Bacterial Systematics I

Species Identification by Chemotaxonomic Methods

Protocol

1st of February 1999
through
6th of February 1999

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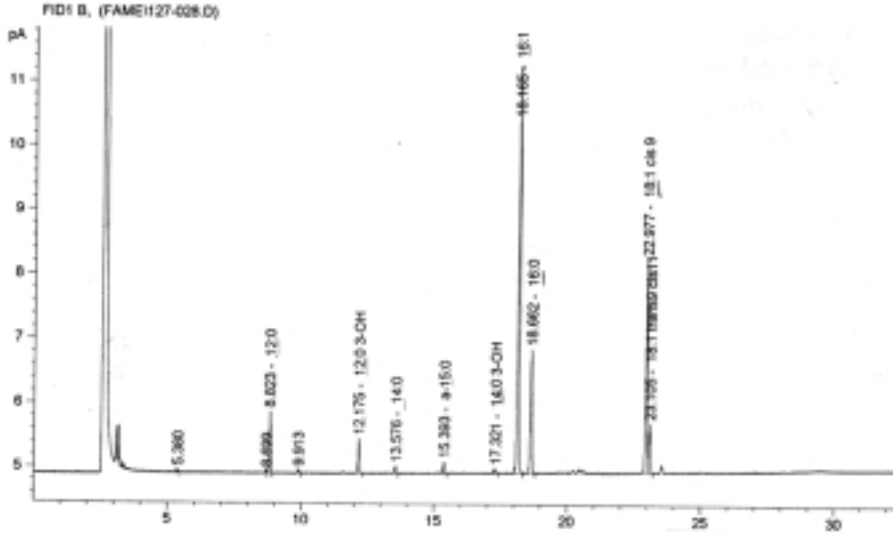
Appendix

Getting started A broad selection of bacterial strains have been collected from the Museum of Natural History in Vienna and cultivated on a several full-medium agar plates using the **spread plane method** on e.g. Trypcase Soy Agar, or other.
Samples of these cultures have been transferred on **streak plane technique** to obtain single colonies (isolated strains) of those unknown species on the agar (see appendix techniques).
To obtain enough bionic material, each of those individual colonies was transferred onto three separate full medium plates, and a single 4-sector plate, and incubated for a few days at room temperature.
Finally every student obtained a set of three spread plane dishes and a single 4-sector dish to execute the taxonomic analysis of a particular strain.

Note: Specimens and bacterial cultures should be considered infectious and handled appropriately by trained and competent staff
Disposal of used Material: After use, all utensils which are not meant to be reused (inoculated agar plates, Pasteur pipettes, mono-use syringes, Greiner containers, ampoules, strips, and incubation boxes of the api-test) should be autoclaved, incinerated, or immersed in a disinfectant for decontamination prior to disposal.

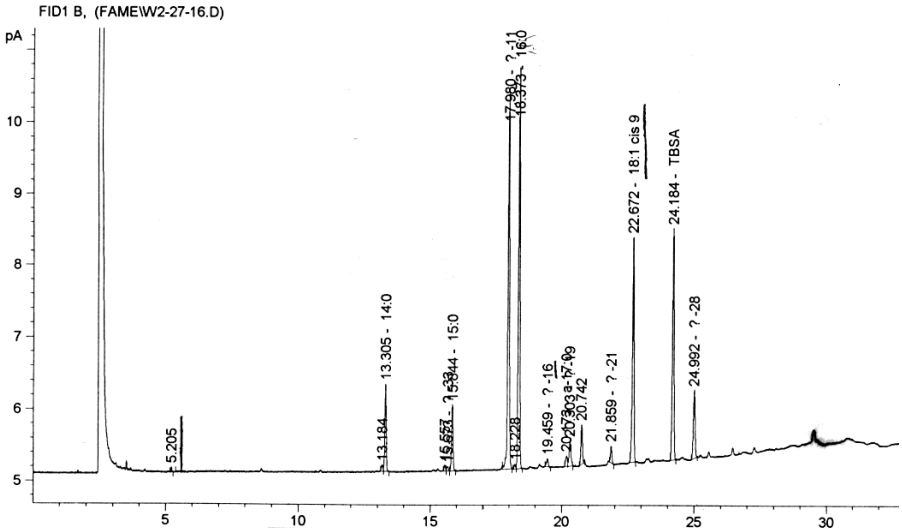
Preliminary Data: A Capillary Gas Chromatographic scan of the strains under investigation has been kindly provided by the students of the Institute of Veterinary Medicine working with Dr. Busse.
Analysis of fatty acids by capillary gas chromatography can be done in the following way:
Using a capillary gas chromatographic analyzer, a high chromatographic efficiency is obtained by using helium as a carrier gas, although hydrogen would yield better results (highly explosive). To fully exploit the resolving power of capillary columns the sample must be applied as a narrow band. This is commonly achieved by using the "hot needle" method, in which the sample is withdrawn into the syringe barrel, the needle is then placed in the injection port and allows to come to temperature before the plunger is rapidly depressed to flash vaporization of the sample.
A sample amount of 1ng/µL has been introduced into the capillary gas chromatographic analyzer; the results of the scan are given as follows:

Capillary Gas Chromatogram of strain ***W1-27-28***



Peak #	Ret. Time [min]	Type [-]	Width [min]	Area [pA·s]	Area [%]	FA-Name [-]
1	5.380	PB	0.0341	$1.37235E^{-1}$	0.23706	?
2	8.699	BP	0.0356	$8.43452E^{-1}$	0.14570	?
3	8.823	VB	0.0522	$31.95280E^{-1}$	5.51959	12:0
4	9.913	BB	0.0403	$1.62378E^{-1}$	0.29049	?
5	12.175	BP	0.0569	$19.76460E^{-1}$	3.41417	12:0 3-OH
6	13.576	PP	0.0446	$4.35169E^{-1}$	0.75172	14:0
7	15.393	BP	0.0517	$7.13514E^{-1}$	1.23254	a-15:0
8	17.321	PP	0.0428	$2.59759E^{-1}$	0.44871	14:0 3-OH
9	18.165	BB	0.0635	$260.32900E^{-1}$	44.96973	16:1
10	18.662	BP	0.0601	$76.43320E^{-1}$	13.20321	16-0
11	22.977	BV	0.0661	$129.69220E^{-1}$	24.13070	18:1 cis 9
12	23.105	VP	0.0681	$32.80260E^{-1}$	5.66638	18:1 trans 9 cis 11

