

## **Methods for the taxonomic description of the Actinobacteria**

The strains were originally cultivated by media and conditions as described by the DSMZ.

### **1. Colony description and formation of melanoid pigment**

Standard media for the colony description were complex medium 5006 (sucrose 3g/l, dextrin 15,0 g/l, meat extract 1,0 g/l, yeast extract 2,0 g/l, tryptone soy broth (Oxoid) 5,0 g/l, NaCl 0,5 g/l, K<sub>2</sub>HPO<sub>4</sub> 0,5 g/l, MgSO<sub>4</sub> x 7H<sub>2</sub>O 0,5 g/l, FeSO<sub>4</sub> x 7H<sub>2</sub>O 0,01 g/l) and the media described by Shirling and Gottlieb (1966) 5265 (ISP2), 5315 (ISP3 = DSM 609), 5317 (ISP4 = DSM 547), 5323 (ISP5 = DSM 993), 5318 (ISP6) and 5322 (ISP7) – (complete media descriptions on pages 30-42). About 1 ml of the shaking flask culture was plated and the plates were incubated for 10 to 14 days at 28°C or any other temperature which was required by the strain. If special media were required these are described with the strains.

The last two media were also used together with the synthetically suter medium (5337 with and without tyrosine - Kutzner 1986 – glycerol 15,0 g/l, tyrosine 1,0 g/l, L-arginine 5,0 g/l, L-glutamic acid 5,0 g/l, L-methionine 0,3 g/l, L-isoleucine 0,3 g/l, K<sub>2</sub>HPO<sub>4</sub> 0,5 g/l, MgSO<sub>4</sub> x 7H<sub>2</sub>O; 1 ml/l trace element solution – CuSO<sub>4</sub> x 5H<sub>2</sub>O 10,0 g/l, CaCl<sub>2</sub> x 2H<sub>2</sub>O 10,0 g/l, FeSO<sub>4</sub> x 7H<sub>2</sub>O 10,0 g/l, ZnSO<sub>4</sub> x 7H<sub>2</sub>O 10,0 g/l, MnSO<sub>4</sub> x 7 H<sub>2</sub>O 40,0 g/l) for the detection of the melanoid pigment formation.

For a faster determination of the colony characteristics a micro plate technique was established, in which 6-well plates were used. The media and their arrangement on the plate are shown on the next but one page. By incubation temperatures higher than 30°C the plates had to be incubated in a humid chamber to prevent shrinking of the agar.

For the description of the strains four parameters were used:

- Growth (G)

It was differentiated between good growth, sparse growth and no growth (none).

- Reverse color (R)

This is the color of the substrate mycelium, which is often influenced by the formation of a soluble pigment. The color is characterized with the help of the RAL-code (edition of 1990 – Reichsausschuß für Lieferbedingungen – Deutsches Institut für Gütesicherung und Kennzeichnung e.V.). It is often not possible to find the color in the card which is 100% identical with the color of the strain, but this code is an easy key for determination and recognition of the color. In addition to the RAL color a strain could be colorless.

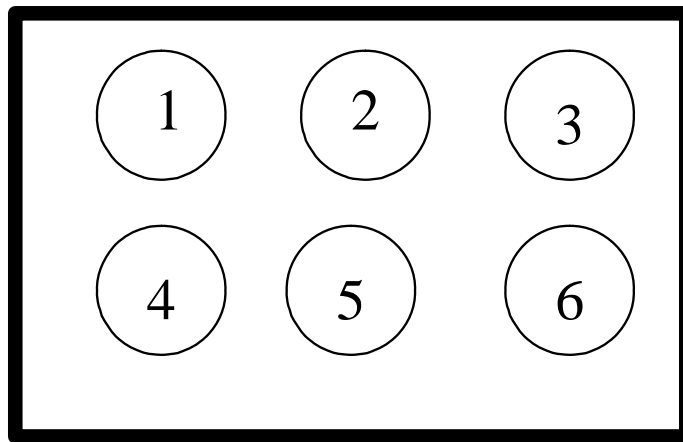
- Aerial Mycelium (A)

If aerial mycelium is formed it is differentiated between a good formation and a sparse formation. When aerial mycelium is well developed the color is described with the help of the RAL color code.

- Soluble Pigment (S)

If a soluble pigment is produced it is also described with the help of the RAL color code.

# Microplate Techniques in Actinomycetes Taxonomy I: growth on ISP-media and melanin formation



Each well of a 6-well plate e.q. Costar (# 25810) will be filled sterile with 5 ml of agar.

Each well is marked by a number for easy identification.

The filling pattern is:

Well 1	=>	agar 5317 => ISP 4
Well 2	=>	agar 5323 => ISP 5
Well 3	=>	agar 5337 => Synt. med. Suter without tyrosine
Well 4	=>	agar 5318 => ISP 6
Well 5	=>	agar 5322 => ISP 7
Well 6	=>	agar 5337 => Synt. med. Suter with tyrosine

The plate will be covered and sealed with parafilm after solidification of the agar and stored in the refrigerator.

On the next but one page are some details:

A

Part of a intensive colored *Streptomyces* isolate showing the color of the substrate mycelium which is influenced by the soluble pigment and a sparse aerial mycelium only formed at the random aerea of the colony. The color of the aerial mycelium is cream (RAL 9001), the soluble pigment oxide red (RAL 3009)

B

Typical growth of a *Streptomyces* isolate on media 5006, 5265, 5315 and 5317 with aerial mycelium on all four media which belongs to the grey series

C

Six well micro plate with FH 1568 *Streptomyces viridochromogenes*, showing the formation of aerial mycelium only on the medium 5317 and the formation and pigmentation of the substrate mycelium on the other media

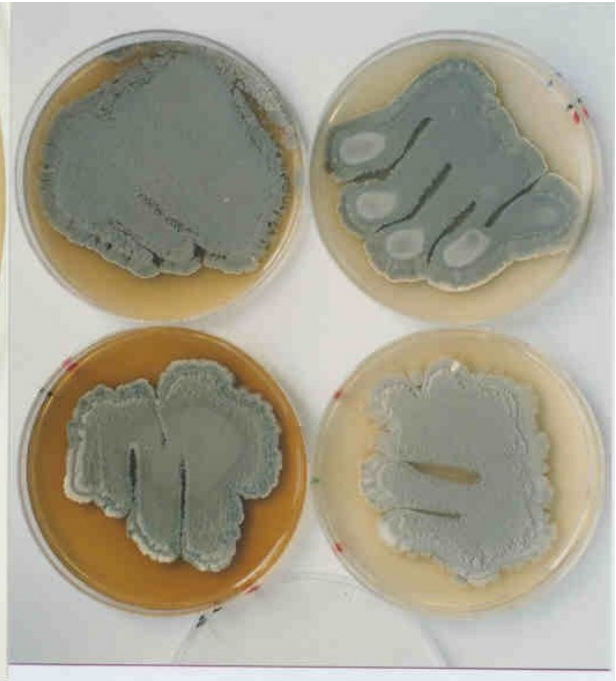
D

RAL color card (Reichsausschuß für Lieferbedingungen – Deutsches Institut für Gütesicherung e.V.)

A



B



C



D

A lot of the *Streptomyces* species, but members of other genera of the *Actinobacteria* too, form a dark brown to black pigment on media containing tyrosine. The amino acid tyrosine is part of the peptones in the media, but could also be found in complex media with meat extract. The enzyme tyrosinase is responsible for the first step in the melanin biosynthesis and could only be found in melanin positive strains.

