NEW α-AMYLASE INHIBITOR, TRESTATINS

IV. TAXONOMY OF THE PRODUCING STRAINS AND FERMENTATION OF TRESTATIN A

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Taxonomic characteristics of strains NR-320-OM7HB and NR-320-OM7HBS producing a homologous series of new α-amylase inhibitors named trestatins are described, together with the comparison of these strains with similar *Streptomyces* species by DNA-DNA hybridization. A new species, *Streptomyces dimorphogenes* sp. nov. Watanabe and Maruyama is proposed as a result of the studies. The type strain of this species is strain NR-320-OM7HB (ATCC 31484), and the morphovar is strain NR-320-OM7HBS (ATCC 31485). The productivity of trestatin A, major and most active component, using these two strains in flask culture is also presented.

As reported in a previous paper[1], a *Streptomyces* strain (NR-320-OM7HB) was found to produce new α-amylase inhibitors named trestatins. The structures of several components have been determined[2,3]. In the present paper, we describe a new species for which the name *Streptomyces dimorphogenes* nov. sp. is proposed, and discuss the productivity difference of trestatin A in a flask culture.

Materials and Methods

Bacterial Strains

Strain NR-320-OM7HB was isolated in our laboratory from a soil sample collected in 1974 in Chichibu-shi, Saitama Prefecture, Japan. The isolation was carried out on oatmeal agar at 37°C, after heating the soil at 100°C for 1 hour. For direct comparison in physiological, morphological and biochemical properties with our new isolate as well as for DNA-DNA hybridization experiments, the following type strains were used: *Streptomyces griseus* NR 0247 (ISP 5236), *Streptomyces nigrifaciens* NR 0157 (ISP 5071), *Streptomyces olivaceus* NR 0165 (ISP 5072), *Streptomyces plicatus* NR 0183 (ISP 5319).

Cell Wall Analyses

Cell wall analyses were performed by the methods of BECKER *et al.*[4] and LECHEVALIER *et al.*[5].

ISP Methods

Procedures and media recommended for the ISP[6] as well as those by WAKSMAN[7] were used for description. Color names and hue numbers are those of the Color Harmony Manual, 4th ed., 1958 (Container Corporation of America).

DNA-DNA Hybridization by the S1 Nuclease Method

The modification of procedures described by BARTH *et al.*[8] and those by COYKENDALL *et al.*[9] was used. The final method adopted for DNA-DNA hybridization in the streptomycetes is as follows: Each reassociation mixture consisted of 10 μg of sheared unlabeled DNA (average size: *ca.* 300 base pairs) and 20 ng labeled DNA (average size: *ca.* 300 base pairs) that had been prepared by nick translation in 400 μl of 0.42 M NaCl and 20% formamide in a capped tube. The mixture was denatured by boiling for 5 minutes, and then incubated for 40 hours at 65°C (*ca.* $2 \times C_0 t^{1/2}$ time). After incubation, 600 μl of S1 buffer (50 mM sodium acetate, pH 4.8, 1.67 mM $ZnSO_4$, 33 μg of sheared and denatured

salmon sperm DNA per ml), and 5 U of nuclease S1 were added, and the mixture was incubated for 30 minutes at 65°C. The enzyme action was stopped by addition of 1 ml of cold 10% trichloroacetic acid (TCA). After standing in ice for 30 minutes, the precipitated DNA was collected onto a Whatman GF/C filter, rinsed twice with 10 ml of 5% TCA containing 200 mM thymine and once with 2.5 ml of acetone, and then dried for 30 minutes at 65°C before counting in a scientillation cocktail. The detailed process which led to this optimization as well as appropriate methods for preparation of labeled DNA by nick translation will be dealt in a subsequent paper[10].