Capillary Gas Chromatogram of strain ***W2-27-16***



Peak #	Ret. Time [min]	Type [-]	Width [min]	Area [pA·s]	Area [%]	FA-Name [-]
1	5.205	PB	0.0283	$1.49850E^{-1}$	0.16332	?
2	13.184	BV	0.0503	$3.53235E^{-1}$	0.38568	?
3	13.305	VP	0.0556	$45.9486E^{-1}$	5.01686	14:0
4	15.557	BV	0.0490	$3.13580E^{-1}$	0.34238	?
5	15.673	VP	0.0489	$2.76104E^{-1}$	0.30146	?-33
6	15.844	VB	0.0602	$36.7148E^{-1}$	4.00868	15:0
7	17.980	BB	0.0651	$222.219E^{-1}$	24.25911	?-11
8	18.228	PV	0.0424	$2.50444E^{-1}$	0.27345	?
9	18.373	VB	0.0610	$222.552E^{-1}$	24.62307	16:0
10	19.459	BB	0.0552	$5.33038E^{-1}$	0.58199	?-16
11	20.173	BV	0.0513	$5.81941E^{-1}$	0.63539	a-17:0
12	20.303	VB	0.0663	$17.16910E^{-1}$	1.87460	?-19
13	20.742	PB	0.0587	$23.22770E^{-1}$	2.52409	?
14	21.859	BP	0.0496	$9.03427E^{-1}$	0.98640	?-21
15	22.672	BB	0.0595	$133.30912E^{-1}$	14.53147	18:1 cis 9
16	24.184	BB	0.0631	$134.66450E^{-1}$	14.70325	TBSA
17	24.992	BB	0.0675	$43.85990E^{-1}$	4.78881	?-28

1. Determining GRAM Reaction (Experiment 1)

Grouping of bacteria is usually based upon their gross morphology and the manner in which they react to staining procedures. The Gram test, performed properly, differentiates nearly all bacteria into two major groups:

- Gram-positive (G^{Pos}), includes causative agents of diseases such as anthrax, diphtheria, etc.
- The Gram-negative (G^{Neg}), includes organisms which cause dysentery, cholera and different types of food poisoning.

The gram non-reactive group of microorganisms includes those which do not stain or which stain very poorly. Experiment split into two section in which the first utilizes the KOH-test to determine Gram reaction, whereas the subsequent Gram-staining procedure is used to confirm those results obtain with the KOH test.

1.1 KOH-Test - Day 1: 1st of Feb 1999:

material: marking pen (water resistant)
1 1ml pipette plus blue tips
2 object slide
1 inoculation needle
1 gas burner
1 igniter
10mL KOH-solution (3%)*
colony: 2 plate with the following colonies:
W1-27-28 (spread plane dish)
W2-27-16 (-"-)
(*)see appendix media and solutions

Purpose: A technique used to rapidly determine The GRAM reaction of an isolated colony. In G^{Neg} colonies, the KOH-solution lysis the bacterial cells wall and causes coagulation of the bacterial DNA. The thick peptidoglycan layer in G^{pos} strains prevents lysis.

Procedure: Mark slide and pipet a drop or two of KOH-solution onto the slide.

- Disinfect the inoculation needle in the burner's flame (until glowing red-hot), and place it at the edge of the collecting agar dish where no colonies are present. Once cooled off, extract a loop-full of raw material from the colony dish and dip it into the drops of KOH solution of your slide. Shake off sample and try to dissolve it completely. Observe if there is any thread-like reaction when lifting the needle.
- Repeat procedure with other strain as well.

Note: Discard the mouth pieces of the pipette each and every time after use.

Results and Evaluation: Day 1: 1st of Feb 1999

As far as regarding the left strain, only at the third attempt, the KOH-test finally yielded a useful result:

Strain	*W1-27-28*	*W2-27-16*
KOH-Test	Coagulation	-
GRAM-Reaction	G^{Neg}	G^{Pos}
visual examination of dish		
Colony shape	circular, slightly elevated	rather a dense continuos carpet
Colony color	white/yellow	yellow/orange

Coagulation scan of sample: As can be seen at the tip of the needle, lysis of the cell broth results in a thread-like viscous property.

1.2 GRAM-Stain: Day 1: 1st of Feb 1999:

Purpose: Confirmation of the observations made in previous experimentation.
Staining is almost essential in identifying an unknown bacterium. According to GRAM reaction, G^{Neg} appear red and G^{Pos} appear purple.

material: marking pen (water resistant)
1ml pipette plus blue tips
2 object slide
microscope w/ 100x objective and flask of lens oil
1 inoculation needle
1 gas burner w/ igniter
pair of latex gloves
flat pan w/ vertical slide holders
10mL NaCl-solution (0.5%)*
0.5L bottle of ethanol
100mL dye (Crystal Violet)
100mL fixing reagent (Lugol-soltn.)
100mL counter-stain (Safranin)
colony: PYE plates w/ the following strains:
W1-27-28 (spread plane dish)
W2-27-16 (-"-)

(*)see appendix media and solutions

Procedure: Mark slide with colony code and pipet a few drops of NaCl solution onto it.

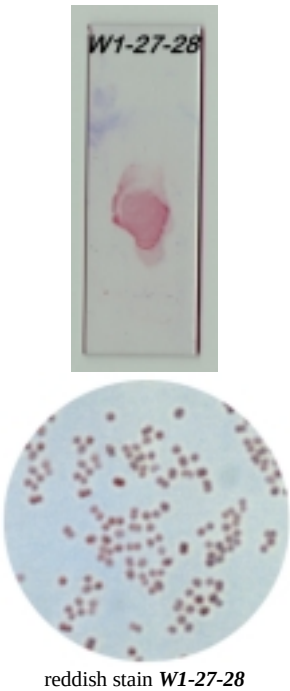
- Disinfect the inoculation needle in the burner's flame (until glowing red-hot), and place it at the edge of the collecting agar dish where no colonies are present. Once cooled off, extract a tiny sample of raw material from the colony dish and widely spread it along with the solution over the slide.
- Dehydrate emulsion gently over the burners flame until it is completely dry.
- Execute Gram Stain (see appendix techniques), examine the dried slide under the microscope at a magnification of 100 (oil-immersion lens) by placing a few drops of lens oil onto the section under investigation.
- Repeat procedure with other strain as well.

Note: Discard the mouth pieces of the pipette each and every time after use.