Four different media were used for the characterization of the *Actinobacteria*. The first two are described by Shirling and Gottlieb (1966) for the ISP project and the third and fourth are from Kutzners' manual of Actinomycetes (1982):

5318 (ISP6), a peptone iron agar with yeast extract as a complex medium

5322 (ISP7), a synthetic medium with tyrosine

5337, a synthetically medium from Suter (1978) which is used with and without tyrosine (see above)

The production of the formation of a brown to black pigment was checked after 5, 10 and 14 days and is reported as + for formation or – for none formation, in addition there are in some cases (+) for a brown exopigment which might be correlated with melanin. With this results the strains could be arranged in four clusters

<u>Melanin production on medium</u>					Cluster
5318	5322	5337 with Tyrosine	5337 without Tyrosine		
+	+	+	-	3	
+	+	-	-	2	
-	-	+	-	1	
-	-	-	-	0	

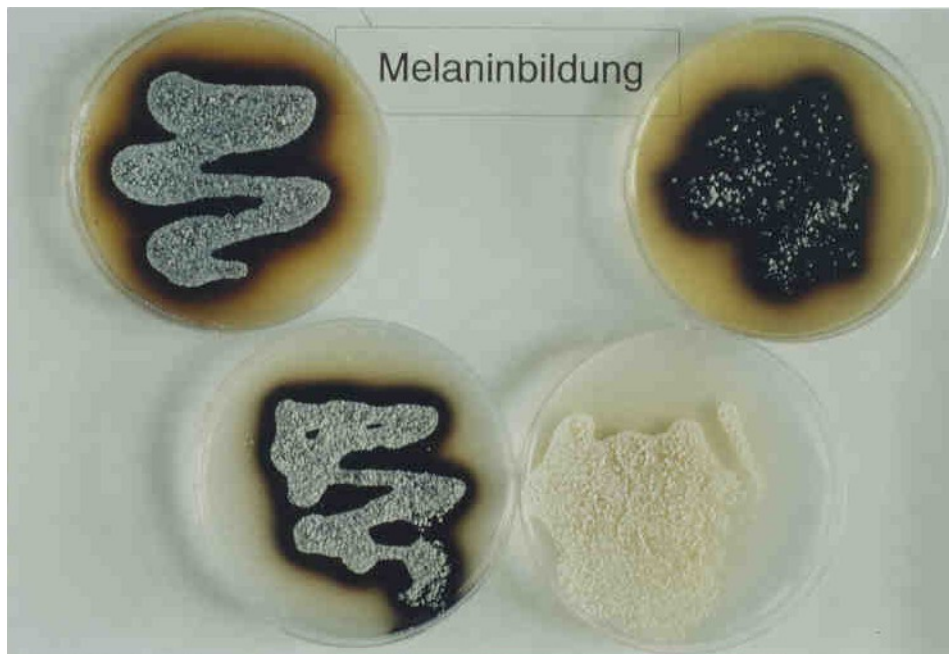
Some examples of melanin formation:

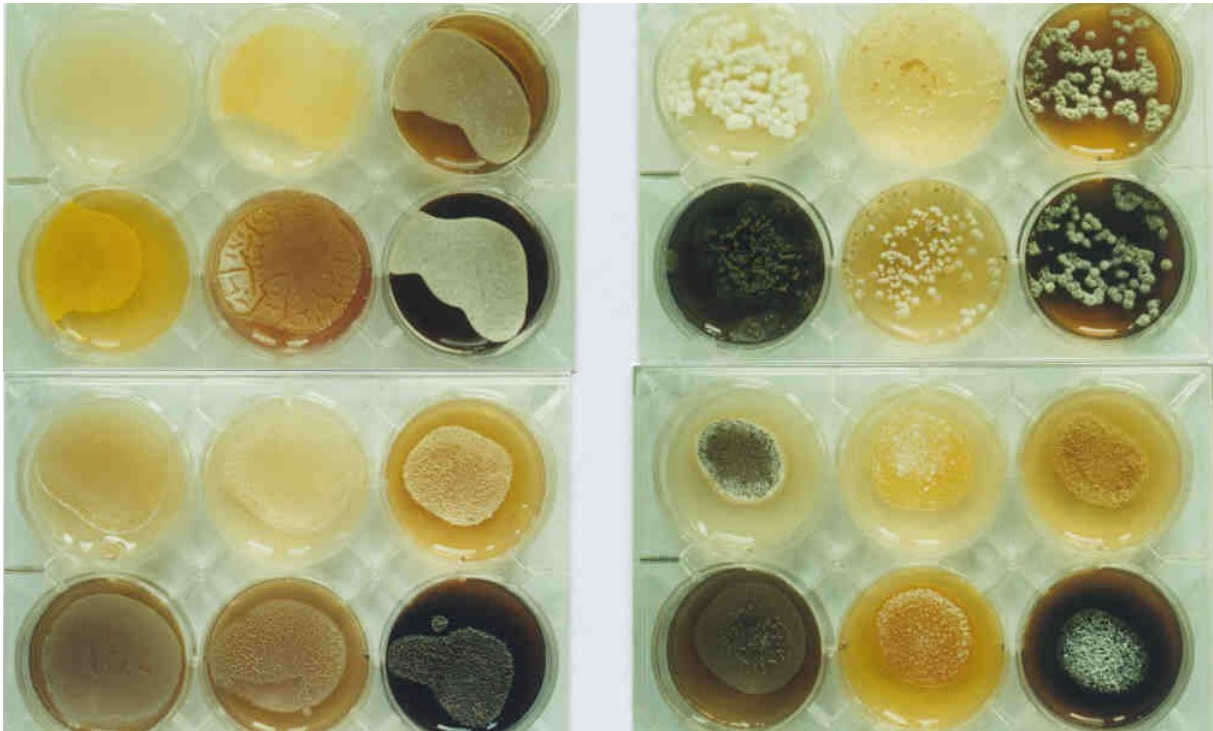
- A - Streptomycete forming no melanoid pigment (cluster 0)
- B - Streptomycete showing a typical 3 cluster with formation of a dark brown to black pigment on 5318 (upper left), 5322 (upper right) and 5337 with tyrosine (lower left)
- C - Two *Streptomyces* of the cluster 1, melanin production significant only on 5337 with tyrosine
- D - A typical production of melanoid pigment or a not to melanin correlated brown pigment



A

B





C

D

## 2. Physiological tests

### 2.1. Resistance toward sodium chloride (Kutzner 1981)

The resistance toward sodium chloride is a helpful tool in differentiating between the species in *Actinobacteria* not only in differentiating between marine or halophilic Actinomycetes.

Like the growth on ISP media a micro plate method has been established. On a six well plate the growth on basal medium 5339 (casein peptone 10,0 g/l, yeast extract 5,0 g/l) with 0, 2.5, 5, 7.5 and 10 % of sodium chloride could be checked. The scheme of the cell culture plate is shown on one of the next pages.

After five to ten days of incubation the highest concentration of salt that allows growth is recorded. In most cases there were no clear-cut borderlines between growth and no growth, so often there was good growth and formation of aerial mycelium without sodium chloride, then first the formation of aerial mycelium was lost by a good growth of substrate mycelium and with higher concentrations of sodium chloride also the growth of substrate mycelium stopped.

Two examples of sodium chloride resistance are shown.

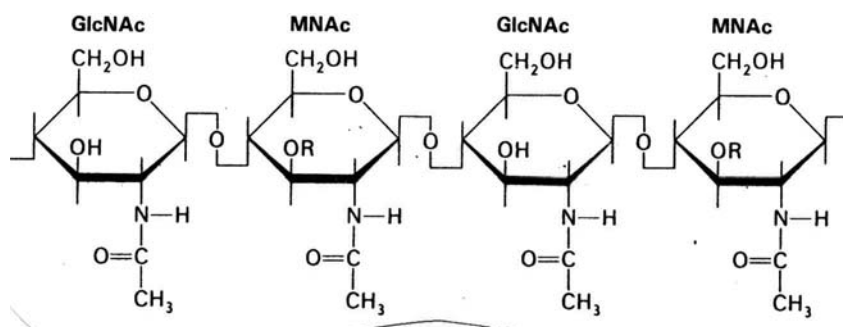
(Upper) Aerial mycelium forming Actinomycetes that growth up to 5 % of sodium chloride, but forms aerial mycelium only on the salt free basal medium.

(Lower) Member of the Actinoplanetes group showing good growth and exopigment formation up to 5 % of sodium chloride.

## 2.2. Resistance to lysozyme (Kutzner 1981)

Gram positive cells are in principle sensitive against lysozyme. Because of additional layers like teichonic acids or modifications of the peptidoglycan molecule some Gram-positive bacteria got resistant against lysozyme. So are *Mycobacterium*, *Nocardia* and „*Streptovercillium*“ lysozyme resistant, most of the *Streptomyces* species are sensitive.

Lysozym hydrolyses the glycosidic bond between MNAc and GlcNAc:



Analogue to the sodium chloride resistance the lysozyme resistance could be detected by a micro plate method which was modified after Kutzner et al (1978). The scheme of this test is shown on one of the next pages.

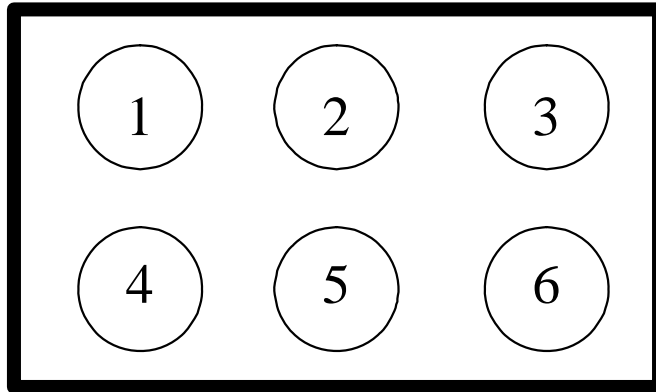
In this case medium 5339 was filled in six wells of a six well cell culture plate (5 ml per well). The lysozyme stock solution is 100 mg lysozyme in 10 ml H<sub>2</sub>O, sterilized by filtration. The final concentrations are 0, 10, 25, 50, 75 and 100 µg/ml of lysozyme in the basal medium 5339.

