Methods for the taxonomic description of the Actinobacteria

The strains were originally cultivated by media and conditiones 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_2HPO_4 0,5 g/l, $MgSO_4$ x $7H_2O$ 0,5 g/l, $FeSO_4$ x $7H_2O$ 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). About 1 ml of the shaking flask culture was plated out 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_2HPO_4 0,5 g/l, $MgSO_4$ x $7H_2O$; 1 ml/l trace element solution – $CuSO_4$ x $5H_2O$ 10,0 g/l, $CaCl_2$ x $2H_2O$ 10,0 g/l, $FeSO_4$ x $7H_2O$ 10,0 g/l, $ZnSO_4$ x $7H_2O$ 10,0 g/l, $MnSO_4$ x 7 H_2O 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 was described by the colors of the RAL-code (edition of 1990 – Reichsausschuß für Lieferbedingungen – Deutsches Instutut 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 refinding of the color. In addition to the RAL color a strain could be colorless.

- Aerial Mycelium (A)

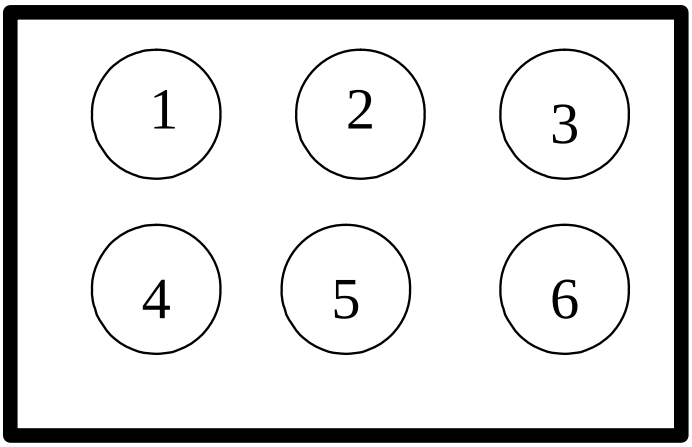
If aerial mycelium is formed it was differentiated between a good formation and a sparse formation. When aerial mycelium was well developed the color is described with the help of the RAL color code.

- Soluble Pigment (S)

If a soluble pigment is produced it was 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 identifikation.

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 *Streptomycetes* isolate showing the color of the substrate mycelium which its influenced by the soluble pigment and a sparse aerial mycelium only formed at the random aeria 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