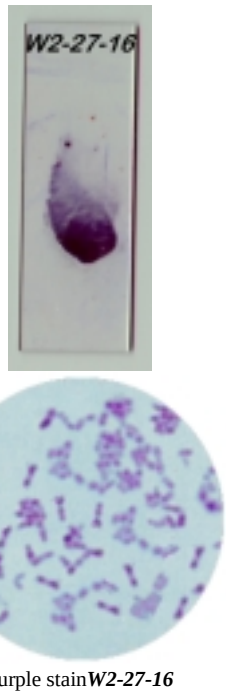
Results and Evaluation: Day 1: 1st of Feb 1999
The first series of slides carried an excess amount of dried material; staining results on those slides revealed a plain dark spot, making identification almost impossible. A second series, using tiny samples of biomass resulted in a weakly stained specimen allowing distinct differentiation with the microscope.

Strain	*W1-27-28*	*W2-27-16*
GRAM-Reaction	reddish	purple
GRAM-Stain	G^{Neg}	G^{Pos}
Cell shape	cocci	coccoidal-chains

staining procedure



reddish stain ***W1-27-28***



purple stain***W2-27-16***

2. Substrate Degradation (Experiment 3)

The ability to attack different substrates consisting of carbon, hydrogen and oxygen varies with the battery of enzymes present in the bacteria being identified. Complex carbohydrates are first hydrolyzed by extracellular enzymes to molecules of sufficiently small size to enter readily into the cell. Further dissimilation results in end-products that can be detected by various methods. The carbohydrate decomposition spectrum determined by production of acid from substrates is now regarded as a secondary tool in classification, but for the characterization of lower taxa and for epidemiological purposes it is still a valuable feature.
A satisfactory basic medium for determining the fermentation reactions of microorganisms must be capable of supporting growth of the organisms under study, and free from fermentable carbohydrates which could give erroneous interpretations. It must be stable, uniform in composition, give distinct reactions and yield accurate results.
This experiment is proceeded by several preparative steps which are needed to determine the strains preferences for certain carbon-sources and mineral utilizing capabilities.

material: marking pen
3 0.2L Erlenmeyer flask
50 20mL test tubes w/ cap
10 50mL Greiner sealable tubes
10 20mL single-use syringe
10 sterile acrodisc (pore-∅: 0.2µm)
10mL pipette
1000µl pipette w/ blue tips
autoclaver
vortex (vx5-genie)
digital flat-pan balance
gas burner w/ igniter
1.5g Phenol red (B203)
1L deionized water (squeeze flask)
0.5% NaCl solution*
sugars: 2.5g D-Glucose ($C_6H_{12}O_6$)
2.5g Gluconat ()
2.5g Inulin ()
2.5g Lactose ($O(CH(COH_2)_3CH_2OH)_2$)
2.5g Mannose ($COH(COH_2)_4CH_2OH$)
2.5g Manitol ()
1.5g Raffinose ()
1.5g Ribose ($COH(COH_2)_3CH_2OH$)
1.5g Trehalose ()
2.5g D-Xylose ($COH(COH_2)_3CH_2OH$)
minerals: 1.6g di-potassium-hydrogen-phosphate ($K_2HPO_4{\cdot}3H_2O$)
0.64g sodium-dihydrogen-hophate ($NaH_2PO_4{\cdot}3H_2O$)
0.04g tri-sodium-citrate ($C_6H_5Na_3O_7{\cdot}2H_2O$)
0.195g ferrous-sulfate ($FeSO_4{\cdot}7H_2O$)
0.22g ammonium-sulfate [$(NH_4)_2SO_4$]
0.02g magnesium-sulfate ($MgSO_4{\cdot}7H_2O$)
other: 2.5g amber ()
2.5g fumarin ()
2.5g citric acid ($COH(CH_2COOH)_2COOH$)
2.5g D-sorbitol ()
colony: PYE plates w/ the following strains:
W1-27-28 (spread plane dish)
W2-27-16 (-"-)

(*)see appendix media and solutions

2.1 Preparation of Carbohydrates - Day 1: 1st of Feb. 1999

Purpose: At least 10 different carbon reactants are required to obtain a better picture of a strains preferences for different sources of sugar.

Procedure: According to the table listed below, weigh the given amount of each sugar and dissolve it in approx. 25mL of deionized water in separate labeled Greiner tubes.
2.5g D-Glucose, filled up w/ H_2O to the 25mL mark
2.5g Gluconat - " -
2.5g Inulin - " -
2.5g Lactose - " -
2.5g Manose - " -
2.5g Manitol - " -
1.5g Raffinose - " -
1.5g Ribose - " -
1.5g Trehalose - " -
2.5g D-Xylose - " -

- For sterilization, each solution is transferred into a syringe and squeezed through a µ-filter disc into a sterile Greiner tube. Sweep both tap and tube before and after filling it with the sterilized solution trough the burner.

Note: Apply gentle pressure to the plunger of the syringe, since the tiny pore-diameter exerts a considerable resistance.

Results and Evaluation: Day 1: 1st of Feb 1999
Manose and inulin solution removed from program due to non-sterile preparation.

Membrane filtration using mono-use syringe and a tough disc, generally composed of cellulose acetate or cellulose nitrate.

2.2 Preparation of Phenol-Red Indicator and Mineral Base - Day 1: 1st of Feb. 1999

Purpose: Phenol red (phenol-sulfon-phthalein) with a pH range of 6.8 to 8.4, is an excellent indicator. Bacterial metabolic activity generates acidic products which cause a reactions from red to yellow. In the case of mineral base, ammonium sulfate is the sole source of nitrogen, and the various types of sugars added later on serve as a source of carbon in this medium. A positive reaction is indicated by a precipitate after several days of incubation at room temperature.

Procedure: Mark all tubes before filling with any substance to avoid any confusion.

- **Indicator**: 1.5g of phenol-red is added to the 0.2L flask and filled up with deionized water. 5mL of this indicator solution is pipetted into each of the 20 test-tubes provided.
- **Mineral Base**: To obtain the desired concentration of ferrous-sulfate of 1.95g/L, 0.195g of it is filled up to the 0.2L flask. Then the remaining amounts of mineral alts are added and filled up to the 0.1L mark with deionized water.
  1.6g di-potassium-hydrogen-phosphate ($K_2HPO_4 \cdot 3H_2O$)
  0.64g sodium-dihydrogen-hophate ($NaH_2PO_4 \cdot 3H_2O$)
  0.04g tri-sodium-citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$)
  0.22g ammonium-sulfate [$(NH_4)_2SO_4$]
  0.02g magnesium-sulfate ($MgSO_4 \cdot 7H_2O$)
  5mL of this mixture is pipetted into other 16 test-tubes.
  2 "0-control" test-tubes (1 for each strain) of the phenol-red is prepared as well.
- **Other**: Each of the four other remaining chemicals are separately placed in Greiner tubes and filled up w/ H_2O to the 25mL mark. Pipette 5mL into each tube (2 tubes of each base/strain)
- **NaCl**-solution: 5mL of this broth is pipetted into two test-tubes (1 for each strain) and mixed vigorously on the vortex.
- All 46 sealed test tubes have to be autoclaved (sterilized) at 120°C for at least two hours.

