* FH 2264 show most similarities to the strain HAG 010964 basing on data of colony morphology, pigmentation and shape of sporangia, so we used this strains for the comparing studies. Between *A. utahensis* the strain FH 2264 shows more similarity to HAG 010964 than the type strain DSM 43147 and therefore we used FH 2264. The strain *A. brasiliensis* FH 2237 showed yellow-orange substrate mycelium, while the mycelium of the other strains was orange. Also *A. brasiliensis* FH 2237 is the only strain which produceed sparse white mycelium on medium ISP 3. All four strains produced red soluble pigment on ISP 7 but only "*A. nipponensis*" FH 2241 also produced brown one on ISP 6. HAG 010964 and *A. utahensis* FH 2264 utilized all carbohydrates. *A. brasiliensis* FH 2237 showed inferior utilization of xylose, inositol and raffinose, and "*A. nipponensis*" FH 2241 did not utilize xylose, mannitol, fructose and raffinose.

With regard to enzymatic activities, API ZYM and API 20E reacted much more positively with HAG 010964 than with the three other strains. In the API 20E pattern *A. brasiliensis* FH 2237, "*A. nipponensis*" FH 2242 and *A. utahensis* FH 2264 looked very similar and only acetoin production and gelatinase activity could be found in all four strains. *A. brasiliensis* FH 2237 and *A. utahensis* FH 2264 showed no antibacterial activity on any of the tested media. Antibacterial activity against *Bacillus subtilis* was found on all four media for the strain "*A. nipponensis*" FH 2242. Strain HAG 010964 showed activity against *Staphylococcus aureus*, *Micrococcus luteus* and *Bacillus subtilis* only on starch-containing production medium. In the sporangial morphology observed with the scanning electron microscope, strain HAG 010964 showed the most irregular shapes. The sporangia of *A. brasiliensis* FH 2237 are much

Table 2. Colonial characteristics of *Actinoplanes brasiliensis* FH 2237, "*A. nipponensis*" FH 2241, *A. utahensis* FH 2264 and *A. friuliensis* sp. nov. HAG 010964.

Culture medium	Strains FH 2237	FH 2241	FH 2264	HAG 010964
ISP 2	SM yellow-orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none
ISP 3	SM yellow-orange AM white SP none	SM orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none
ISP 4	SM yellow-orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none
ISP 5	SM yellow-orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none	SM orange AM none SP none
ISP 6	SM yellow-orange AM none SP none	SM orange AM none SP brown	SM orange AM none SP none	SM orange AM none SP none
ISP 7	SM yellow-orange AM none SP red	SM orange AM none SP red	SM orange AM none SP red	SM orange AM none SP red

Formation and color of: SM, substrate mycelium; AM, aerial mycelium; SP, soluble exopigment

Table 3. Utilization of carbohydrates by *Actinoplanes brasiliensis* FH 2237, "*A. nipponensis*" FH 2241, *A. utahensis* FH 2264 and *A. friuliensis* sp. nov. HAG 010964.

Carbohydrate	Strains FH 2237	FH 2241	FH 2264	HAG 010964
Glucose	+	+	+	+
Arabinose	+	+	+	+
Sucrose	+	+	+	+
Xylose	(+)	-	+	+
Inositol	(+)	(+)	+	+
Mannitol	+	-	+	+
Fructose	+	-	+	+
Rhamnose	+	+	+	+
Raffinose	(+)	-	+	+

-, growth no better than the negative control (basal medium with water);
(+), growth better than the negative control but not as good as the positive control;
+, growth as good as the positive control (basal medium with glucose).

Table 4. Enzymatic activities of *Actinoplanes brasiliensis* FH 2237, "*A. nipponensis*" FH 2241, *A. utahensis* FH 2264 and *A. friuliensis* sp. nov. HAG 010964.