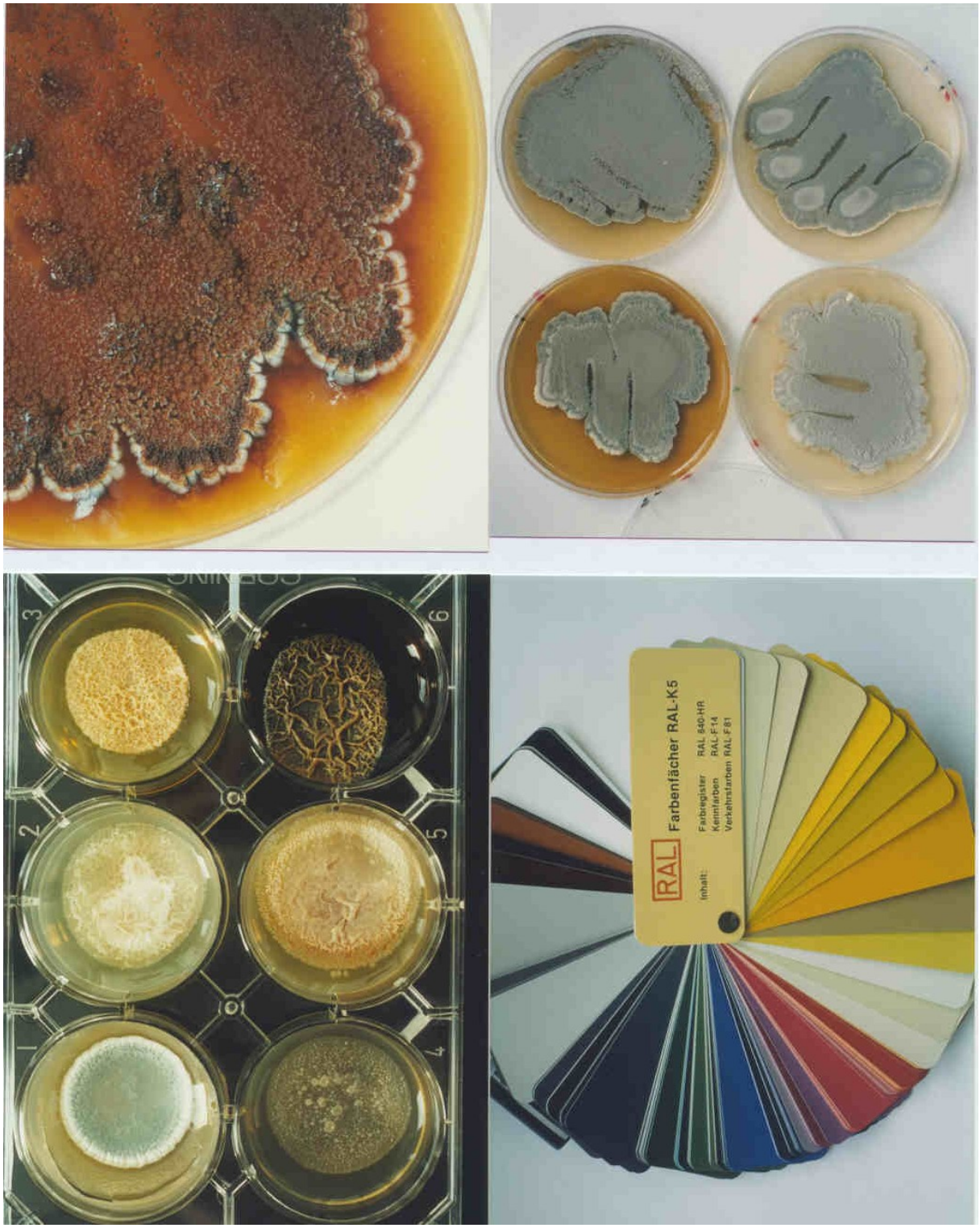
C

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

The 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´s 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

Melanin production on medium

5318	5322	5337 with Tyrosine	5337 without	Cluster
+	+	+	-	3
+	+	-	-	2
-	-	+	-	1
-	-	-	-	0

Some examples of melanin formation:

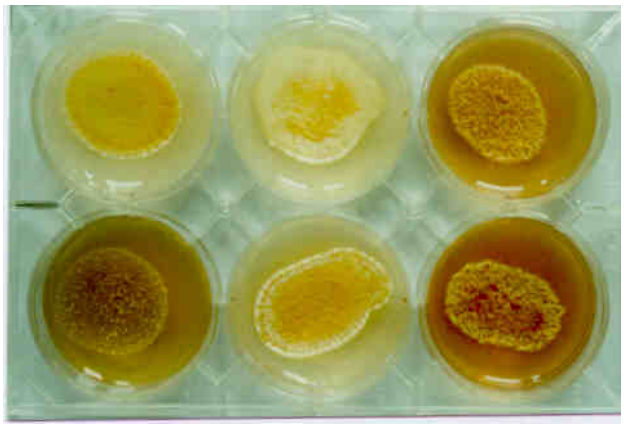
A - Streptomycete forming no melanoid pigment (cluster 0)

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)

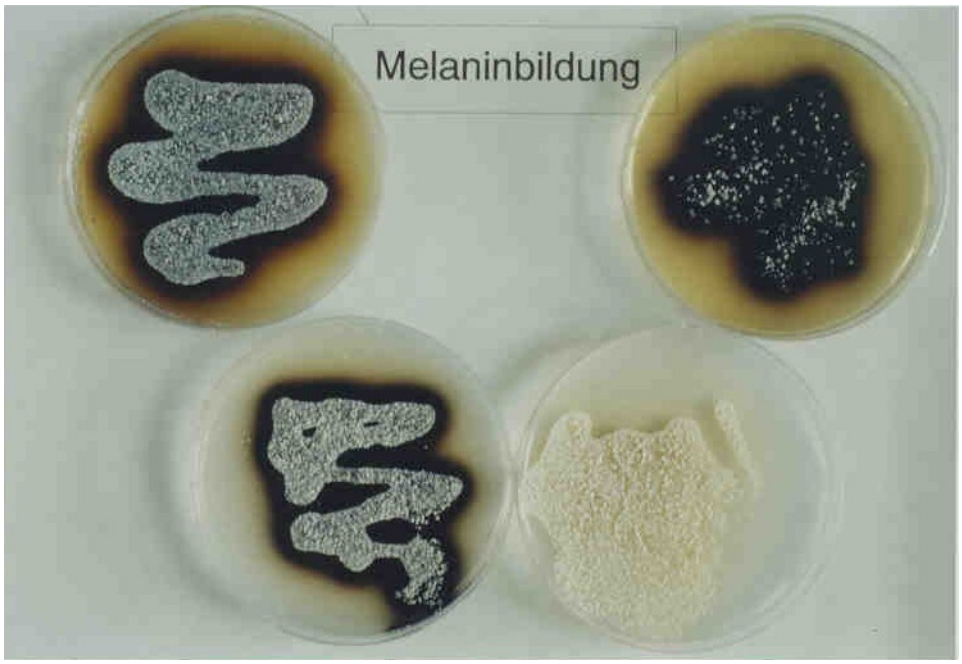
C - Two *Streptomycetes* of the cluster 1, melanin production significant only on 5337 with tyrosine