2.4 Inoculating the Test solutions - Day 1: 1st of Feb. 1999

Purpose: Transferring a sample from the solid medium into solution to inoculate each of the mineral and sugar probes with the test strain under investigation to further convert the liquidized broth to the culture media prepared before.

Procedure: Each strain obtains 23 test tubes (2 NaCl, 4 other, 8 mineral, and of 10 phenol-red). After disinfecting the inoculation loop in the burner's flame (until glowing red-hot), and placing it at the edge of the source plate where no colonies are present, an abundant sample should be extracted, dissolved in 1 NaCl test-tube, and shaken vigorously on the vortex.

- To each of the 4 *other* tubes, to each of the 8 mineral tubes, and to each of the 10 phenol-red tubes, 100µL of the liquidated broth is added.
- Each of the phenol-red and mineral base obtains a 500µL dose of one type of sugar, except for a "0-control" test-tubes of phenol-red (reference control).
- Repeat procedure for the other strain.

Note: To keep all test-tubes sterile, uncap tubes, sweep both cap and tube through the burner's flame, pipette the liquidized colony into the tube, sweep again tube and cape through flame and close inoculated tubes.

Results and Evaluation: Day 5: 5th of Feb 1999

Fermentation of the added reactant b the inoculated culture causes phenol red to change its color from red to yellow. The "0-control" growth broth remained clear and bright red, indicating that sterile working procedure was maintained during the entire procedure.

	Phenol-red base											Mineral base										Other			
Strain	0-control	D-Glucose	Gluconat	Lactose	Inulin	Manitol	Mannose	Raffinose	Ribose	Trehalose	D-Xylose	D-Glucose	Gluconat	Lactose	Inulin	Manitol	Mannose	Raffinose	Ribose	Trehalose	D-Xylose	Amber	Citric acid	D-Sorbitol	Fumarin
W2-27-16	-	+	-	-		-		+	+	-	+	+	+	+		+		+	+	+	+	+	+	+	+
W1-27-28	-	-	-	-		-		-	+	-	+	+	+	+		+		-	+	+	+	-	+	+	-

Legend

Positive	+	*negative*	-	*precipitate*	+	*not executed*	

3. Quinone Analysis (Experiment 4)

Isoprenoid quinones are free lipids that can be readily extracted from bacterial cells using lipid solvents such as acetone, chloroform and hexane. It is normally achieved with any one of these solvents or with a mixture of any two of them. They are also susceptible to photo-oxidation in the presence of oxygen and strong light but it is not necessary to work in a nitrogen atmosphere or dim light. It is, however, good practice to conduct extraction and subsequent purification procedures fairly rapidly, only storage should be carried out under protected conditions. It is now well established that isoprenoid quinones occur in the cytoplasmic membranes of most prokaryotes. Different bacteria not only synthesized different quinone classes (e.g. menaquinones, ubiquinones) but the number of isoprene units in the multiprenyl side-chain often vary amongst taxa and that this structural variation can be of value in prokaryote systematics.

3.1 Quinone extraction of both Strains- Day 2: 2nd of Feb. 1999

Purpose: Lysis of the cells to obtain the quinone containing portion.

material: marking pen and soft pencil
spatula and scalpel
water-jet pump
gas-burner w/ igniter
magnetic stirrer w/ magnetic rod
6 15mL sealable Pyrex bottle w/ Teflon-lined screw cap
4 5mL sealable Pyrex bottle w/ Teflon-lined screw cap
3 0.3L sealable flasks
5 Pasteur pipettes (230mm) w/ rubber bulb
1000µL pipette + blue tips
N_2-outlet (source) w/ rubber-hose
Centrifuge w/ tubes + adapter
digital flat-pan balance
TLC-chamber and
1 TLC plate (silica gel 60 F_{254})
HPTLC chamber and
2 HPTLC plates (RP18 F_{254})
0.1L petroleum benzine (-)
0.1L di-ethyl ether ($C_4H_{10}O$)
0.1L tetra-hydrofurane (C_4H_8O)
0.1L acetonitril (CH_3CON)
0.1L acetone (CH_3COCH_3)
0.1L deionized water
UV-lamp (254/366nm)
0.1L hexane (C_6H_{14})
0.1L methanol (CH_3OH)
0.1L of 0.3% NaCl solution*
ice bath in Styrofoam container
colony: PYE plates w/ following strains:
W1-27-28 (spread plane dish)
W2-27-16 (-"-)

(*)see appendix media and solutions

Procedure: Both strains need to be prepared simultaneously in separate tubes.

- Pipette 2mL of methanol and 1mL of hexane into each Pyrex tube to obtain a desired ratio of 2:1.
- Gently extract some biomass from the agar and add it to the alcohol mixture (one strain per flask, clean spatula before dipping it into the other culture plate). Add a small magnetic.
- Before tapping the flask tightly, slowly exchange the trapped air with N_2 (use Pasteur pipette to blow out the remaining air). Mark flask properly.
- Place flasks on a magnetic stirrer for ≈ 30min, before popping them in an ice bath to allow phase separation. Once separated, pipette an extra 1mL of hexane to the mixture and shake moderately.
- Transfer entire mixture (of both strains) into separate **marked** centrifugal test tubes, dock them into their rubber adapters and make sure that they are equally balanced (add some of the 1:1 methanol-hexane mixture to balance both tubes) and centrifuge for 5mins at 3000rpm.
- After centrifugation extract the upper hexane phase (w/ Pasteur pipette) and pour it into the 5mL sealable flask (one strain per flask). Swap trapped air with N_2, tap them and mark them properly.
- Replace extracted hexane by adding 2mL of chilled hexane and 2mL of 0.3% NaCl solution to both centrifugal tubes to obtain a solute ratio of 1:1:1 (methanol:hexane:NaCl). Balance both tubes accurately and adapters before executing another centrifugation (5mins at 3000rpm).
- After the 2nd centrifugation, extract the upper hexane-phase from the tubes and add it to the 5mL flasks of the 1st extraction made (don't mix the flasks and their extracts). Replace trapped air with the extracted hexane-phase with N_2, using a Pasteur pipette and rubber hose.
- Transfer the remaining methanol phases into sealable 15mL Pyrex tubes, and swap air against N_2 gas (same procedure as above). *Since these samples can be used for the polar lipid analysis (experiment 4), store those containers at -20°C under darkness.*

Note: Since quinones are light and oxygen sensitive, entire procedure should be done under dim and O_2 free conditions. If extract is not used the same day, store them at -20°C in a freezer.