Evaluation was carried out in the same way like the sodium chloride tolerance.

## 2.3. pH tolerance (Kutzner 1981)

This physiological parameter is tested in tubes with medium 5265 (ISP2) with the pH levels of 2, 3, 4, 5, 6, 7, 8, 9 and 10. Incubation took place in a roller incubator. The visible growth is documented. On one of the next pages a row of medium slants with the different pH levels, starting with the pH of 2 at the left is shown. Good growth could be detected at a pH range from 5 to 8.

## Microplate Techniques in Actinomycetes Taxonomy II: **sodium chloride tolerance**



Each well of a 6-well Plate e.g. Costar (# 25810) will be filled sterile with 5 ml of agar.

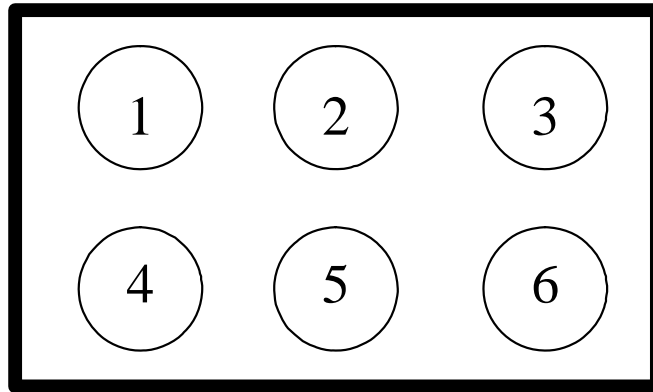
Each well is marked by a number for easy identification.

The filling pattern is:

Well 1	=>	agar 5339 with 0 % sodium chloride
Well 2	=>	agar 5339 with 2,5% sodium chloride
Well 3	=>	agar 5339 with 5 % sodium chloride
Well 4	=>	agar 5339 with 7,5% sodium chloride
Well 5	=>	agar 5339 with 10 % sodium chloride
Well 6	=>	leave empty

The plate will be covered and sealed with parafilm after solidification of the agar and stored in the refrigerator.

## Microplate Techniques in Actinomycetes Taxonomy III: **resistance to lysozyme**



Each well of a 6-well Plate e.g. Costar (# 25810) will be filled sterile with 5 ml of agar.  
Each well is marked by a number for easy identification.

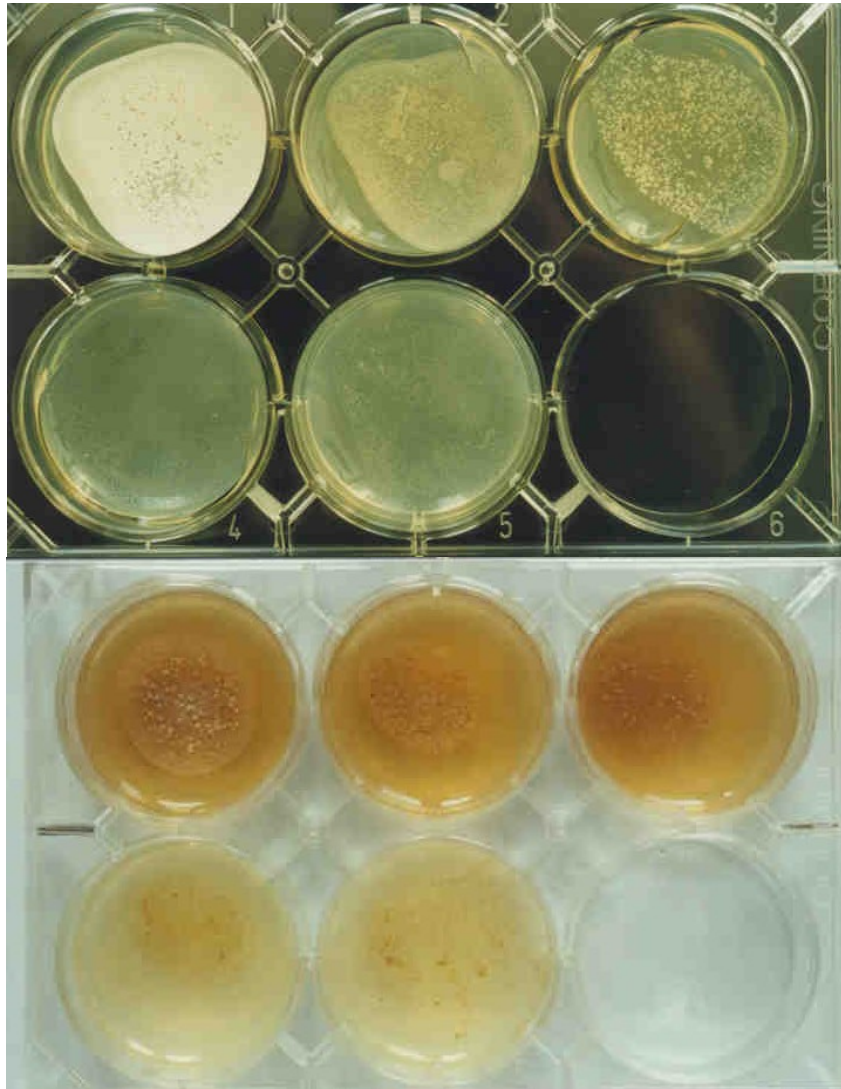
The filling pattern is:

Well 1	=>	agar 5339 without lysozyme
Well 2	=>	agar 5339 with 5,0 µl lysozyme solution
Well 3	=>	agar 5339 with 12,5 µl lysozyme solution
Well 4	=>	agar 5339 with 25 µl lysozyme solution
Well 5	=>	agar 5339 with 37,5 µl lysozyme solution
Well 6	=>	agar 5339 with 50 µl lysozyme solution

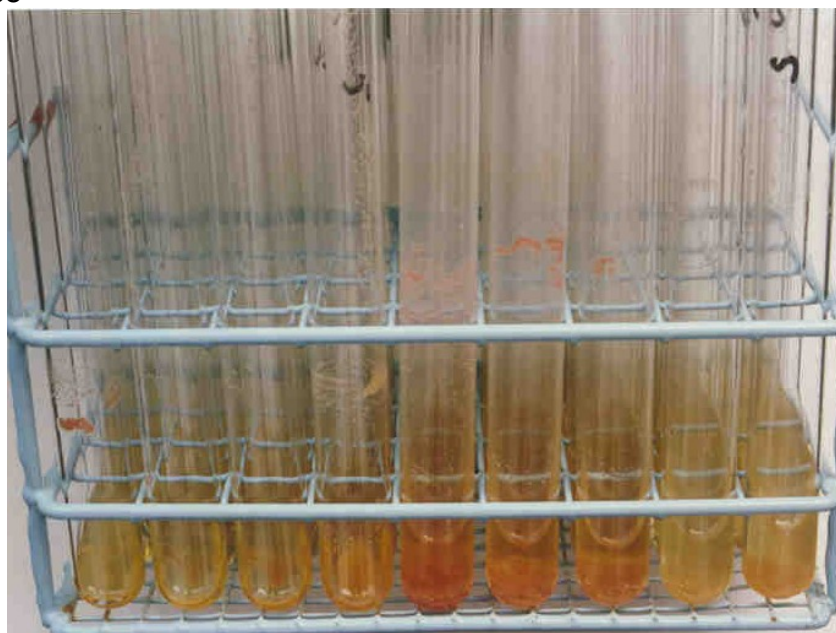
Lysozyme stock solution: 100 mg lysozyme in 10 ml water; sterilized by filtration.

The plate will be covered and sealed with parafilm after solidification of the agar and stored in the refrigerator.

Sodium chloride tolerance



pH tolerance



## 2.4. Utilization of carbohydrates

The utilization of carbon sources plays an important role in species differentiation by bacteria and so also by the *Actinobacteria* (Benedickt et. al 1955).

The ability of strains to use 10 compounds is tested in a micro plate (12 well) technique basing on the method of Shirling and Gottlieb (1966) with the basal agar 5338 ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 2,64 g/l, KH<sub>2</sub>PO<sub>4</sub> 2,38 g/l, K<sub>2</sub>HPO<sub>4</sub> 4,31 g/l, MgSO<sub>4</sub> x 7 H<sub>2</sub>O 1,0 g/l and 1,0 ml/l trace element solution – CuSO<sub>4</sub> x 5H<sub>2</sub>O 0,64 g/l, FeSO<sub>4</sub> x 7H<sub>2</sub>O 0,11 g/l, ZnSO<sub>4</sub> x 7 H<sub>2</sub>O 0,15 g/l, MnCl<sub>2</sub> x 4 H<sub>2</sub>O).