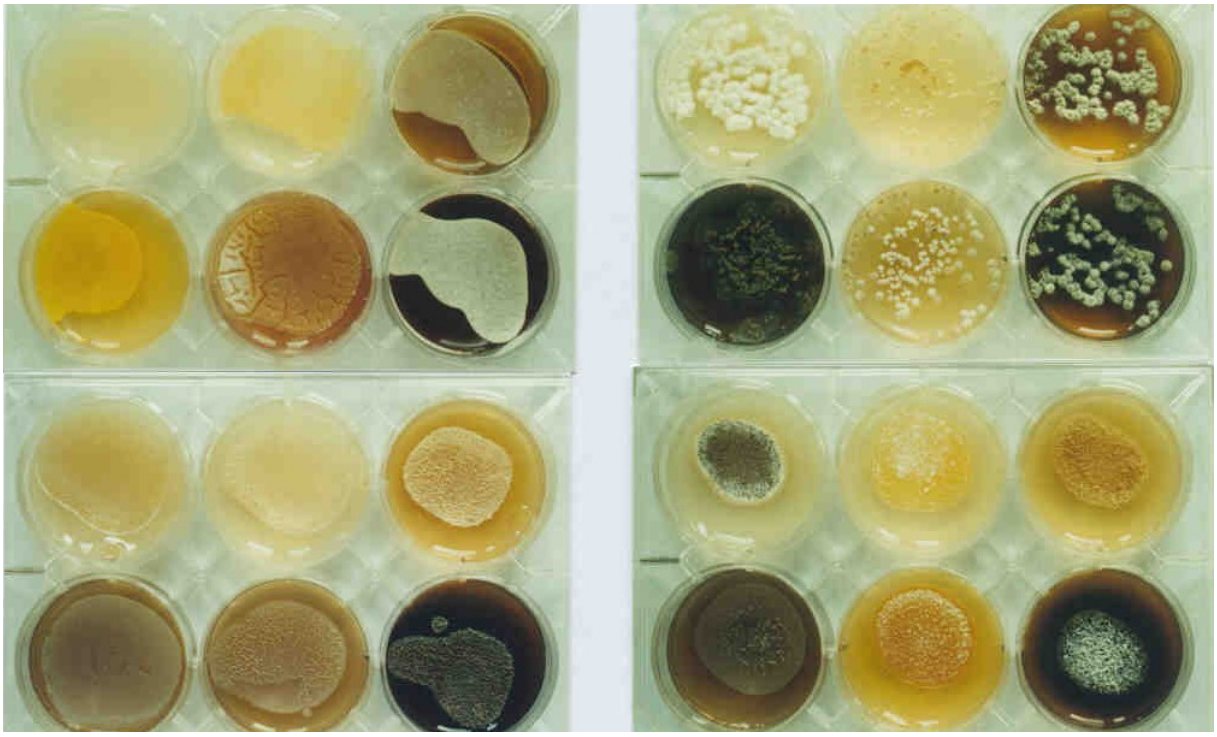
D - A typical production of melanoid pigment or a not to melanin correlated brown pigment

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 of the next pages.

After fife 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, than 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.

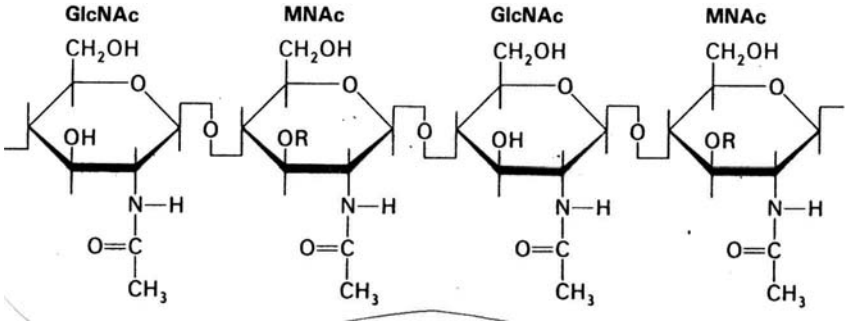
Two examples of sodium chloride resistance are shown.

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

(Lower) A 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 teiconic acids or modifications of the peptidoglycan molecule some Gram-positive bacteria got resistant against lysozyme. So are *Mycobacterium*, *Nocardia* and *„Streptoverticillium“* lysozyme resistant, most of the *Streptomyces* species are sensitive.
Lysozym hydrolyses the glycosidic bond between MNAc and GlcNAc:



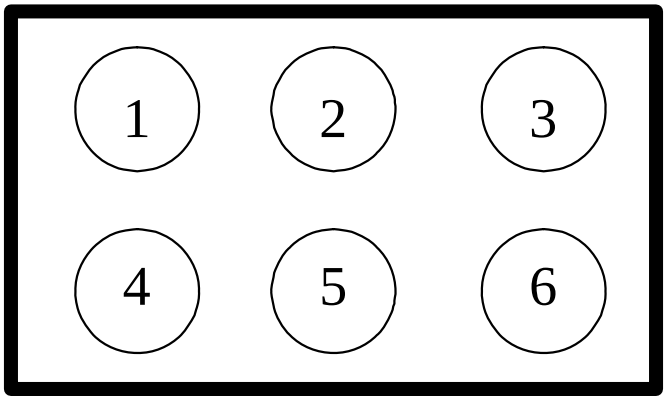
Analogue to the sodium chloride resistance the lysozyme resistance could be detected by a micro plate method which was modified after Kutzner et al (1978). The scheme of this test is shown on of the next pages.
In this case medium 5339 was filled in six wells of a six well cell culture plate (5 ml per well). The lysozyme stock solution was 100 mg lysozyme in 10 ml H_2O, sterilized by filtration. The final concentrations were 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 was 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 was 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.q. Costar (# 25810) will be filled sterile with 5 ml of agar.

Each well is marked by a number for easy identification.