Physiological parameter	Strains FH 2237	FH 2241	FH 2264	HAG 010964
API ZYM				
Alkaline phosphatase	+	+	+	+
Esterase (C 4)	+	+	+	+
Esterase (C 8)	-	+	+	+
Lipase (C 14)	+	-	-	-
Leucine arylamidase	-	+	+	+
Valine arylamidase	-	-	-	+
Cystine arylamidase	+	-	-	+
Trypsin	+	-	-	+
Chymotrypsin	+	-	+	+
Phosphatase acid	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	-	+	+
α-Galactosidase	+	-	+	+
β-Galactosidase	-	-	+	+
β-Glucuronidase	+	-	-	+
α-Glucosidase	+	+	+	+
β-Glucosidase	+	+	+	+
N-Acetyl-β-glucoseamidase	+	+	+	+
α-Mannosidase	+	-	-	+
α-Fucosidase	-	-	-	-
API 20E				
β-Galactosidase	-	-	-	+
Arginine dihydrolase	-	-	-	+
Lysine decarboxylase	-	-	-	+
Ornithine decarboxylase	-	-	-	+
Citrate utilization	+	-	-	+
H_2S production	-	-	-	-
Urease	-	-	-	+
Tryptophan deaminase	-	-	-	-
Indole production	-	-	-	-
Acetoin production	+	+	+	+
Gelatinase	+	+	+	+

more compact and regular and similar to the sporangia of *A. utahensis* FH 2264 as shown in Fig. 1. For "*A. nipponensis*" FH 2242, production of sporangia was undetectable. In all four *Actinoplanes* strains *meso*-diaminopimelic acid was found in whole cell extracts as well as xylose as characteristic sugar. The phospholipide type of all strains was PII containing phosphatidyl ethanolamine. Main fatty acids of all four strains were 15 : 0 Iso, 15 : 0 Anteiso, 16 : 0 Iso, 17 : 0 Anteiso, 17 : 1 Cis 9, 17 : 0, 18 : 1 Cis 9 and 18 : 0.

Fermentation

Beside a suitable C-source and phosphate the wild type strain needs a N-source like meat extract or soya peptone and magnesium ions seems to be necessary for a good production of the lipopeptides. In contrast to magnesium calcium salts at 10 mM decreased the lipopeptide production to 15%. The strain does not exhibit a strong phosphate or ammonia inhibition. Amino acids like L-valine, L-leucine and L-isoleucine or the corresponding keto-acids or acids, which are precursor substances of the fatty acid biosynthesis, lead to an increased production of the desired components of the lipopeptide complex (see

Table 5. Antimicrobial activities from *Actinoplanes brasiliensis* FH 2237, "*A. nipponensis*" FH 2241, *A. utahensis* FH 2264 and *A. friuliensis* sp. nov. HAG 010964.

Culture broth	*Actinoplanes brasiliensis* FH 2237				*Actinoplanes nipponensis* FH 2241			
	Soy meal	ISP 2	Starch	ISP 3	Soy meal	ISP 2	Starch	ISP 3
Staphylococcus aureus	0	0	0	0	0	11	0	0
Escherichia coli	0	0	0	0	0	0	0	0
Micrococcus luteus	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0
Streptomyces murinus	0	0	0	0	0	0	0	0
Bacillus subtilis	0	0	0	0	21	21	15	15
Candida albicans	0	0	0	0	0	0	0	0
Saccharomyces cerevisiae	0	0	0	0	0	0	0	0
Aspergillus niger	0	0	0	0	0	0	0	0
Culture broth	*Actinoplanes utahensis* FH 2264				*Actinoplanes friuliensis* HAG 010964			
	Soy meal	ISP 2	Starch	ISP 3	Soy meal	ISP 2	Starch	ISP 3
Staphylococcus aureus	0	0	0	0	0	0	16	0
Escherichia coli	0	0	0	0	0	0	0	0
Micrococcus luteus	0	0	0	0	0	0	15	0
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0
Streptomyces murinus	0	0	0	0	0	0	0	0
Bacillus subtilis	0	0	0	0	0	0	16	0
Candida albicans	0	0	0	0	0	0	0	8
Saccharomyces cerevisiae	0	0	0	0	0	0	0	0
Aspergillus niger	0	0	0	0	0	0	0	0