Additional Tests for Taxonomy

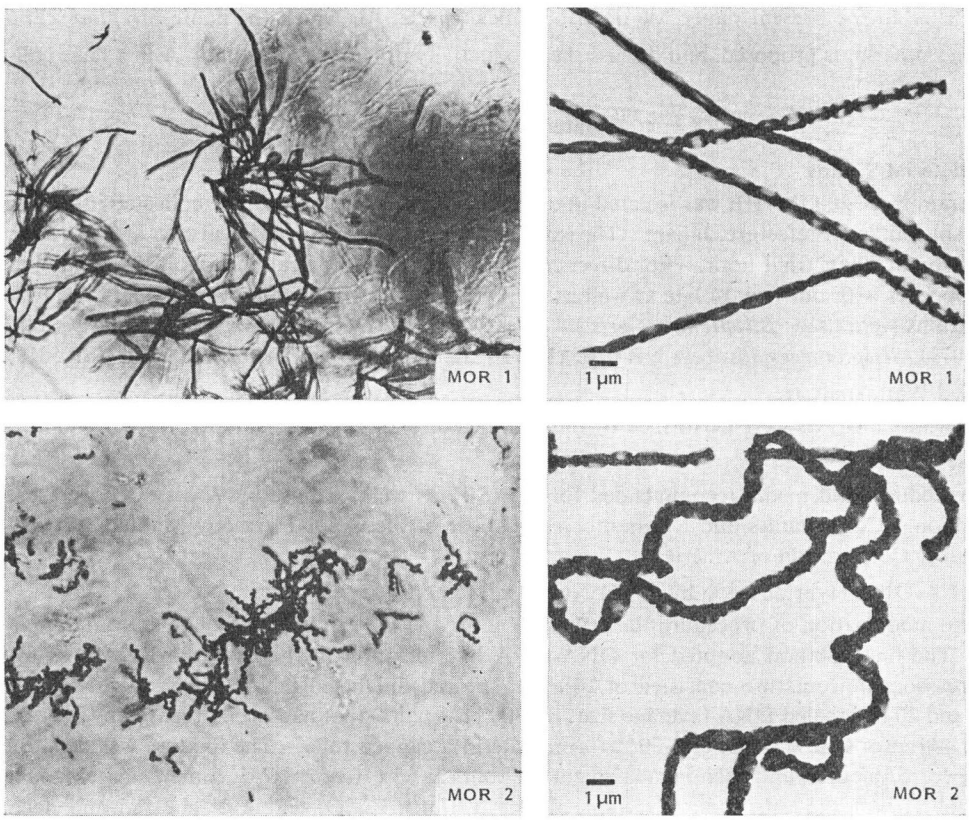
The temperature range for growth and aerial mycelium formation were examined on ISP-3 and -4 media at 10, 15, 20, 27, 37, 40, 45, 52 and 55°C. The heat tolerance of spores suspended in skim milk (10%) was examined in an oil bath at various temperature (95, 100, 105 and 110°C) for 5 minutes. The tolerance to sodium chloride was tested using ISP-1 medium at 27°C for 10 days cultivation with shaking. Antibiotic susceptibility was determined with 3 point susceptibility discs (Eiken Co., Ltd.) placed onto the surface of Trypticase soy agar plates (BBL) seeded with 10% vegetative inoculum.

Results and Discussion

Morphological and Cultural Properties

Strain NR-320-OM7HB formed gray aerial mycelia (Gray color series) with white patches on the well-branched substrate mycelia. Sporangia and flagellated spores were not observed. By microscopic observation, the spore chains of the gray aerial mycelium contained more than 10 spores, and were

Fig. 1. Light micrographs (×320) and electron micrographs of strain NR-320-OM7HB (MOR 1) and NR-320-OM7HBS (MOR 2) on ISP medium 4, 27°C for 14 days.



mainly straight to flexuous (*Rectiflexibiles*) and occasionally spirals (*Spirales*). Therefore, single spore isolation based on the spore chain morphology were made to proceed for selection of spiral forming-morphovar. This was obtained by single spore isolation from the gray part of the colony forming-spirals. Thus, the morphovar designated as NR-320-OM7HBS, was found to maintain spiral spore chain morphology (MOR 2) by forming constantly in a low frequency (less than 2%) straight spore chain morphology (Fig. 1). As can be seen in Table 1, this morphovar is interesting in that it forms morphological variations depending on the cultivation temperature. When a few hundreds spores of strain NR-320-OM7HBS (MOR 2) were plated on ISP-4 medium, and then cultivated for 10 days at 37°C, the progeny showed straight morphology (MOR 1, 10%) and parental morphology (MOR 2, 90%), while at 45°C MOR 1 and MOR 2 consisted of 60% and 40% respectively. Spores of both strains were smooth in surface and ranged 0.4 to 0.6 by 0.8 to 1.2 μm (Fig. 1). Color of aerial mycelia, substrate mycelia, reverse-substrate mycelia and diffusible pigmentation properties of both strains were found to be exactly the same as shown in Table 2.

Table 1. Variation in spore chain morphology of strain NR-320-OM7HB (MOR 1) and strain NR-320-OM7HBS (MOR 2) by cultivation temperatures.