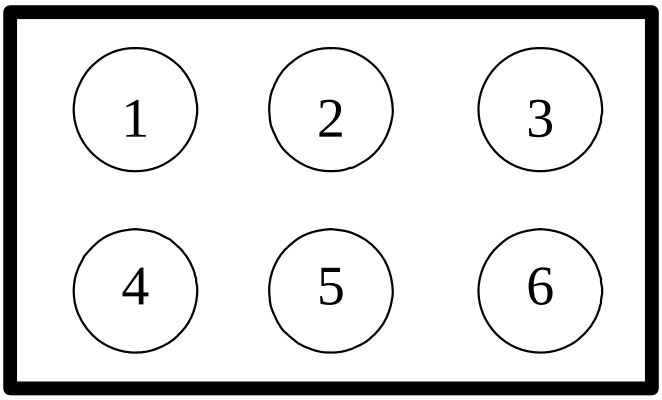
The filling pattern is:

Well 1	=>	agar 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.q. Costar (# 25810) will be filled sterile with 5 ml of agar.

Each well is marked by a number for easy identification.

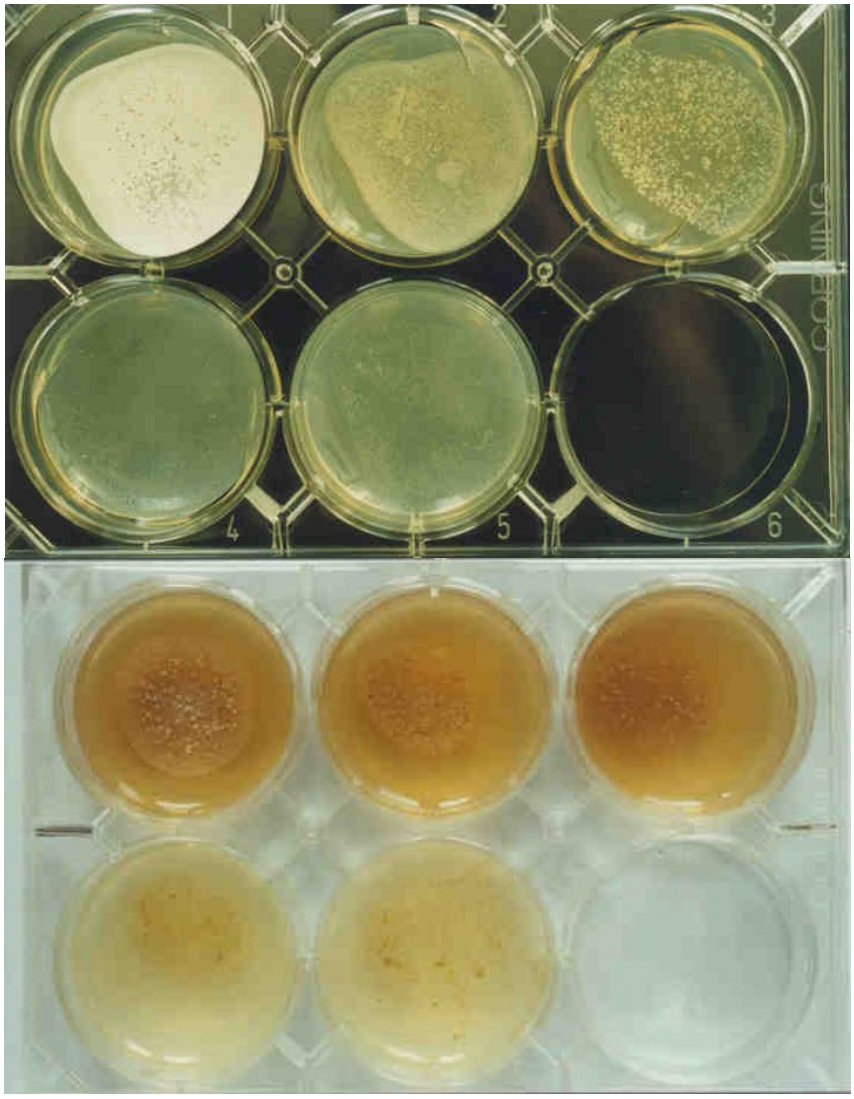
The filling pattern is:

Well 1	=>	agar 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

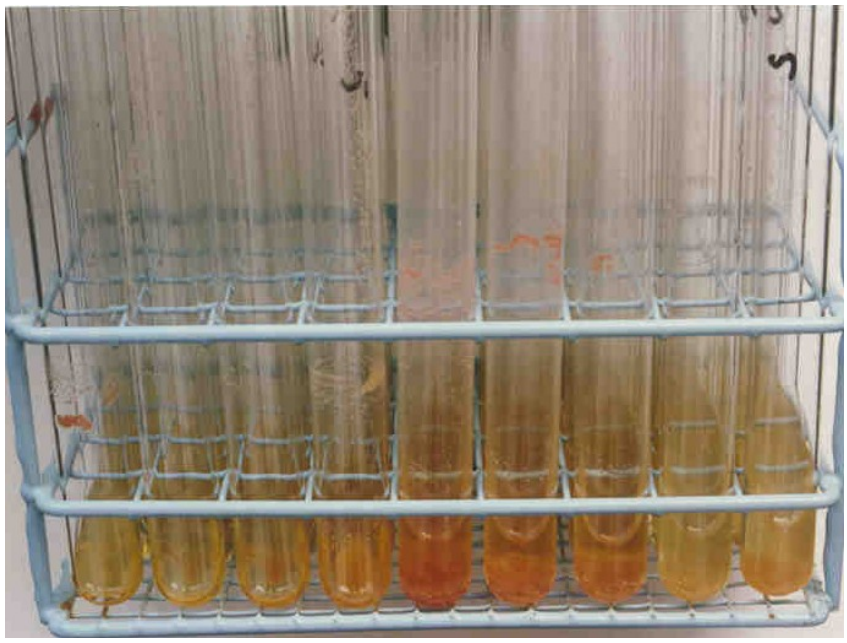
Lysozyme stock solution: 100 mg lysozyme in 10 ml water; sterilized by filtration.