3.2 Quinone Separation - Day 2: 2nd of Feb. 1999

Menaquinone (vitamin K2): A naturally occurring class of molecules in which the length of C3 isoprene side-chain which varies from one up to 15 isoprene units. Recent studies indicate that the position of hydrogenation in multiprenyl side-chains can be very specific and consequently of taxonomic value.
Ubiquinones (coenzyme Q): Their forms are widely distributed amongst animals, plants, and microorganisms. Ubiquinones have a more restricted distribution amongst prokaryotes than menaquinones. They are present in many Gram-negative bacteria; they do not exhibit the same degree of structural variation as components of the menaquinones series.

Purpose: Separation of ubiquinone from menaquinone via Thin Layer Chromatography (TLC).

Procedure: Using the extracts made in the previous steps.

- Reduce the hexane (solute) of the solution to about 0.5mL under a gentle flow of N_2 gas (using Pasteur pipette and rubber hose - Evaporation chills the flask considerably).
- Mix about 100mL of running agent in the following ratio and store it in a 0.3L sealable container:
  petroleum benzine:di-ethyl-ether (0.85:0.15) (see appendix techniques).
- Pipette the extracts in a narrow band (roughly 1cm in length) onto a $60F_{254}$ silica gel plate. Place it into the TLC chamber and let chromatographic separation proceed under dim light until the running agent almost reaches the upper limit of the plate (3-4 cm before that).
- Once separation is complete, briefly evaluate chromatogram under the UV-lamp and mark the designated areas of interest with a soft pencil.

Note: Quinones are light sensitive. Therefore, work under dim light conditions and limit observations under UV to as short as possible.

Results and Evaluation: Day 2: 2nd of Feb. 1999

Separation was distinct and clearly visible in both strains. Menaquinone, as the more agile molecule is situated on the top, whereas ubiquinone (more adherent to the stationary phase) is found at the lower end of the plate.

Strain	Quinone	
W1-27-28	**Q**	few **Q**
W2-27-16	-	large spot of **Mk**

Scan of the TLC-plate showing the separated quinones:

Upper limit of mobile phase

....Menaquinone

....Ubiquinone

starting position

3.3 Ubiquinone Separation - Day 2: 2nd of Feb. 1999

Purpose: Separating the sub-classes of ubiquinone from each other by using the *W1-27-28* extract only; (separation according to their isoprenoid side-chains).

Procedure: Prepare a tiny amount of hexane-methanol mixture (approx. 0.05L).

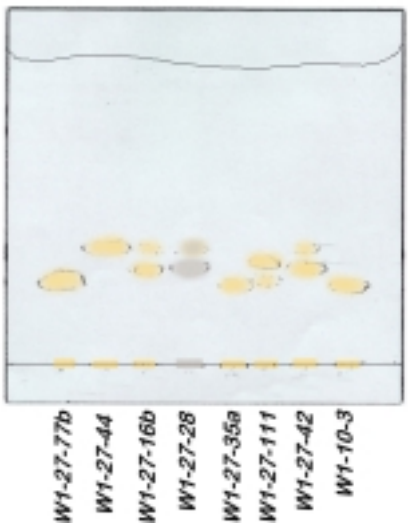
- Use a glass-wool popped Pasteur pipette attached via a rubber hose to a water-jet pump to gently scratch the outlined **ubiquonone** portion (Q) of the TLC-silica gel plate with a scalpel (break off the narrow part of the pipette and stick that side into the hose, popping the base of the wide portion with glass wool).
- Suck the extracted debris into the pipette, and flush them with a approx.0.5mL of the hexane-methanol solution to obtain the filtrate for further analysis into a sealable 15mL Pyrex flask.
- Place the elute into the ice bath until phase separation takes place. Add 5 drops of 0.3%NaCl and 0.45mL of chilled hexane to the mixture.
- Extract the upper hexane-phase with the dissolved **ubiquinones** with a Pasteur pipette into a 5mL Pyrex flask.
- Reduce the hexane (solute) of the solution to about 0.5mL under a gentle flow of N_2 gas (use Pasteur pipette and rubber hose - Evaporation chills the flask considerably).
- Mix about 100mL of running agent in the following ratio and store it in a 0.3L sealable container: acetonitril:tetrahydrofurane (0.65:0.35) (see appendix techniques).
- Pipette the extracts in a narrow band (roughly 1cm in length) onto the 1st $18F_{254}$ silica gel plate. Place it into the HPTLC chamber and let chromatographic separation proceed under dim light until the running agent almost reaches the upper limit of the plate.
- Once separation is complete, briefly evaluate chromatogram under the UV-lamp and mark the designated areas of interest with a soft pencil.

Note: If extract is not used the same day, exchange the oxygen containing air of the 5mL flask with a N_2-atmosphere, seal properly and mark flask store them at -20°C in a freezer preferably under darkness.

Results and Evaluation: Day 4: 24th of April 1997

Separation was distinct and clearly visible in both strains. According to the professional advice given, The more agile Ubiquinone-8 (above Q-9) is slightly lower in concentration than the more adherent Ubiquinone-9 (at the bottom).

Strain	**Q-8**	**Q-9**
W1-27-28	approx. 40%	approx. 60%



upper limit

Q-8
Q-9
Q-10

Staring position

Ubiquinone separation

3.4 Menaquinone Separation - Day 3: 3rd of Feb. 1999

Purpose: Separating the sub-classes of Menaquinone from each other by using the *W2-27-16* extract only; (separation according to their isoprenoid side-chains).

Procedure: Prepare a tiny amount of hexan-methanol mixture (approx. 0.05L).