The following carbon sources are used:

Glucose	Glu
Arabinose	Ara
Sucrose	Suc
Xylose	Xyl
Inositol	Ino
Mannitol	Man
Fructose	Fra
Rhamnose	Rha
Raffinose	Raf
Cellulose	Cel

The plate scheme of the 12 well micro plate is shown on the next but one page. 10 % solutions of the above compounds are sterilized by filtration and added to the basal medium 5338 after autoclaving and cooling to 60°C to give a final concentration of 1 %. The well with glucose (1) serves as positive control and the well with pure basal medium and water (11) as negative control.

The valuation is carried out after the following scheme:

Growth not better than the negative control	-
Growth better like the negative control but not like the positive control	(+)
Growth like the positive control	+
Growth better like the positive control	++

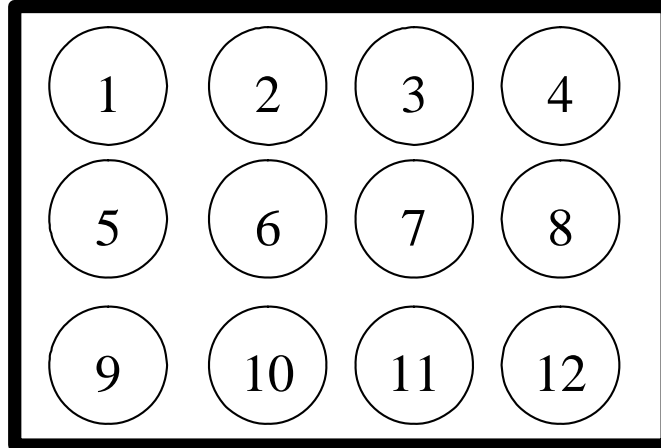
On one of the next pages are four examples of „sugar plates“ with the following spectra:

	Glu	Ara	Suc	Xyl	Ino	Man	Frau	Rha	Raf	Cel
(A)	+	+	+	-	-	-	+	-	+	-
(B)	+	+	+	-	-	-	+	+	+	-
(C)	+	+	-	-	-	+	+	-	-	-
(D)	+	+	+	-	++	+	+	+	+	-

## Microplate Techniques in Actinomycetes Taxonomy

### IV:

#### carbon utilization



Each well of a 12-well Plate e.g. Costar (# 430345) will be filled sterile with 2 ml of the agar.

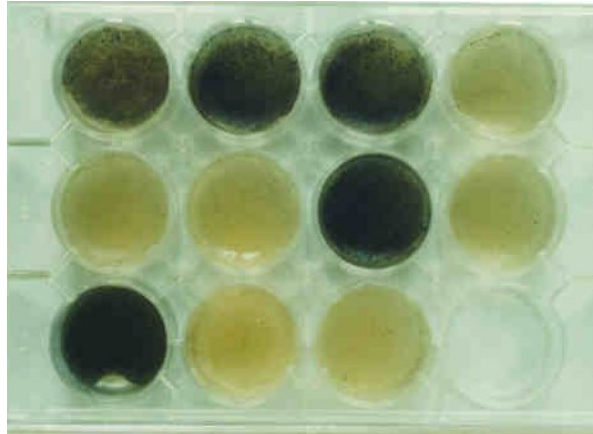
Each well is marked by a number for easy identification.

The filling pattern is :

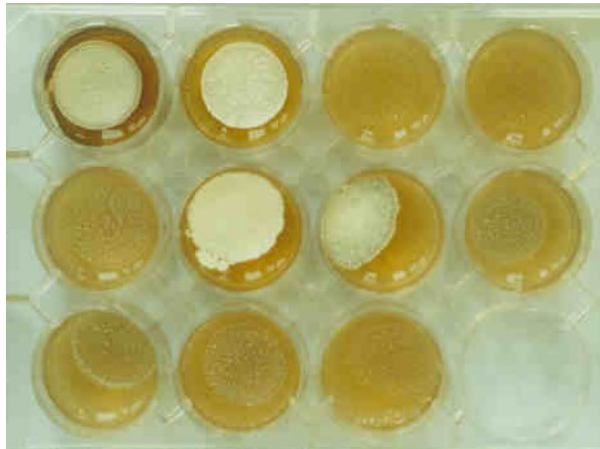
Well 1	=>	agar 5338 with 1% glucose
Well 2	=>	agar 5338 with 1% arabinose
Well 3	=>	agar 5338 with 1% sucrose
Well 4	=>	agar 5338 with 1% xylose
Well 5	=>	agar 5338 with 1% inositol
Well 6	=>	agar 5338 with 1% mannitol
Well 7	=>	agar 5338 with 1% fructose
Well 8	=>	agar 5338 with 1% rhamnose
Well 9	=>	agar 5338 with 1% raffinose
Well 10	=>	agar 5338 with 1% cellulose
Well 11	=>	agar 5338 with water (control)
Well 12	=>	leave empty

The plate will be covered and sealed with parafilm after solidification of the agar and stored in the refrigerator.

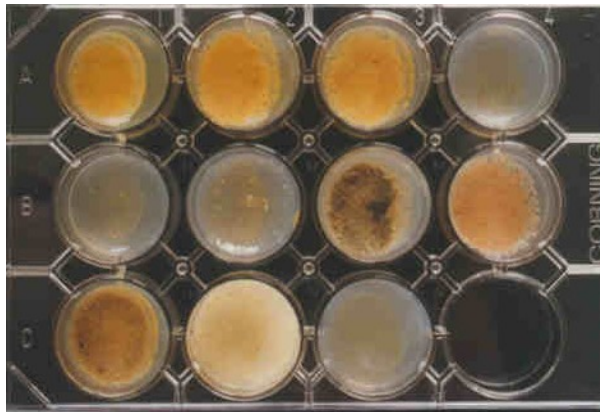
Carbon utilization



A



B



C



D

## 2.5. Physiological fingerprints with the api® stripes

BioMérieux is offering a number of different identification stripes which contain different tests. Three of these are used in routine description of the *Actinobacteria*. The **api Coryne**, a test cit especially for the *Propionibacterineae*, *Micrococcineae* and *Corynebacterinae* (MacFaddin 1980).

This contains the following tests and reactions:

Test	Reaction
Nit	Nitrate reduction
Pyz	Pyrazinamidase
PyrA	Pyrrolidonyl Arylamidase
Pal	Alkaline phosphatase
β Gur	beta Glucuronidase
β Gal	beta Galactosidase
α Glu	alpha Glucosidase
β Nag	N-Acetyl-beta Glucosamidase
Esc	Esculin (beta Glucosidase)
Ure	Urease
Gel	Gelatine(hydrolysis)
Glu	Glucose fermentation
Rib	Ribose fermentation
Xyl	Xylose fermentation
Man	Mannitol fermentation
Lac	Lactose fermentation
Sac	Sucrose fermentation
Glyg	Glycogen fermentation

The **api Zym**, a simple rapid system for the detection of bacterial enzymes (Humble et al. 1977) which has been successfully used for identification of *Actinomycetaceae* and related bacteria (Kilian 1978).

No	Enzyme assayed for	Substrate
2	Phosphatase alcaline	2-naphthyl phosphate
3	Esterase (C 4)	2-naphthyl butyrate
4	Esterase Lipase (C 8)	2-naphthyl caprylate
5	Lipase (C 14)	2-naphthyl myristate
6	Leucine arylamidase	L-leucyl-2-naphthylamide
7	Valine arylamidase	L-valyl-2-naphthylamide
8	Cystine arylamidase	L-cystyl-2-naphthylamide
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide
10	Chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamide
11	Phosphatase acid	2-naphthyl phosphate
12	Naphthol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate
13	α galactosidase	6-Br-2-naphthyl-αD-galactopyranoside
14	β galactosidase	2-naphthyl-βD-galactopyranoside
15	β glucuronidase	Naphthol-AS-BI-βD-glucuronide
16	α glucosidase	2-naphthyl-αD-glucopyranoside
17	β glucosidase	6-Br-2-naphthyl-βD-

18	N-acetyl- $\beta$ -glucoseamidase	glucopyranoside 1-naphthyl-N-acetyl- $\beta$ D- glucoseaminide
19	$\alpha$ mannosidase	6-Br-2-naphthyl- $\alpha$ D- mannopyranoside
20	$\alpha$ fucosidase	2-naphthyl- $\alpha$ L-fucopyranoside

The **api 20 E** is a micromethod for identification of *Enterobacteriaceae* (Smith et al. 1972) which also could be used for fingerprint of physiology of the *Actinobacteria*.