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* (Bennedickt et. al 1955).
The ability of strains to use 10 compounds was tested in a micro plate (12 well) technique basing on the method of Shirling and Gottlieb (1966) with the basal agar 5338 ($(NH_4)_2SO_4$ 2,64 g/l, KH_2PO_4 2,38 g/l, K_2HPO_4 4,31 g/l, $MgSO_4$ x 7 H_2O 1,0 g/l and1,0 ml/l trace element solution – $CuSO_4$ x $5H_2O$ 0,64 g/l, $FeSO_4$ x $7H_2O$ 0,11 g/l, $ZnSO_4$ x 7 H_2O 0,15 g/l, $MnCl_2$ x 4 H_2O).
The following carbon sources were 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 were 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 was carried out after the following scheme:

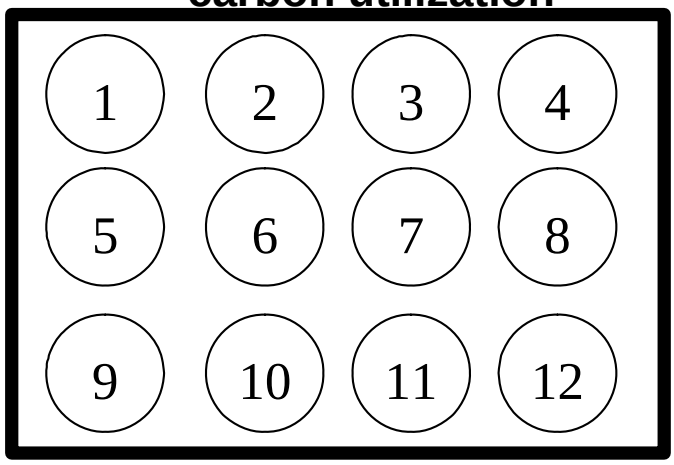
Growth not better than the negative control	-
Growth better like the negative control but not like the positive control	(+)
Growth like the positive control	+
Growth better like the positive control	++

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.q. Costar (# 430345) will be filled sterile with 2 ml of the agar.

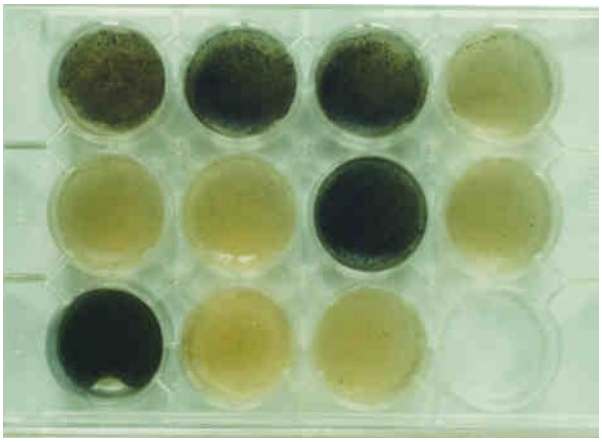
Each well is marked by a number for easy identification.

The filling pattern is :

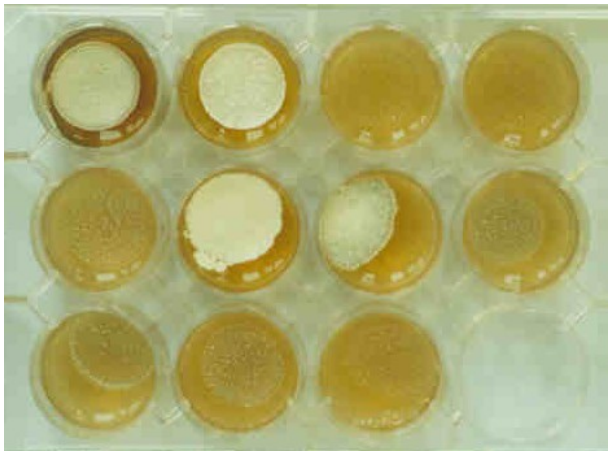
Well 1	=>	agar 5338 with 1% glucose
Well 2	=>	agar 5338 with 1% arabinose
Well 3	=>	agar 5338 with 1% sucrose
Well 4	=>	agar 5338 with 1% xylose
Well 5	=>	agar 5338 with 1% inositol
Well 6	=>	agar 5338 with 1% mannitol
Well 7	=>	agar 5338 with 1% fructose
Well 8	=>	agar 5338 with 1% rhamnose
Well 9	=>	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.