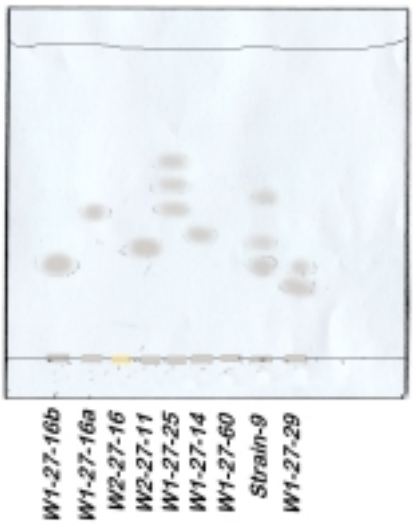
- Use a glass-wool popped Pasteur pipette attached via a rubber hose to a water-jet pump to gently scratch the outlined **menaquinone** portion (Mk) of the TLC-silica gel plate with a scalpel (break off the narrow part of the pipette and stick that side into the hose, popping the base of the wide portion with glass wool).
- Suck the extracted debris into the pipette, and flush them with a approx.0.5mL of the hexane-methanol solution to obtain the filtrate for further analysis into a sealable 15mL Pyrex flask.
- Place the elute into the ice bath until phase separation occurs. Add 5 drops of 0.3%NaCl and 0.45mL of chilled hexane to the mixture.
- Extract the upper hexane-phase with the dissolved **menaquinones** with a Pasteur pipette into a 5mL Pyrex flask.
- Reduce the hexane (solute) of the solution to about 0.5mL under a gentle flow of N_2 gas (use Pasteur pipette and rubber hose - Evaporation chills the flask considerably).
- Mix about 100mL of running agent in the following ratio (acetone : water - 0.99:0.01) and store it in a 0.3L sealable container; see appendix techniques.
- Pipette the extracts in a narrow band (roughly 1cm in length) onto the 2nd $18F_{254}$ silica gel plate. Place it into the HPTLC chamber and let chromatographic separation proceed under dim light until the running agent almost reaches the upper limit of the.
- Once separation is complete, briefly evaluate chromatogram under the UV-lamp and mark the designated areas of interest with a soft pencil.

Note: If extract is not used the same day, exchange the trapped air of the 5mL flask with a N_2, seal properly and mark flask store them at -20°C in a freezer preferably under darkness.

Results and Evaluation: Day 3: 3rd of Feb. 1997

Although extracts where obtained and adequately applied onto the HPTLC-plate, no traces of menaquinone where found. This could have been due to an improperly prepared sample or to the fact that colleagues within the course that accidentally worked on our sample (similar strain-ID) did not execute the necessary precaution in preparing the sample.
Observations made with fellow colleagues working on a similar strain, we found surprising parallels with our sample (*W2-27-11*); therefore, we concluded that their results gave us a clue of what the menaquinone analysis could have been:*

Strain	**Mk-10**	**Mk-9H_2**
W2-27-16	None	None
(*) *W2-17-11*	<10%	>90%



Upper limit

Mk-7
Mk-8 or Mk-7H_2
Mk-9
Mk-10 or Mk-9H_2

Starting position

Menaquinone separation

4. Polar Lipid Analysis (Experiment 2)

Polar lipids are essential components of the plasma membrane and play an important role in its regulation and permeability. Both polar and non-polar lipids are free lipids and as such are readily extractable into organic solvents of the correct polarity. The most common polar lipids are phospholipids but glycolpids and amino acid amide lipids are also found. Phospholipids such as phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol are widely distributed, hence of great taxonomic value.

4.1 Increasing Biomass - Day 1: 1st of Feb. 1999

Purpose: To obtain enough raw material from each strain for lipid analysis, more plates have to be prepared.

Procedure: Disinfect the inoculation needle in the burner's flame (until glowing red-hot), and place it at the edge of the source plate where no colonies are present. Once cooled off, localize at least three single colonies from the 4th sector plate, extract and transfer one colony at a time to one of the three provided agar dishes using the spread plane technique (see appendix techniques). Mark plates with colony-ID and date.
Repeat procedure for the remaining strain and incubate them at room temperature for two days.

Note: Use a light touch, try not to dig into the agar.

material: marking pen and soft pencil
6 PYE culture plates
inoculation needle
gas burner w/ igniter
heating block stove (0-200°C)
incubator
vortex
1000µL pipette w/ blue tips
20mL pipette w/ yellow tips
digital flat-pan balance
centrifuge
2 5mL sealable Pyrex bottle w/ Teflon-lined screw cap
4 centrifugation tubes w/ adapters
N_2-outlet (source) w/ rubber-hose + 4 Pasteur pipettes
10µL glass syringe
pair of latex gloves
TLC-chamber and 4 TLC plate (silica gel 60 F_{254})
UV-lamp (254/366nm)
0.1mL chloroform ($CHCL_3$)
0.1mL methanol (CH_3OH)
0.1L acetic acid (CH_3COOH)
10mL 0.3% NaCl solution*
0.1L deionized water
spraying reagents in spraying-flask:
molybdenum-blue (MbO_2 in H_2SO_4)
ninhydrine (dissolved $C_9H_6O_4$)
α-naphtol (in H_2SO_4)
colony: PYE plates w/ following strains:
W1-27-28 (4-sector dish)
W2-27-16 (-"-)
15mL bottle w/ methanolic phase of strain *W1-27-28* (from exp. 3)
15mL bottle w/ methanolic phase of strain *W2-27-16* (from exp. 3)
(*)see appendix media and solutions

4.2 Extraction of Polar Lipids - Day 3: 3rd of Feb. 1999

Purpose: To obtain the lipids from cell material, it is necessary to isolate the lipids and to transfer them into a volatile medium.

Procedure: Gently remove bionic material from the three extra cultivation dishes avoiding any abrasion of agar and dissolve it in the 15mL Pyrex bottle containing the aqueous, methanolic phase obtained from experimentation 3 (approx. 4mL total volume and 3 dishes per strain).

- Add 5.5mL of chloroform:methanol solution (volumetric ratio of 2.5:3) to the aqueous methonalic suspension to obtain a mixing ratio of chloroform:methanol:0.3%NaCl of 1:2:0.8, briefly gas both bottles with N_2, seal, heat them for 15mins at 80°C in a heating block, and allow to cool down at room temperature thereafter.
- Before transferring each sample into separate centrifugation tubes verify that phase separation *did not* occur. Phase separation does occur if there is an excess of hexane in the extracts - to obtain a homogeneous mixture add a small amount of methanol (approx. 2mL).
- Place them on balance and add a few drops of chloroform:methanol:NaCl solution to equalize tubes, and remove cell debris by centrifugation at 3000rpm for 5 to 10mins.
- Extract the upper layer from the biphasic mixtures and transfer them into a second set of centrifugation tubes. Pipette 2.5mL of 0.3%NaCl and 2.5mL of chloroform to both tubes.
- Before running a second centrifugation at 3000rpm for 5mins, mix suspension well on the vortex, and balance tubes again afterwards.
- Transfer the lower chloroform phase of both tubes containing the lipids with Pasteur pipettes into separate 5mL Pyrex bottles and bring them to dryness under a stream of nitrogen.
- Re-dissolve the dried lipid material in 250µL of chloroform:methanol (mixing ratio 2:1) and store at -20°C if not used on the same day.