Morphology		Cultivation temperature			
Parent	Progeny	20°C	27°C	37°C	45°C
MOR 1 (RF)	MOR 1 (RF)	98%	98%	99%	100%
	MOR 2 (S)	2	2	1	0
MOR 2 (S)	MOR 1 (RF)	2	2	10	60
	MOR 2 (S)	98	98	90	40

RF: *Rectiflexibiles*.
S: *Spirales*.

Physiological Characteristics

Both strains were aerobic and mesophilic. The optimum temperature for growth and aerial mycelium formation was between 27°C and 37°C; the growth was also fairly good at the temperature range from 15 to 52°C, but there was no growth at 55°C (Table 5). No difference between both strains was recognized in all tests carried out (Table 3). All carbohydrates tested as the sole carbon sources were well utilized.

Cell Wall Composition Analysis

Cell walls of both strains contained LL-diaminopimelic acid and glycine. Arabinose and galactose were not detected in hydrolysates. Based on these results, the cells of the strain were judged to be of Type I of BECKER *et al.*[4].

Identification and Classification

A comparison of the characteristics of strain NR-320-OM7HB and its morphovar with the published descriptions of *Streptomyces* species indicated that the strain resembles *Streptomyces nigrifaciens* Waksman, 1961[7,11,12,13], *Streptomyces olivaceus* Waksman and Henrici, 1948[7,11,12,13] and *Streptomyces plicatus* Pridham, Hesseltine and Benedict, 1958[11,14] in most properties as described above. Direct comparative studies of strain NR-320-OM7HB with the type cultures of the strains cited were undertaken. Results shown in Table 4 indicate that of the three species *S. olivaceus* was most closely related to strain NR-320-OM7HB. However, in the temperature range for growth and aerial mycelium formation, heat tolerance and sodium chloride tolerance of mycelia and antibiotic sensitivity, there were clear differences in physiological characteristics between these two strains as is summarized in Tables 5 and 6.

These distinctions were further supported by a DNA-DNA hybridization experiment[10]. As can be seen in Table 7, the DNA-DNA homology values in reciprocal hybridization between strain NR-

Table 2. Cultural characteristics of strain NR-320-OM7HB and strain NR-320-OM7HBS.

Medium		NR-320-OM7HB (MOR 1)	NR-320-OM7HBS (MOR 2)
Sucrose - nitrate agar	AM	Natural (3dc)	Natural (3dc)
	SM	Light brown (31g)~oak brown (4pi)	Dark luggage tan (4pg)~oak brown (4pi)
	R	Yellow maple (3ng)~dark luggage tan (4pg)	Yellow maple (3ng)~dark luggage tan (4pg)
	DP	Topaz (3ne)	Topaz (3ne)
Glucose asparagine agar	AM	Covert gray (2fe)~silver gray (3fe)	Natural (2dc~3dc)
	SM	Old gold (21e)	Old gold (21e)
	R	Light gold (2ic)~old gold (2le)	Light gold (2ic)
	DP	None	None
Glycerol asparagine agar (ISP medium 5)	AM	Natural (2dc)~covert gray (2fe)	Natural (2dc)~covert gray (2fe)
	SM	Topaz (3ne)~yellow maple (3ng)	Topaz (3ne)~yellow maple (3ng)
	R	Topaz (3ne)	Topaz (3ne)
	DP	Light maize (2ea), slightly	Light maize (2ea), slightly
Inorganic salts - starch agar (ISP medium 4)	AM	Covert gray (2fe)~silver gray (3fe)	Natural (3dc)~covert gray (2fe)
	SM	Yellow maple (3le)~light brown (3lg)	Light amber (3ic)~light brown (3lg)
	R	Camel (3ie)~light brown (3lg)	Light amber (3ic)~light brown (3lg)
	DP	Bamboo (2gc)	Light mustard tan (2ie)
Tyrosin agar (ISP medium 7)	AM	Natural (2dc)~covert gray (2fe)	Natural (2dc)~covert gray (2fe)
	SM	Yellow maple (3ng)	Yellow maple (3ng)~golden brown (3pg)
	R	Yellow maple (3le~3ng)	Yellow maple (3le~3ng)
	DP	Bamboo (2gc), slightly	Bamboo (2ge), slightly
Nutrient agar	AM	None	None
	SM	Colorless~light gold (2ic)	Colorless~light gold (2ic)
	R	Colorless~bamboo (2gc)	Colorless~bamboo (2gc)
	DP	None	None
Yeast - malt extract agar (ISP medium 2)	AM	Covert gray (2fe)~silver gray (3fe)	Oyster white (b)~silver gray (3fe)
	SM	Light brown (31g)	Yellow maple (3ng)
	R	Light brown (31g)~yellow maple (3ng)	Yellow maple (3ng)
	DP	Yellow maple (3le)	Bamboo (2gc), slightly
Oatmeal agar (ISP medium 3)	AM	Covert gray (2fe)~silver gray (3fe)	Covert gray (2fe)~silver gray (3fe)
	SM	Bamboo (2gc)~light mustard tan (2ie)	Bamboo (2gc)~light mustard tan (2ie)
	R	Bamboo (2gc)~light mustard tan (2ie)	Bamboo (2gc)~light mustard tan (2ie)
	DP	Light gold (2ic), slightly	Light gold (2ic), slightly