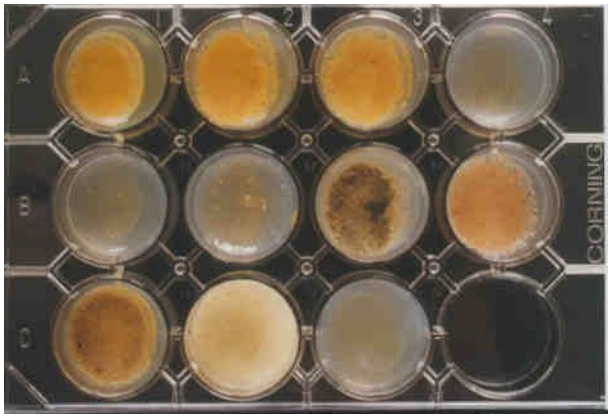
Carbon utilization



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 were used in routine description of the *Actinobacteria*.

The **api Coryne**, a test cit especially for the *Propionibacterineae*, *Micrococcineae* and *Corynebacterinae* (MacFaddin 1980).

This contain 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 hase 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-glucopyranoside
18	N-acetyl-β-glucoseamidase	1-naphthyl-N-acetyl-βD-glucoseaminide
19	α mannosidase	6-Br-2-naphthyl-αD-mannopyranoside
20	α fucosidase	2-naphthyl-αL-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_2S	sodium thiosulfate	H_2S 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 were grown in shaking flasks and in most cases had 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 had 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 keratiniphila*

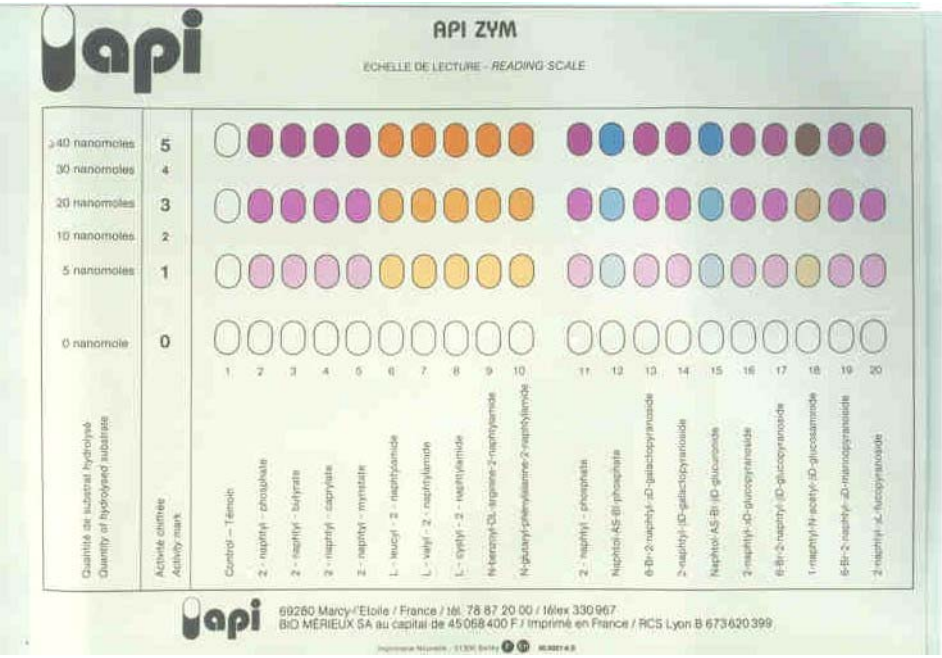
(B) Comparison of three Streptomyces species with api ZYM strips – FH 6304 *S. albolongus*, FH 6303 *S. albofaciens*, FH 6302 *S. afghaniensis*

(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). Biologs´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-Pyroglutamic 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

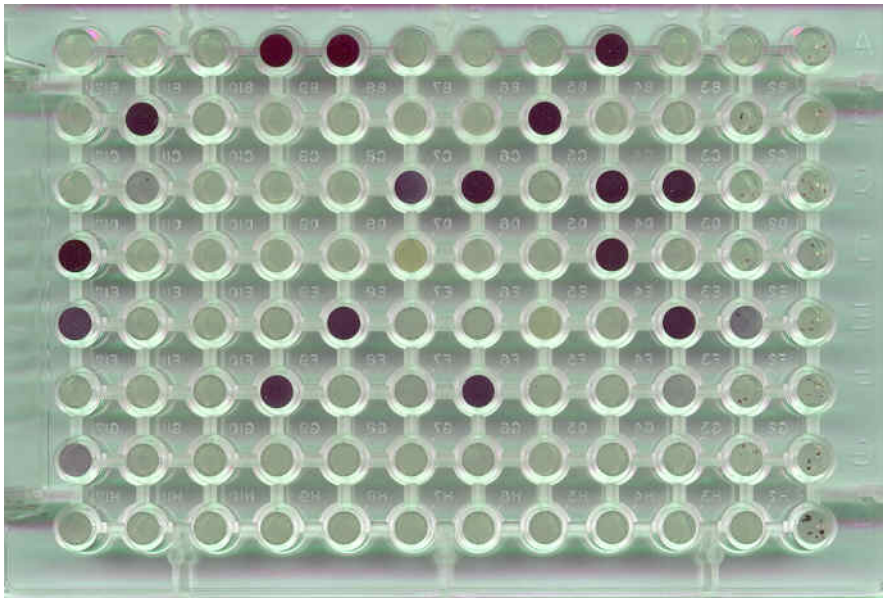
FIGURE 1. Carbon Sources in GP2 MicroPlate