Note: Sterilize inoculation needle before use in the burner's flame. Swap Pasteur pipette any time a different sample is gassed.

4.3 Thin Layer Chromatography - Day 4: 4th of Feb. 1999

Purpose:The classic method of polar lipid extraction utilizes a monophasic mixture of chloroform, methanol and water for extraction. Addition of more chloroform and water forces a phase separation. The lower, mainly chloroform layer contains the polar lipids whereas non-lipid components remain in the upper aqueous phase. A modified procedure in which an initial extraction with hexane removes non-polar compounds such as isoprenoid quinones. In this way quinones and polar lipids can be extracted from a single sample of biomass (as done in experiment 3). 2D thin layer chromatography can be used to determine simple 2-dimensional patterns of polar lipids which may be characteristic of individual taxa.

Procedure: 5µL of each of the merely dissolved probes are spotted onto the lower left corner of the square TLC plates (two plates per strain / bottle).

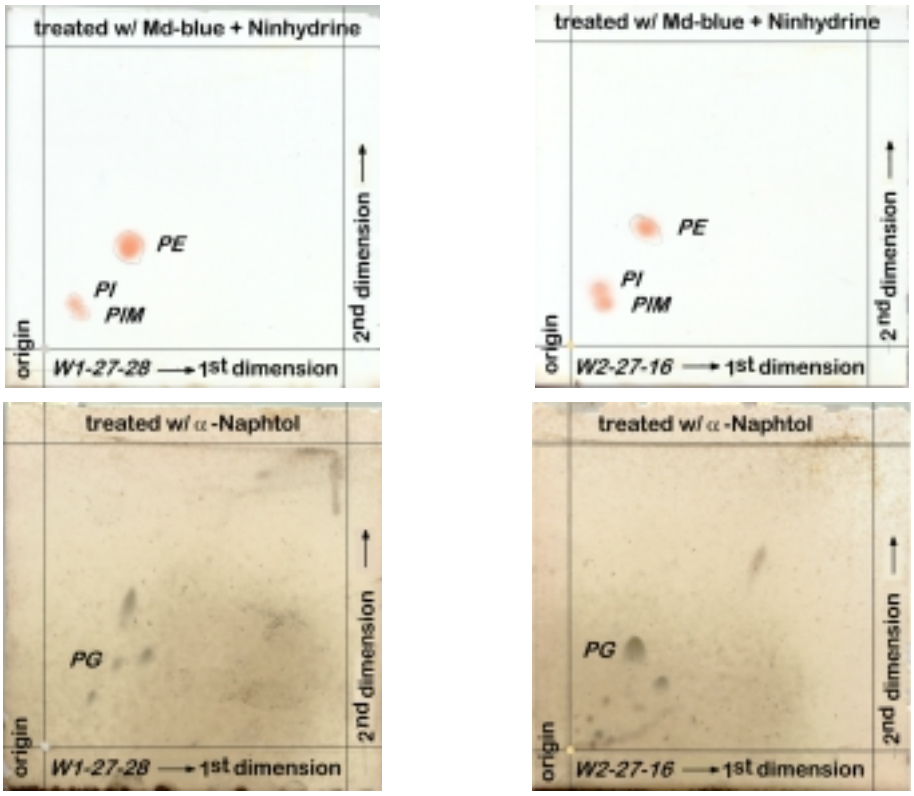
- Develop the plates in 2 dimensions using as mobile phase:
  i) in the first dimension a mixture of chloroform:methanol:water with a volumetric ratio of 65:25:4. Once the mobile phase reaches the upper limits of the plates, remove and dray them at room temperature for about 10mins;
  i) in the second dimension a mixture of chloroform:methanol:acetic acid:water with a volumetric ratio of 80:12:15:4. Once the mobile phase reaches the upper limits of the plates, remove and dray them at room temperature for about 10mins.
- Treat 2 plates (one per strain) with Mb-Blue (detects phospho-lipids). Dry in incubator at 80°C, and outline reaction sites with soft pencil before executing a second spraying treatment with ninhydrin (detects lipids with free amino groups).
- Spray the remaining two plates (one per strain) with α-naphtol (reacts with lipids containing sugars) and develop them at 120-160°C in incubator.

Note: Avoid opening of TLC-chamber while separation is in progress. Due to high toxicity of spray reagents, execute spraying of TLC plates only under aspirator; wear protection gloves.

Results and Evaluation: Day 4: 4th of Feb. 1999

Due to problems regarding the mobile phases, chromatographic procedure has been repeated for a laborious three runs (wasting 4 TLC plates) until a suitable chromatogram was obtained. PE: phosphatidyl-ethanolamine; PI: phosphatidyl-inositol; PIM: phosphatidyl-inositol-mannoside; PG: phosphatidyl-glycerol;

strain *W1-27-28* strain *W2-27-16*



2D-TLC of polar lipid extractions

5. Di-Amino Acid Determination (Experiment 5)

The value of peptidoglycan structure in taxonomic has been widely recognized. Although there is considerable inter-species variation in the detailed structure of peptidoglycan, its chemical architecture remains constant. A β-1,4 linked disaccharides of N-acetylglucosamine and N-acetylmuramic acid form glycan chains up to 100 units long, which are covalently cross-linked in three dimensions by oligopeptides interconnecting the 3-O-lactoyl groups of muramic acid residues in different glycan chains. This structure is very stable. Detection of the presence of diamino-pimelic acid (DAP) and identification of the isomer is one of the most useful chemo-taxonomic procedures for Gram-positive bacteria, and can be carried out conveniently with whole bacteria.

5.1 Isolating Di-Amino Acids - Day 2 and 3: 2nd/3rd of Feb. 1999

Purpose: To hydrolyse the glycosidic links in the glycan chains efficiently, a 6M HCl at 100°C for 16 h under the same conditions is needed to degrade the oligopeptides to their constituent amino acids. By which time, considerable degradation of the aminosugars of the glycan remains insoluble and intact.
A G^{Pos} cell wall consists chiefly of peptidoglycan (90%). Therefore this test is executed with the G^{Pos} strain only.