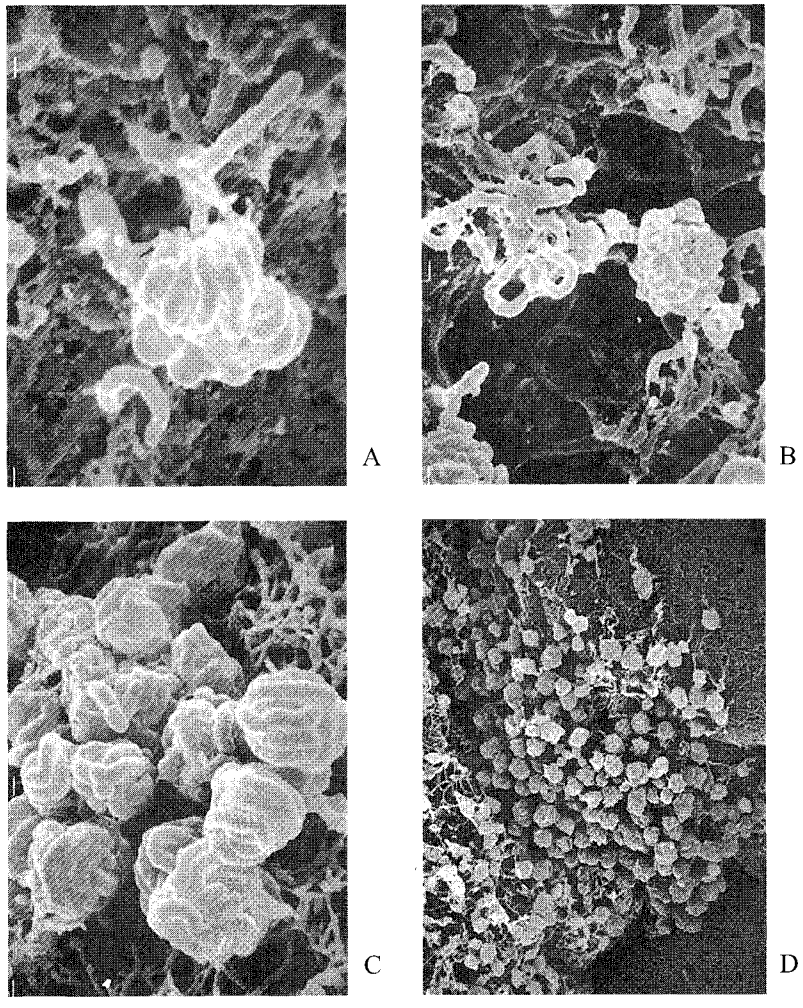
Table 7). Using L-valine as a precursor the compounds B and D are exclusively produced. Using L-leucine the compounds A and C and by using L-isoleucine the compounds E, F, G and H are produced. With this results it is possible to produce the compounds B and D in a yield of 1.5 g/liter in the medium which has been described above. The time course of the production of this two compounds of the friulimicin/amphomycin complex is shown in Fig 2.

Discussion

On the base of chemotaxonomic and morphological properties, strain HAG 010964 can be classified as a member of the genus *Actinoplanes*. Strains of the genus *Actinoplanes* present cell wall chemotype II according to the classification of LECHEVALLIER and LECHEVALLIER[17], with *meso*- and/or 3-hydroxydiaminopimelic acid and glycine, in combination with xylose and arabinose as characteristic sugar in the hydrolysate of whole organisms, and glycolyl type according to UCHIDA and AIDA[18]; phospholipid type PII according to the classification of LECHEVALLIER[19]; fatty acid type 2 c with characteristic *iso*- and *anteiso*-branched fatty acids according to KROPPENSTEDT[20]. Mycolic acid is absent. In strain HAG 010964 we found *meso*-diaminopimelic acid and xylose. The phospholipid type of the strain is PII and the *iso*- and *anteiso*-branched fatty acids are characteristic. A significant cultural marker is the orange substrate mycelium and the absence of aerial mycelium, combined with the production of sporangia on the surface of the colony. Each sporangium contains numerous globose to subglobose spores, which become flagellated in aqueous habitats. In addition the sporangial development which could be studied in SEM showed the branching and septa formation before spore formation indicating affiliation to the genus *Actinoplanes* (VOBIS[8]). The irregular arrangement of the spore chains excludes the former *Ampullariella* species, which have strict parallel rows of spore chains and distinct rod-shaped sporangiospores. Thus only "*A. nipponensis*", *A.*

Fig. 1. Scanning electron micrograph of sporangia formation of the different *Actinoplanes* species.

A) strain HAG 010964 grown on ISP 3 agar for 10 days at 28°C (magnification ×10,000).
B) strain HAG 010964 grown on ISP 3 agar for 14 days at 28°C (magnification ×5,000).
C) strain *A. brasiliensis* FH 2237 grown on ISP 3 agar for 14 days at 28°C (magnification ×5,000).
D) strain *A. utahensis* FH 2264 grown on ISP 3 agar for 14 days at 28°C (magnification ×1,500).



brasiliensis and *A. utahensis* showed very similar colony morphology and pigmentation. In carbohydrate utilization and enzymatic activities strain HAG 010964 shows a lot of differences to *A. brasiliensis* FH 2237, "*A. nipponensis*" FH 2241 and *A. utahensis* FH 2264.