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 theses products is the limited number of species and organism types that they can identify. Furthermore, theses 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 were incubated in shaking cultures medium 5006 at 28° and 35°C. The cultures were diluted with water and directly used with the GP2 MicroPlates. The resulting plates were 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 formular of the strain datasheet of *Nesterenkonia aethiopica* is shown, where all the data from the methods which are described in the chapters 1 and 2 (1-5) can be 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 cases that a test was not carried out it is marked as nd (not determinated). 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
ISP 2	good	zinc yellow
	A	SP
	none	none
	G	R
ISP 3	good	zinc yellow
	A	SP
	none	none
	G	R
ISP 4	good	zinc yellow
	A	SP
	none	none
	G	R
ISP 5	good	zinc yellow
	A	SP
	none	none
	G	R
ISP 6	good	zinc yellow
	A	SP
	none	none
	G	R
ISP 7	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			
-	-	-	-	-	-	-	-			

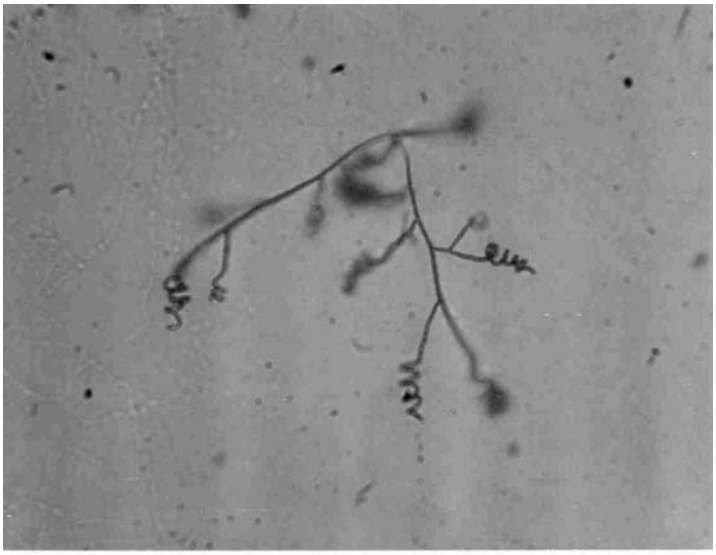
Comments:

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 were used. This objectives allow to work with a long distance between objective and object, so that the agar plates could directly be used as praeparates. The strains were cultivated on the media as described in part 1 of this chapter. For light microscopy mainly media with low turbidity were used.

The following characteristics were checked:

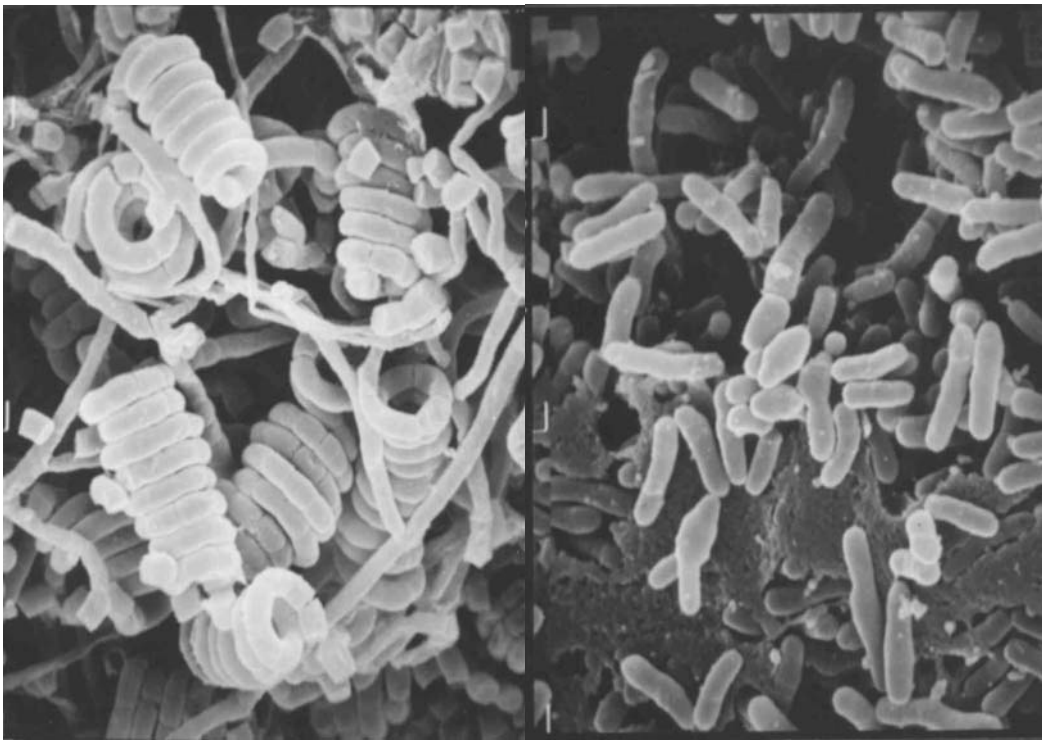
Spore chains	yes or no
Sporangia	yes or no
Single spores	yes or no
Fragmentation of aerial or substrate mycelium	yes or no
Spore chain morphology	Recti flexibilis, Retinaculum-apertum Spira, Verticillus