Test	Substrates	Reactions/Enzymes
Onp	ortho-nitro-phenyl-galactoside	beta-galactosidase
Arg	arginine	arginine dihydrolase
Lys	lysine	lysine decarboxylase
Odc	ornithine	ornithine dacarboxylase
Cit	sodium citrate	citrate utilization
H <sub>2</sub> S	sodium thiosulfate	H <sub>2</sub> S production
Ure	urea	urease
Trp	tryptophane	tryptophane desaminase
Ind	tryptophane	indole production
VP	sodium pyruvate	acetoin production
Gel	Kohn´s gelatin	gelatinase
Glu	glucose	fermentation
Man	mannose	fermentation
Ino	inositol	fermentation
Sor	sorbitol	fermentation
Rha	rhamnose	fermentation
Sac	sucrose	fermentation
Mel	melibiose	fermentation
Amy	amygdalin	fermentation
Ara	arabinose	fermentation

For the api tests the cultures are grown in shaking flasks and in most cases have to be diluted with water because the biomass or the produced exopigments could interfere with the color production in the test tube. If a strain grows in big pellets it has to be homogenized with an ultra turrax.

Description of results:

- + significant color reaction
- (+) color reaction interfered by the strain sample
- no color reaction

Examples and additional information about the api stripes:

- (A) Comparison of api 20E (upper row), api ZYM (mid) and api Coryne (lower row) of FH 2999 *Amycolatopsis keratiniphila* subsp. *keratiniphila*
- (B) Comparison of three *Streptomyces* species with api ZYM strips – FH 6304 *S. albolongus*, FH 6303 *S. albofaciens*, FH 6302 *S. afghaniensis*
- (C) Reading scale of api ZYM

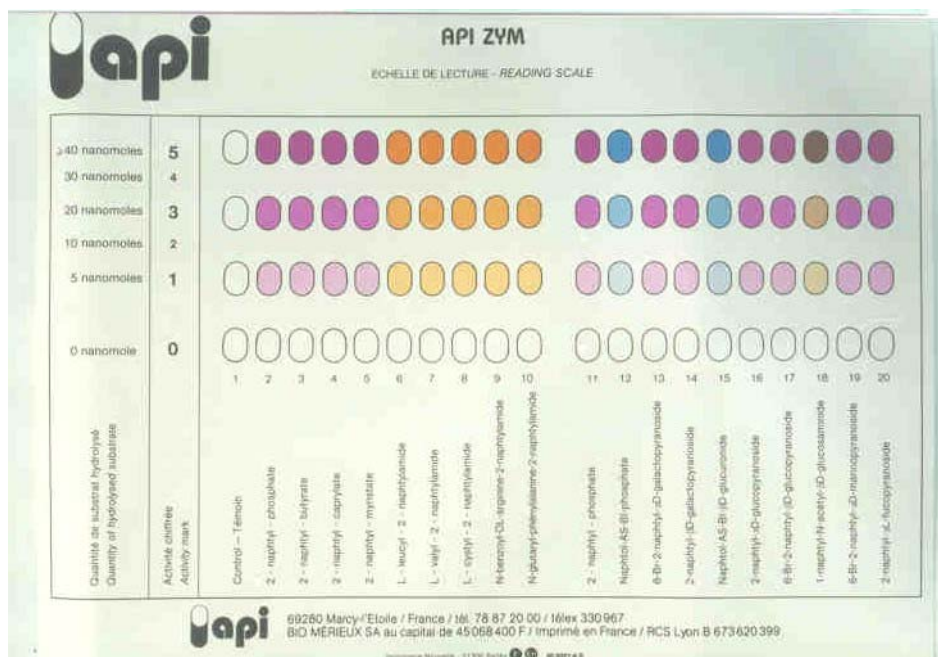
(A)



(B)



(C)



## 2.6. The Biolog Plate

The Biolog GP2 MicroPlate (Figure) is designed for identification and characterization of a very wide range of aerobic gram-positive bacteria. Biolog's MicroPlates and databases were first introduced in 1989, employing a novel, patented redox chemistry. This chemistry, based in reduction of tetrazolium, responds to the process of metabolism (i.e. respiration) rather than to metabolic by-products (e.g. acid). Biolog's chemistry works as an universal reporter of metabolism and simplifies the testing process as color developing chemicals do not need to be added. Since the GP2 MicroPlate is not dependent upon growth to produce identifications, it provides superior capability for all types of gram positive organisms: cocci, rods, and spore-forming rods all are identified with a single panel. The database for the GP2 MicroPlate is now over 310 species. It is by far the largest kit-based identification database available.

**GP2 MicroPlate™**

A1 Water	A2 α-Cyclodextrin	A3 β-Cyclodextrin	A4 Dextrin	A5 Glycogen	A6 Inulin	A7 Mannan	A8 Tween 40	A9 Tween 80	A10 N-Acetyl-D-Glucosamine	A11 N-Acetyl-D-Mannosamine	A12 Amygdalin
B1 L-Arabinose	B2 D-Arabitol	B3 Arbutin	B4 D-Cellobiose	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 α-D-Glucose	B12 m-inositol
C1 α-D-Lactose	C2 Lactulose	C3 Maltose	C4 Maltotriose	C5 D-Mannitol	C6 D-Mannose	C7 D-Melezitose	C8 D-Melibiose	C9 α-Methyl D-Galactoside	C10 β-Methyl D-Galactoside	C11 3-Methyl Glucose	C12 α-Methyl D-Glucoside
D1 β-Methyl D-Glucoside	D2 α-Methyl D-Mannoside	D3 Palatinose	D4 D- Psicose	D5 D-Raffinose	D6 L-Rhamnose	D7 D-Ribose	D8 Salicin	D9 Sedoheptulosan	D10 D-Sorbitol	D11 Stachyose	D12 Sucrose
E1 D-Tagatose	E2 D-Trehalose	E3 Turanose	E4 Xylitol	E5 D-Xylose	E6 Acetic Acid	E7 α-Hydroxy Butyric Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 p-Hydroxy Phenyl Acetic Acid	E11 α-Keto Glutaric Acid	E12 α-Keto Valeric Acid
F1 Lactamide	F2 D-Lactic Acid Methyl Ester	F3 L-Lactic Acid	F4 D-Malic Acid	F5 L-Malic Acid	F6 Methyl Pyruvate	F7 Mono-methyl Succinate	F8 Propionic Acid	F9 Pyruvic Acid	F10 Succinamic Acid	F11 Succinic Acid	F12 N-Acetyl L-Glutamic Acid
G1 L-Alaninamide	G2 D-Alanine	G3 L-Alanine	G4 L-Alanyl-glycine	G5 L-Asparagine	G6 L-Glutamic Acid	G7 Glycyl- L- Glutamic Acid	G8 L-Pyrroglutamic Acid	G9 L-Serine	G10 Putrescine	G11 2,3-Butanediol	G12 Glycerol
H1 Adenosine	H2 2-Deoxy Adenosine	H3 Inosine	H4 Thymidine	H5 Uridine	H6 Adenosine-5'- Monophosphate	H7 Thymidine-5'- Monophosphate	H8 Uridine-5'- Monophosphate	H9 Fructose-6- Phosphate	H10 Glucose-1- Phosphate	H11 Glucose-6- Phosphate	H12 D-L-α-Glycerol Phosphate

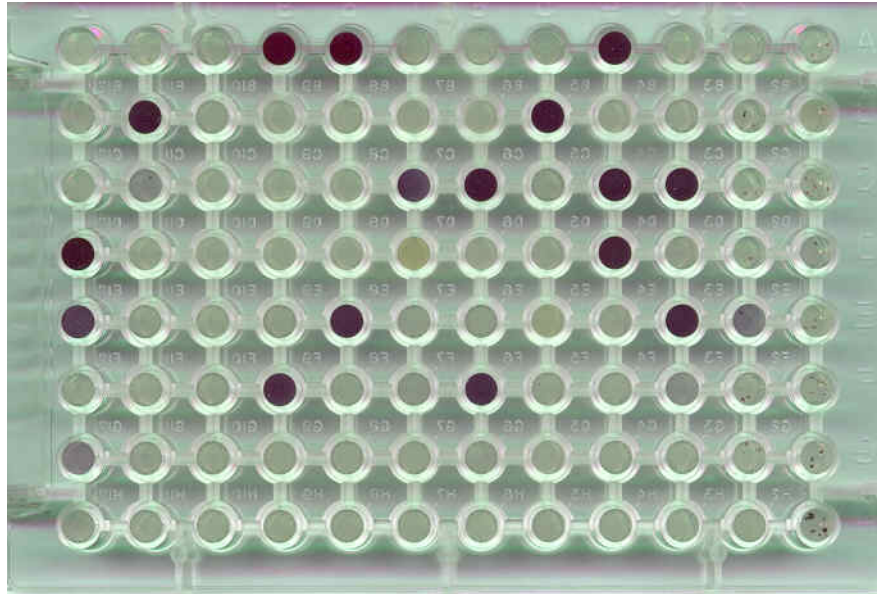
FIGURE 1. Carbon Sources in GP2 MicroPlate

The Biolog GP2 MicroPlate performs 95 discrete tests simultaneously and gives a characteristic reaction pattern called a “metabolic fingerprint”. These fingerprint reaction patterns provide a vast amount of information conveniently contained in a single Biolog MicroPlate. The metabolic fingerprint patterns are compared and identified using the MicroLog™ database software.