Abbreviations: AM, aerial mycelium; SM, substrate mycelium; R, reverse-substrate mycelium; DP, diffusible pigment.

Table 3. Physiological properties of strain NR-320-OM7HB and strain NR-320-OM7HBS.

Test	Result	Test	Result
Melanin formation[a]		Utilization of[c]	
on ISP medium 1	(−)	L-Arabinose	++
on ISP medium 6	−	D-Xylose	++
on ISP medium 7	(−)	D-Glucose	++
Starch hydrolysis[b]	+	D-Fructose	++
Gelatin liquefaction	+	Sucrose	++
Nitrate reduction	−	*i*-Inositol	++
Milk coagulation	−	Rhamnose	++
Milk peptonization	+	Raffinose	++
		D-Mannitol	++

[a] −: Not formed, (−): probably not formed.
[b] +: Positive, −: negative.
[c] ++: Good utilization.

Table 4. Comparison of strain NR-320-OM7HB with related species.

Characters	Organisms			
	NR-320-OM7HB (MOR 1)	*S. nigrifaciens* NR 0157	*S. plicatus* NR 0183	*S. olivaceus* NR 0165
Spore chain morphology	RF, S	RF	RA, S	RF, RA, S
Color of aerial mycelium	Covert gray (2fe)~silver gray (3fe)	Sand (3cb)	Pussywillow gray (5dc)	Natural (3dc)~covert gray (2fe)
Color of substrate mycelium	Yellow maple (3le)~light brown (3lg)	Pastel yellow (1fb)	Colorless	Yellow maple (3ng)~clove brown (3ni)
Color of diffusible pigment	Bamboo (2gc)	None	None	None
Melanin formation	−	−	−	−
Starch hydrolysis	+	+	+	+
Gelatin liquefaction	+	+	−	+
Nitrate reduction	−	−	−	−
Milk coagulation	−	+	+	−
Utilization of				
L-Arabinose	++	+	++	++
D-Xylose	++	+	++	+
D-Fructose	++	±	++	+
Sucrose	++	−	−	−
i-Inositol	++	−	++	±
Raffinose	++	−	−	−

RF: *Rectiflexibiles*, S: *Spirales*, RA: *Rectinaculiaperti*.

320-OM7HB and *S. olivaceus* were found to be less than 35%. These degrees of DNA-DNA homology were almost the same as those between strain NR-320-OM7HB and *S. griseus* which was used as a negative control.

Considering this binding homology of strain NR-320-OM7HB DNA to the DNA of *S. olivaceus*, *S. plicatus* and *S. nigrifaciens*, we have concluded that it is suitable to recognize this taxon as a new species in the genus *Streptomyces*. We propose to name it *Streptomyces dimorphogenes* nov. sp. Watanabe and Maruyama. The type strain of this new species, NR-320-OM7HB, was deposited in the American Type Culture Collection as ATCC 31484; strain NR-320-OM7HBS, the morphovar, was deposited as ATCC 31485. The proposed specific epithet "dimorphogenes (di, morpho, genes: Gr.

Table 5. Difference in physiological characteristics between strain NR-320-OM7HB and *S. olivaceus* NR 0165.

Physiological characteristics	Organisms	
	NR-320-OM7HB	NR 0165
Temperature range for growth	15~52°C	15~40°C
Temperature range for aerial mycelium formation[a]		
15°C	(+)	(+)
20°C	+	+
27°C	++	++
37°C	++	+
40°C	++	(+)
45°C	+	No growth
52°C	−	No growth
Heat tolerance[b]		
95°C	+	+
100°C	+	−
105°C	+	−
110°C	+	−
NaCl tolerance[c]		
1%	+	+
5%	+	+
8%	−	+
12%	−	+

[a] (+): Probably formed. +: Formed. ++: Well formed. −: Not formed.
[b,c] +: Growth. −: No growth.

Table 6. Difference in antibiotic susceptibility patterns between strain NR-320-OM7HB and *S. olivaceus* NR 0165.