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

Scanning electron microscopy

A B



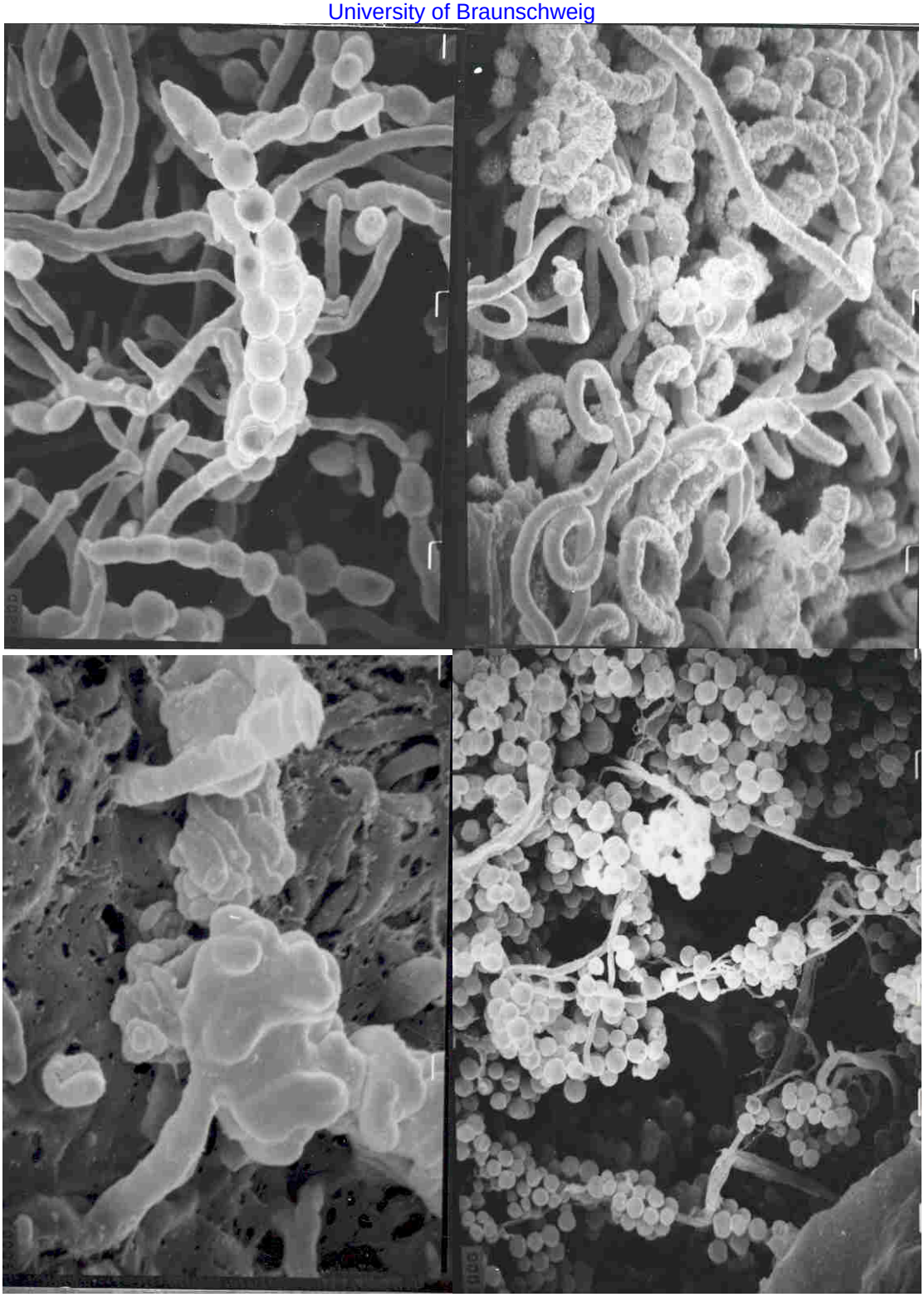
4. Scanning electron microscopy

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

Preparations of the strains were performed using a modified method of VOBIS and KOTHE (1985) described by WINK (1992). The strains were 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^2-pieces were 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 were dehydrated by incubation in 2-methylglycerol (three times, 10 minutes each). The methyl glycerol was replaced by dry acetone (three times, 10 minutes each) and the samples were dried at the critical point using CO_2. The samples were fixed on a brass cylinder and were covered with gold using the sputtering system. This method could 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 were fixed on lysine coated glass plates. Therefore the in glutaraldehyde fixed samples were 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 were transferred to the lysine plate and dried for a minute in the air. Than the plate is washed for two times for 10 minutes in pure ethanol. This samples were also dried at the critical point using CO_2, 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, formation of single spores. The spore surface is characterized as smooth, warty, rugose, knobby, hairy and spiny.
By the scanning electron microscopic investigation the samples could 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 determinated using the following scheme:
Magnification at SEM x 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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