Other aerobic kit-based identification methods rely on much smaller number of tests. Consequently, the significant limitation of these products is the limited number of species and organism types that they can identify. Furthermore, these products were designed to address the needs of routine clinical/hospital testing. The Biolog GP2 MicroPlate was designed to address the needs of much wider range of users including environmental testing labs and animal and plant disease labs as well as clinical reference labs.

The strains are incubated in shaking cultures medium 5006 at 28° and 37°C. The cultures are diluted with water and directly used with the GP2 MicroPlates. The resulting plates are scanned (picture is side-inverted) and read with a Spectrafluor from Tecan.

FH 2813 *Gordonia sputi*



## 2.7. Strain data sheet

On the next page but one the strain datasheet of *Nesterenkonia aethiopica* is shown, here all the data from the methods which are described in the chapters 1 and 2 (1-5) are filled in. The format and kind of information is described in each chapter. All data of the different species of *Actinobacteria* are completely described in this way. In the case that a test was not carried out it is marked as nd (not determinate). In other cases the strains also did not grow on the basal media or, basing on color production, it was not possible to interpret some API results.

**Genus:** *Nesterenkonia* **FH 6773**  
**Species:** *aethiopica*  
**Numbers in other collections:** **DSM 17773**

Morphology:

	G	R
<u>ISP 2</u>	good	zinc yellow
	A	SP
	none	none
	G	R
<u>ISP 3</u>	good	zinc yellow
	A	SP
	none	none
	G	R
<u>ISP 4</u>	good	zinc yellow
	A	SP
	none	none
	G	R
<u>ISP 5</u>	good	zinc yellow
	A	SP
	none	none
	G	R
<u>ISP 6</u>	good	zinc yellow
	A	SP
	none	none
	G	R
<u>ISP 7</u>	good	zinc yellow
	A	SP
	none	none

Melanoid pigment: - - - -

NaCl resistance: %

Lysozyme resistance:

pH: Value-

Optimum-

Temperature : Value-

Optimum- 30 °C

Carbon utilization:

Glu	Ara	Suc	Xyl	Ino	Man	Fru	Rha	Raf	Cel
				n.d.					

Enzymes:

2+	3+	4+	5+	6+	7(+)	8-	9-	10-	11+	
12-	13-	14-	15-	16-	17-	18-	19-	20-		
Nit	Pyz	Pyr	Pal	βGur	βGal	αGlu	βNag	Esc	Ure	Gel
-	-	-	+	-	+	-	-	-	+	+
Glu	Rib	Xyl	Man	Mal	Lac	Sac	Glyg			
-	-	-	-	-	-	-	-			

Comments:

### **3. Light microscopic characterization**

For the light microscopic studies of the strains a Leitz Ortholux microscope with a EF L32/0,40 and a EF L20/0,32 objective is used. These objectives allow working with a long distance between objective and object, so that the agar plates could directly be used for observation. The strains are cultivated on the media as described in part 1 of this chapter. For light microscopy mainly media with low turbidity are used.

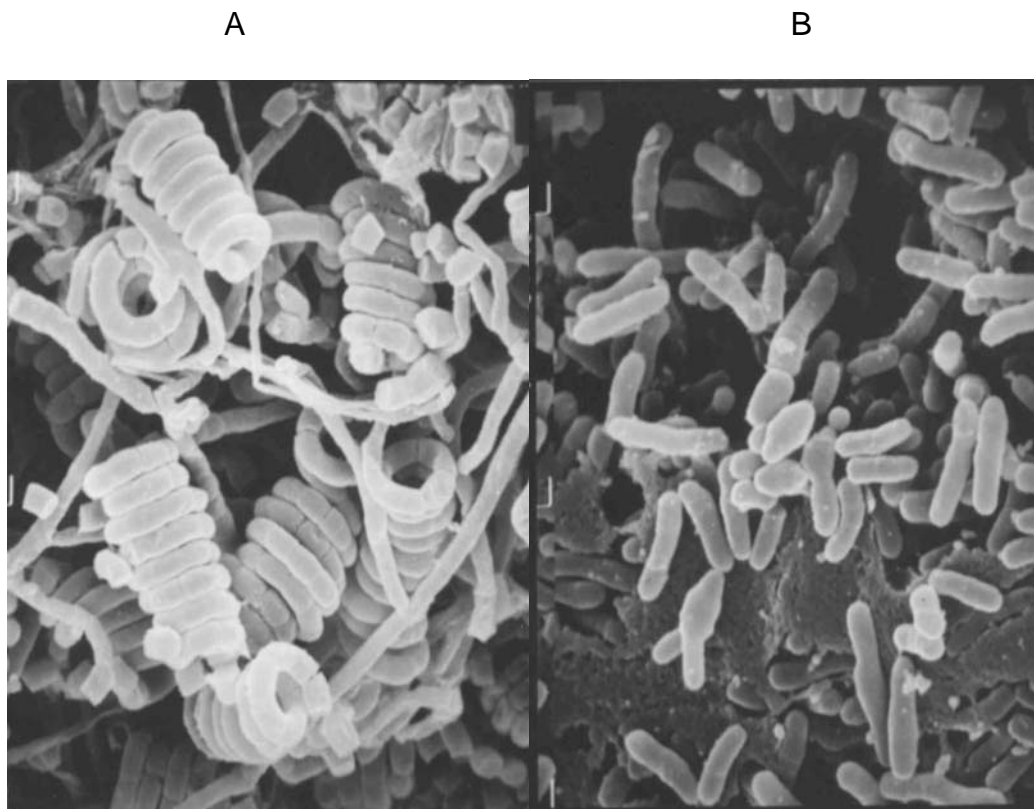
The following characteristics are checked:

Spore chains	yes or no
Sporangia	yes or no
Single spores	yes or no
Fragmentation of aerial or substrate mycelium	yes or no
Spore chain morphologie	Recti flexibilis, Retinaculum- apertum Spira, Verticillus



Spiral spore chains of *Actinomadura namibiensis*, magnification x 350

## Scanning electron microscopy



### **4. Scanning electron microscopy**

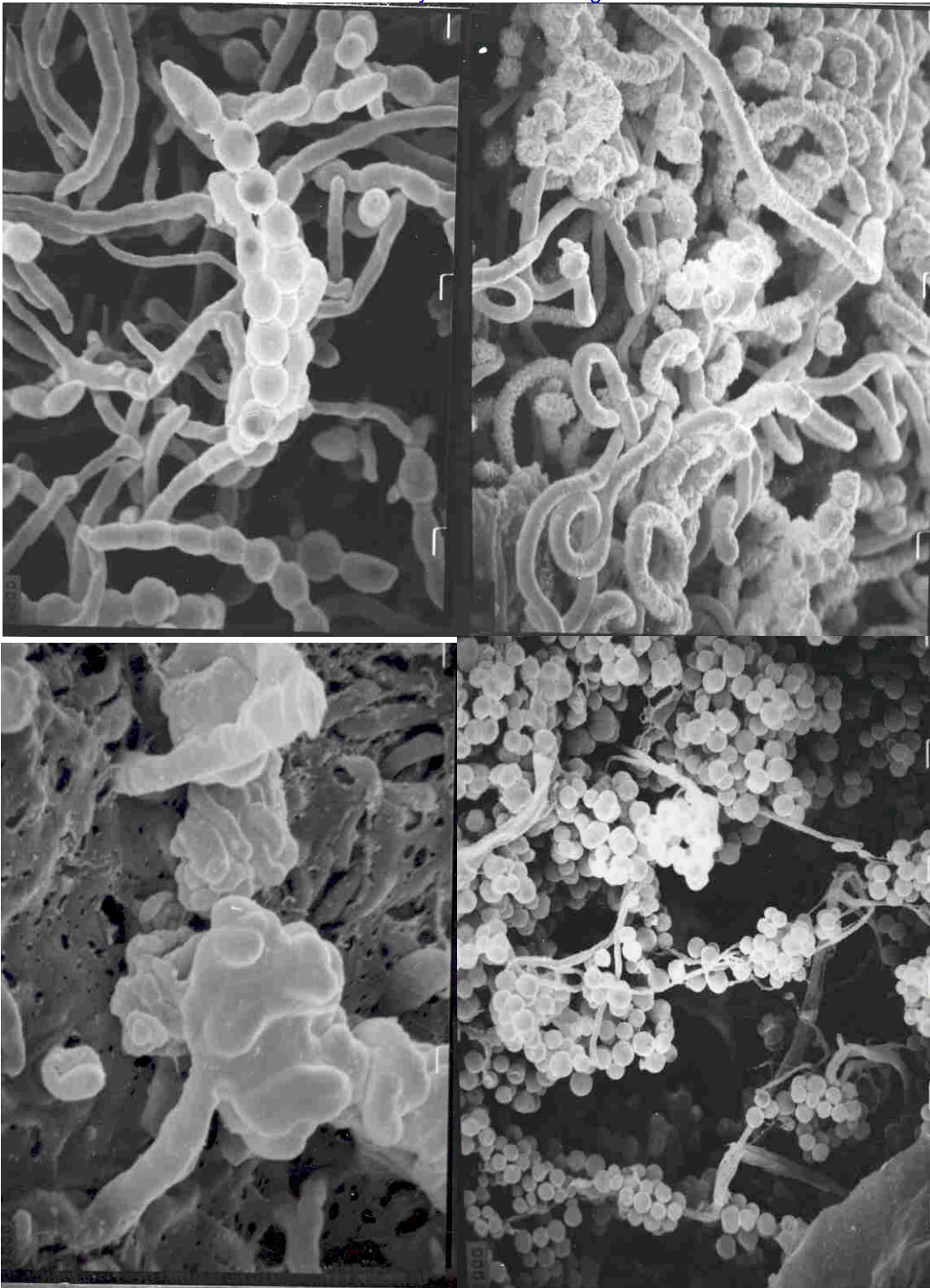
For preparation of the samples a critical point dryer PELCO CPD2 and a HUMMER X sputtering system from ANATECH LTD are used. The scanning electron microscope is a JEOL JSM T-20.