In this group of species closely related to HAG 010964 the species *A. utahensis* and *A. brasiliensis* form sporangia with a regular globose shape which is different from that of HAG 010964. In difference to *A. brasiliensis*, *A. nipponensis* and *A. utahensis* the strain HAG 010964 contain 15 : 1 IsoG and 16 : 1 IsoG as typical fatty acids but has no 16 : 0 acid. All these studies and results as well as the selective fermentation of new lipopetide compounds which have not been described from *Actinoplanes* lead us to conclude, that HAG 010964 is a new species, which we named *Actinoplanes friuliensis* sp.nov.

Description of *Actinoplanes friuliensis* sp. nov.: Produces orange to yellow orange substrate mycelium on the different ISP media. Aerial mycelium is not formed. A red soluble pigment is only produced on medium ISP 7. Melanoid pigment is not produced. Glucose, arabinose, sucrose, xylose, inositol, mannitol, fructose, rhamnose and raffinose could be utilized. Sporangia are formed on the different ISP media. The shape of sporangia is irregular to globose. Cells contain *meso*-diaminopimelic acid and xylose. Characteristic phospholipid is phosphatidyl

Table 6. Fatty acid pattern of *Actinoplanes brasiliensis* FH 2237, "*A. nipponensis*" FH 2241, *A. utahensis* FH 2264 and *A. friuliensis* sp. nov. HAG 010964.

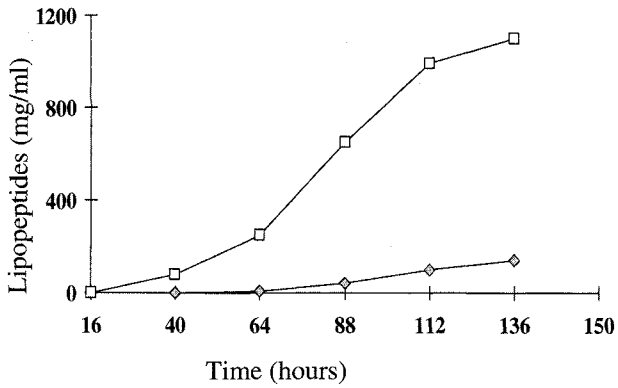
Fatty Acid	Strains FH 2237	FH 2241	FH 2264	HAG 010964
14 : 0 Iso	3.0	4.0	-	2.7
15 : 1 Iso G	-	-	-	5.5
15 : 0 Iso	13.0	9.5	3.6	15.4
15 : 0 Anteiso	18.5	15.5	1.0	12.0
16 : 1 Iso G	-	-	-	3.5
16 : 0 Iso	17.3	21.4	8.0	14.0
16 : 1 Cis 9	3.0	2.2	27.0	4.5
16 : 0	5.0	7.2	22.0	-
17 : 0 Iso	3.4	1.2	6.0	1.8
17 : 0 Anteiso	8.9	8.0	3.0	5.0
17 : 1 Cis 9	4.9	3.0	6.0	10.0
17 : 0	9.4	6.0	3.0	4.5
18 : 1 Cis 9	5.0	9.0	8.0	8.2
18 : 0	6.0	10.0	3.0	3.5

Table 7. Lipopeptides from *Actinoplanes friuliensis* sp. nov. HAG 010964.

Compound A	Amphomycin Type
Compound B	Amphomycin Type
Compound C	Friulimycin A
Compound D	Friulimycin B
Compound E	Amphomycin Type
Compound F	Friulimycin C
Compound G	Amphomycin Type
Compound H	Friulimycin D

Fig. 2. Time course of a selective fermentation in a 20-liter fermenter with additional 10 mmol Val.

-□- peptide B and -◇- peptide D.



ethanolamine. Fatty acids are 15 : 1 IsoG, 15 : 0 Iso, 15 : 0 Anteiso, 16 : 0 Iso, 17 : 0 Iso, 17 : 0 Anteiso, 17 : 1 Cis 9, 17 : 0, 18 : 1 Cis 9 and 18 : 0. Type strain is HAG 010964 (DSM 7358), a antibacterial lipopeptides producing strain.

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