Antibiotics	Organisms[a]	
	NR-320-OM7HB	NR 0165
Penicillin	++	−
Ampicillin	+++	−
Carbenicillin	+++	−
Cephalexin	+++	−
Oleandomycin	+	+
Erythromycin	+++	++
Lincomycin	+	++
Clindamycin	+	++
Chloramphenicol	+++	++
Thiamphenicol	++	−
Tetracycline	+++	+++
Colistin	++	−
Polymyxin B	+++	+
Kanamycin	+++	+++
Streptomycin	+++	+++
Gentamicin	+++	+++
Fusidic acid	+++	−

[a] +++: Sensitive at the low dose disc.
++: Sensitive at the medium and high dose discs.
+: Sensitive only at the high dose disc.
−: No inhibitory zone at all dose discs.

Table 7. Degree of DNA-DNA homology between strains tested.

Unlabeled DNA	Index DNA					
	MOR 1	MOR 2	NR 0165	NR 0183	NR 0157	NR 0247
S. dimorphogenes NR-320-OM7HB (MOR 1)	100 %	107.4%	33.1%	25.4%	27.0%	22.8%
S. dimorphogenes NR-320-OM7HBS (MOR 2)	93.9	100	27.2	23.2	34.0	20.8
S. olivaceus NR 0165	27.6	21.8	100	42.0	26.6	19.7
S. plicatus NR 0183	30.8	29.7	38.9	100	18.3	18.0
S. nigrifaciens NR 0157	28.1	23.6	40.3	21.3	100	26.3
S. griseus NR 0247	21.8	20.1	16.9	14.3	26.0	100
Rate of homoduplex formation (%)	69.7	62.3	68.5	66.3	69.3	62.9

Hybridization was expressed as a percentage of the isohomologous reassociation value taken as 100.

adj. *di*, dual; Gr. n. *morphe*, form; Gr. v. suff.-*genes*, producing; M. L. adj., two forms producing)" refers to the occurrence of two forms in spore chain morphology, *i.e.* the patchy distribution of spiral forms as a low frequency in the straight spore chains.

Differential Production of Trestatin A (Ro 09-0183) among Two Morphovars

As described in previous papers[1,2], the trestatins complex was composed of trestatin A, B and C as major components. Among these components, trestatin A showed the strongest α-amylase inhibitory activity[1]. We therefore studied the productivity of trestatin A using two trestatins-producing strains,

NR-320-OM7HB (RF) and NR-320-OM7HBS (*Spirales*), which have different spore chain morphology.

Seed cultures for trestatin A production were prepared in 500-ml Erlenmeyer flasks, each containing 100 ml of the medium composed of potato starch 3%, maltose 1%, soybean meal 2%, fresh yeast 0.5%, NaCl 0.25%, $CaCO_3$ 0.32%, $ZnSO_4 \cdot 7H_2O$ 0.005%, $CuSO_4 \cdot 5H_2O$ 0.0005% and $MnCl_2 \cdot 4H_2O$ 0.0005%.

Table 8. Time course of trestatin A production.

Strain	Temperature	Trestatin A (μg/ml)		
		3 day	4 day	5 day
NR-320-OM7HB (MOR 1)	24°C	10	55	190
	27°C	160	170	120
NR-320-OM7HBS (MOR 2)	24°C	20	100	340
	27°C	180	220	255

Spores inoculated into each flask were incubated at 27°C on a rotary shaker for 3 days. Five ml each of the seed cultures was transferred to 500-ml Erlenmeyer flasks containing 100 ml of the same medium and incubated further at given temperatures.

The amount of trestatin A produced was assessed by high pressure liquid chromatography as follows: Column: YMCpak A-312 (6 mm×150 mm; Yamamura Chemical Lab. Co., Ltd.). Carrier: 0.05 M phosphate buffer (pH 7.5) - MeOH (85: 15) with 50 mM LiCl. Flow rate: 2.0 ml/minute. Detection: UV absorption at 210 nm.

Interestingly strain NR-320-OM7HBS, forming *Spirales* spore chain morphology showed a higher productivity of trestatin A than strain NR-320-OM7HB forming RF spore chain at either 24°C or 27°C (Table 8). This difference was reproducibly found in several experiments and furthermore, when monospore selection or nitrosoguanidine mutation were carried out on both morphovars, MOR 2 derived descendants or mutants constantly showed a significantly larger population of high trestatin A producers (Not shown). From these facts, we resume that this property may well be chemotaxonomically significant to MOR 2, even if not intrinsic.

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