Preparations of the strains are performed using a modified method of VOBIS and KOTHE (1985) described by WINK (1992). The strains are cultivated on ISP 3 (5315) or ISP 2 (5265) or any special agar required for growth and differentiation at 28°C (or any other temperature required) over a period of 10 to 14 days. Out of this plates 1 cm<sup>2</sup>-piece are cut and incubated in a solution of 4 % glutaraldehyd in water for 24 hours or longer. After washing with water (five times, 10 minutes each), the samples are dehydrated by incubation in 2-methylglycerol (three times, 10 minutes each). The methyl glycerol is replaced by dry acetone (three times, 10 minutes each) and the samples are dried at the critical point using CO<sub>2</sub>. The samples are fixed on a brass cylinder and are covered with gold using the sputtering system. This method can be used for all strains which form stable mycelium and are connected to the agar which is shown in the figure on the page before (A *Streptomyces* species with smooth spores in spiral spore chains). Strains which grow in single cells like coryneform bacteria are fixed on lysine coated glass plates. Therefore the in glutaraldehyde fixed samples are washed for 10 minutes in 30 % ethanol, than for 10 minutes in 60 % ethanol and at last for 10 min in 90 % ethanol. The suspension is decanted and the cells are transferred to the lysine plate and dried for a minute in the air. Afterwards the plate is washed for two times for 10 minutes in pure ethanol. These samples are also dried at the critical point using CO<sub>2</sub>, fixed on brass cylinders and covered with gold. Figure (B) on the page before shows the single cells of a *Corynebacterium glutamicum* species.

With the SEM it is possible to get a lot of information on fine structures of the strains, spore chain morphology, spore surface, shape of sporangia, and formation of single spores. The spore surface is characterized as smooth, warty, rugose, knobby, hairy and spiny.

During the scanning electron microscopic investigation the samples can be photographed with a POLAROID camera. On the pictures the magnification is represented by a bar row at the top of the picture and the size of a structure could be determinate using the following scheme:

Magnification at SEM  $\times$  length D = Real magnification  
(D=length of one repeating bar unit at top of the picture).



Some examples of spore chains, sporangia and single spores in Scanning Electron Microscopy (SEM) from different Actinomycetales (*Actinomadura* spec., *Streptomyces* spec., *Actinoplanes* spec. and *Micromonospora* spec.)

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## Composition of Media

**Medium:** 5006

Name:

Composition	(g/l)
Sucrose	3,0
Dextrin	15,0
Meat extract	1,0
Yeast extract	2,0
Tryptone soy broth (Oxoid)	5,0
NaCl	0,5
K <sub>2</sub> HPO <sub>4</sub>	0,5
MgSO <sub>4</sub> x 7 H <sub>2</sub> O	0,5
FeSO <sub>4</sub> x 7 H <sub>2</sub> O	0,01
Agar	20,0

Sterilization:

20 minutes at 121°C

pH before sterilization:

7,3

Usage:

Maintenance

Organisms:

All Actinomycetes

**Medium:** 5265

Name: ISP 2 / Yeast Malt Agar

Composition	(g/l)
Malt extract	10,0
Yeast extract	4,0
Glucose	4,0
Agar	15,0

Sterilization:

20 minutes at 121°C

pH before sterilization:

7,0

Usage:

Maintenance and Taxonomy

Organisms:

All Actinomycetes

**Medium:** 5294

Name:

Composition	(g/l)
Starch (soluble)	10,0
Yeast extract	2,0
Glucose	10,0
Glycerol	10,0
Corn steep liquor	2,5
Peptone	2,0
NaCl	1,0
CaCO <sub>3</sub>	3,0

Sterilization:

20 minutes at 121°C

pH before sterilization:

7,2

Usage:

Agar plates for fatty acid and MALDI TOF analysis

Organisms:

All Actinomycetes

**Medium:** 5314

Name: Trace element solution

Composition	(g/l)
CaCl <sub>2</sub> x H <sub>2</sub> O	3,0
Fe-III-citrate	1,0
MnSO <sub>4</sub>	0,2
ZnCl <sub>2</sub>	0,1
CuSO <sub>4</sub> x 5 H <sub>2</sub> O	0,025
Sodium tetra borate	0,2
CoCl <sub>2</sub> x 6 H <sub>2</sub> O	0,004
Sodium molybdate	0,01

Preparation:

Use double distillate water.

Sterilisation:

20 minutes at 121°C

Usage:

Trace element solution for different media

Organisms:

**Medium:** 5315

Name: ISP 3

Composition	(g/l)
Dog oat flakes	20,0
Trace élément solution (5314)	2,5 ml/l
Agar	18,0

Preparation:

Oat flakes are cooked for 20 minutes, trace element solution and agar are added (in the case of non rolled oat flakes the suspension has to be filtered).

Sterilization:

20 minutes at 121°C

pH before sterilization:

7,8

Usage:

Maintenance and taxonomy (e.g. SEM)

Organisms:

All Actinomycetes

**Medium:** 5322

Name: ISP 7

Composition	(g/l)
Glycerol	15,0
L-Tyrosine	0,5
L-Asparagine	1,0
K <sub>2</sub> HPO <sub>4</sub>	0,5
NaCl	0,5
FeSO <sub>4</sub> x 7 H <sub>2</sub> O	0,01
Trace element solution 5343	1,0 ml/l
Agar	20,0

Sterilization:

20 minutes at 121°C

pH before sterilization:

7,3

Usage:

Production of melanin pigments

Organisms:

All Actinomycetes

**Medium:** 5323

Name: ISP 5

Composition	(g/l)
L-Asparagine	1,0
Glycerol	10,0
K <sub>2</sub> HPO <sub>4</sub>	1,0
Salt solution (see preparation)	1,0 ml/l
Agar	20,0

Preparation:

Salt solution - 1,0 g FeSO<sub>4</sub> x 7 H<sub>2</sub>O, 1,0 g MnCl<sub>2</sub> x 4 H<sub>2</sub>O, 1,0 g ZnSO<sub>4</sub> x 7 H<sub>2</sub>O in 100 ml water

Sterilization:

20 minutes at 121°C

pH before sterilization:

7,2

Usage:

Maintenance and taxonomy

Organisms:

All Actinomycetes

**Medium:** 5327

Name:

Composition	(g/l)
Glucose	100,0
Yeast extract	10,0
CaCO <sub>3</sub>	20,0
Agar	18,0

Sterilization:

20 minutes at 121°C

pH before sterilization:

6,8

Usage:

Maintenance and taxonomy

Organisms:

All Actinomycetes

**Medium:** 5333

Name:

Composition	(g/l)
Yeast extract	4,0
Soluble starch	15,0
K <sub>2</sub> HPO <sub>4</sub>	1,0
MgSO <sub>4</sub> x 7 H <sub>2</sub> O	0,5

Sterilization:

20 minutes at 121°C

pH before sterilization:

7,0

Usage:

Liquid medium for metabolite production

Organisms:

All Actinomycetes

**Medium:** 5337

Name: Synthetically suter medium

Composition	(g/l)
Glycerol	15,0
Tyrosine	1,0
L-arginine	5,0
L-glutamic acid	5,0
L-methionine	0,3
L-isoleucine	0,3
K <sub>2</sub> HPO <sub>4</sub>	0,5
MgSO <sub>4</sub> x 7 H <sub>2</sub> O	0,2
Trace element solution (5341)	1,0 ml
Agar	20,0

Preparation:

Control medium is prepared without tyrosine

Sterilization:

20 minutes at 121°C

pH before sterilization:

Usage:

Production of melanin pigment

Organisms:

All Actinomycetes

**Medium:** 5338

Name:

Composition	(g/l)
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2,64
KH <sub>2</sub> PO <sub>4</sub>	2,38
K <sub>2</sub> HPO <sub>4</sub>	4,31
MgSO <sub>4</sub> x 7 H <sub>2</sub> O	1,0
Agar	15,0
Trace élément solution 5342	1,0 ml/l

Preparation:

Medium is solved in 900 ml water and is autoclaved. Afterwards 100 ml of a sterile filtrated solution of a carbohydrate is added. Solutions with 10 % of the following carbohydrates are prepared: glucose, arabinose, sucrose, xylose, inositol, mannose, fructose, rhamnose, raffinose and cellulose

Sterilization:

20 minutes at 121°C

pH before sterilization:

7,3

Usage:

Use of carbohydrates

Organisms:

All Actinomycetes

**Medium:** 5339

Name: Sodium chloride tolerance

Composition	(g/l)
Casein peptone	10,0
Yeast extract	5,0
Agar	20,0

Preparation:

The following concentrations of sodium chloride are added to the above medium: 0%; 2,5%; 5,0%, 7,5%; 10,0%

Sterilization:

20 minutes at 121°C

pH before sterilization:

7,0

Usage:

Determination of sodium chloride tolerance

Organisms:

All Actinomycetes

**Medium:** 5341

Name: Trace elemt solution

Composition	(g/l)
$\text{CuSO}_4 \times 5 \text{H}_2\text{O}$	10,0
$\text{CaCl}_2 \times 2 \text{H}_2\text{O}$	10,0
$\text{FeSO}_4 \times 7\text{H}_2\text{O}$	10,0
$\text{ZnSO}_4 \times 7\text{H}_2\text{O}$	10,0
$\text{MnSO}_4 \times 7 \text{H}_2\text{O}$	40,0

Preparation:

The trace elements are solved in double distillate water.

Sterilisation:

20 minutes at 121°C

pH before sterilisation:

Usage:

Trace element solution for medium 5337

Organisms:

**Medium:** 5342

Name: Trace element solution

Composition	(g/l)
$\text{CuSO}_4 \times 5 \text{H}_2\text{O}$	0,64
$\text{FeSO}_4 \times 7\text{H}_2\text{O}$	0,11
$\text{ZnSO}_4 \times 7\text{H}_2\text{O}$	0,15
$\text{MnCl}_2 \times 4 \text{H}_2\text{O}$	0,79

Preparation:

The trace elements are solved in double distillate water.

Sterilisation:

20 minutes at 121°C

pH before sterilisation:

Usage:

Trace element solution for medium 5338

Organisms:

**Medium:** 5343

Name: Trace element solution

Composition	(g/l)
FeSO <sub>4</sub> x 7H <sub>2</sub> O	1,0
ZnSO <sub>4</sub> x 7H <sub>2</sub> O	1,0
MnCl <sub>2</sub> x 7H <sub>2</sub> O	1,0

Preparation:

The trace elements are solved in double distilled water.

Sterilization:

20 minutes at 121°C

pH before sterilization:

Usage:

Trace element solution for medium 5322

Organisms:

**Medium:** 5415

Name: Marine Rhodococcus Medium

Composition	(g/l)
Yeast extract	10,0
Malt extract	4,0
Glucose	4,0
Agar	25,0
Artificial sea water	750 ml (see 5416)

Sterilization:

20 minutes at 121°C

pH before sterilization:

7,0

Usage:

Maintenance and Revitalization

Organisms:

Marine *Actinobacteria*

**Medium:** 5416

Name: Artificial Sea Water

Composition	(g/l)
NaCl	23,477
MgCl <sub>2</sub>	4,981
Na <sub>2</sub> SO <sub>4</sub>	3,917
CaCl <sub>2</sub>	1,102
KCl	0,664
NaHCO <sub>3</sub>	0,192
KBr	0,096
H <sub>3</sub> BO <sub>3</sub>	0,026
SrCl <sub>2</sub>	0,024
NaF	0,003

Sterilization:

20 minutes at 121°C

pH before sterilization:

Usage:

Additive to marine media

**Medium:** 5425

Name: Brain Heart Infusion (BHI) Agar

Composition	(g/l)
Calf brain infusion	200,0
Beef heart infusion	250,0
Casein (meat) peptone	10,0
Glucose	2,0
NaCl	5,0
Na <sub>2</sub> HPO <sub>4</sub>	2,5
Agar	15,0

Preparation:

Sterilization:

20 minutes at 121°C

pH before sterilization:

7,4

Usage:

Maintenance and revitalization

Organisms:

*Dermatophilus congolensis*, *Nocardia brasiliensis*, *N. asteroides*,  
*Rhodococcus equi*, *R. fascians*, *R. rhodochrous*  
*Streptomyces thermoviolaceus* subsp. *apingens*  
*Coryneform species*

**Medium:** 5426

Name: Trypticase Soy Agar

Composition	(g/l)
Casein peptone	15,0
Soy peptone	5,0
NaCl	5,0
Agar	15,0

Preparation:

Sterilization:

20 minutes at 121°C

final pH:

7,3

Usage:

Maintenance and revitalization

Organisms:

*Rhodococcus equi*, *R. rhodochrous*,  
*Nocardia asteroides*, *Coryneform species*

**Medium:** 5429

Name: Streptomycetes Medium

Composition	(g/l)
Glucose	4,0
Yeast extract	4,0
Malt extract	10,0
CaCO <sub>3</sub>	2,0
Agar	12,0

Preparation:

Sterilization:

20 minutes at 121°C

pH before sterilization:

Adjust pH to 7,2 with KOH before adding agar.

Usage:

Maintenance and revitalization

Organisms:

All Actinomycetes

**Medium:** 5436

Name: Trypticase Soy Yeast Extract Medium

Composition	(g/l)
Trypticase soy broth	30,0
Yeast extract	3,0
Agar	15,0

Preparation:

Sterilization:

20 minutes at 121°C

pH before sterilization:

7,0 -7,2

Usage:

Maintenance and revitalization

Organisms:

Coryneform Actinomycetes

**Medium:** 5630

Name: Middlebrook 7H9

Composition	(g/l)
Middelbrook 7H9 Broth Base	4,7
Glycerol	2,0
Casamino acids	11,0
Yeast extract	5,5
Tween80 (20%)	2,5
OADC-Enrichment	100 ml/l
Agar	15,0

Preparation:

Dissolve in distilled water. Before Use add 10% OADC stock solution

Sterilization:

20 minutes at 121°C

Usage:

Maintenance and revitalization

Organisms:

*Mycobacteria*

**Medium:** 5631

Name: Middlebrook 7H10

Composition	(g/l)
Middelbrook 7H10 Agar	19,0
Glycerol	5,0
OADC-Enrichment	100 ml

Preparation:

Dissolve in distilled water. Add 10% OADC stock solution after autoclaving and cooling to 42°C.

Sterilization:

20 minutes at 121°C

Usage:

Maintenance and revitalization

Organisms:

*Mycobacteria*

**Medium:** 5632

Name: Middlebrook 7H11

Composition	(g/l)
Middelbrook 7H10 Agar	20,0
Glycerol	5,0
OADC-Enrichment	100 ml

Preparation:

Dissolve in distilled water. Add 10% OADC stock solution after autoclaving and cooling to 42°C.

Sterilization:

20 minutes at 121°C

Usage:

Maintenance and revitalization

Organisms:

*Mycobacteria*

**Medium:** 5633

Name: OADC-Stock

Composition	(g/l)
Bovine Serum Albumin fraction V50,0	
1% Leic acid in 0,2N NaOH	50,0
Glucose	20,0
NaCl	8,5

Comments: Melt 1% Oleic Acid in 65°C water bath. Dissolve all ingredients in 700ml of ddH<sub>2</sub>O. Adjust volume to 1l. Filter sterilizes and store at 4°C. To make 1% Oleic Acid in 0,2N NaOH: Dissolve 5g oleic acid (ampoule) in 500ml 0,2N NaOH (300ml ddH<sub>2</sub>O + 20ml NaOH + adjust volume to 500ml). Heat solution to 55°C for oleic acid to melt. Store at -20°C in 50ml aliquots. There is no need to sterilize this.