Procedure: Sterilize inoculation needle with burner.

- Collect a sample from the 4-sector dish with the needle and place it into the Pyrex tube.
- Pipette 0.2mL of 6M HCl into the tube, and seal. Hydrolyze suspension for ≈16h in heating block.
- The day after, prepare a filtering tool by using a yellow pipetting tip with a bit of glass wool and a few grams of charcoal on top of it. Transfer the hydrolyzed suspension on top of the charcoal popped pipetting-tip and apply gentle pressure with the pipette by squeezing the filtered suspension through the glass wool into a plastic bullet.
- Allow evaporation of aqueous portion of HCl by placing the bullet (with unplugged stopper) into the heating block at 60°C over night.

Note: Use protection glasses when handling HCl.

material: marking pen
burner w/ igniter
heating block stove
incubator
inoculation needle
200µL pipette w/ 2 yellow tips
2 15mL sealable Pyrex bottle w/ Teflon-lined screw cap
plastic bullet
few grams of charcoal
tiny bit of glass wool
pair of latex gloves
DC-chamber and 1 DC plate (cellulose - silica gel 60 F_{254})
10mL 6M HCl*
10mL 10M HCl*
50 mL of deionized water
0.1L methanol (CH_3OH)
10mL pyridine ($C_5H_5N{\cdot}H2O$)
spraying reagent w/ spraying flask
ninhydrine (dissolved $C_9H_6O_4$)
Reference solutions:
Dap (di-amino-pimelic acid)
Dab (2.4-di-amino-buttric acid)
Orn (ornithin)
Lys (lysine)
colony: 2 plate with the following colonies:
W2-27-16 (4-sector dish)

(*)see appendix media and solutions

5.2 Separating Di-Amino Acids - Day 4 and 5: 4th/5th of Feb. 1997

Purpose: Separation of the optically active isomer (Dap) from the inactive counterpart *meso*-Dap. Reference solutions placed onto the same DC-plate allow appropriate interpretation of the chromatogram obtained.

Procedure: Add one drop of deionized water to the dehydrated material

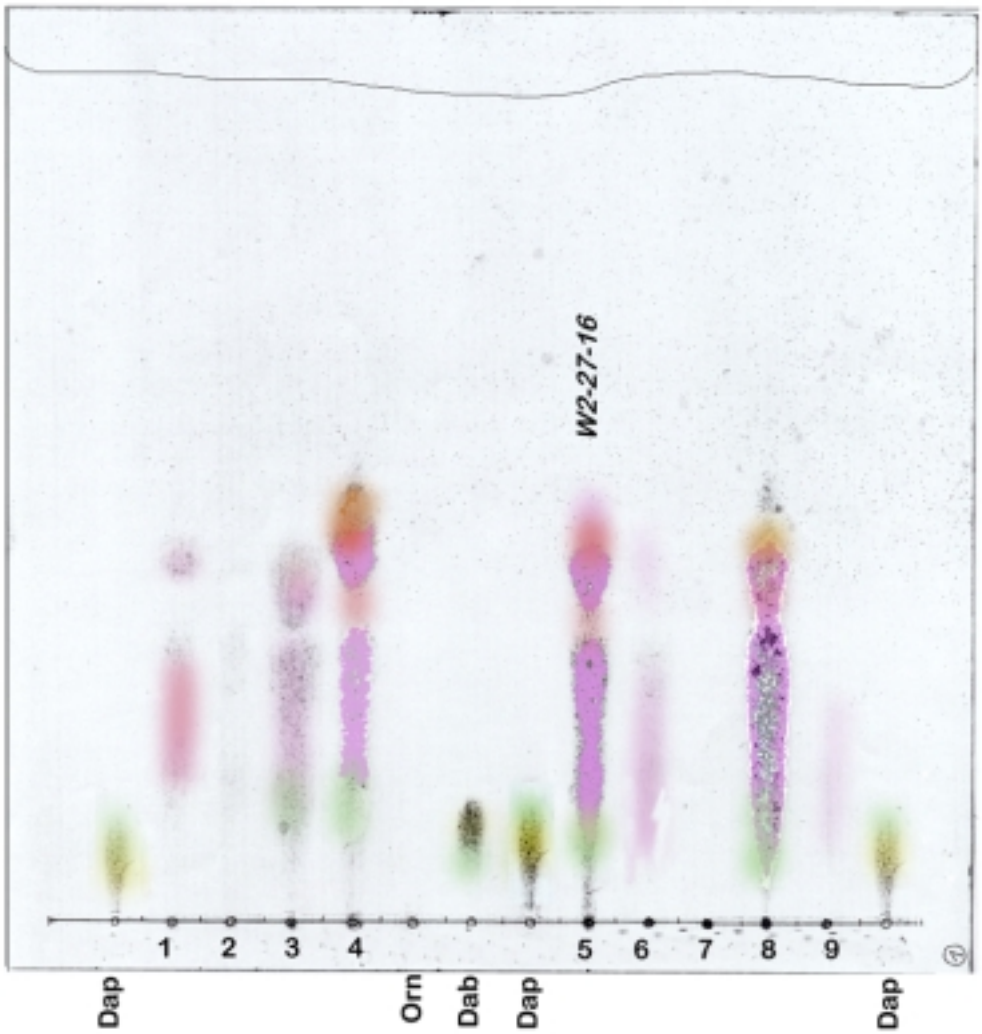
- Extract 8 to 9µL of the aqueous suspension from the bullet and place it onto the assigned position on the DC-plate. Make sure to place the pipetted volume step-wise onto the plate to create a small circular aggregation (not larger than approx.1.5mm in diameter). Use a hairdryer to accelerate evaporation during application.
- In the same manner as above, apply reference solutions (Dap, Dab, Orn) onto the assigned locations (Lys was not available - left out).
- Place plate into DC chamber and run procedure in a mobile phase consisting of a volumetric ratio of 320:40:70:10 with the following: methanol:pyridin:water:10MHCl.
- Once the mobile phase reaches the upper limits, remove from DC chamber, dry plate for 10mins, and spray with ninhydrine reagent.
- Process plate for 24hours in incubator at 100°C.

Note: Spraying reagent to be used under aspirator and latex gloves only - highly toxic!

Results and Evaluation: Day 5: 5th of Feb. 1997

Due to an improper handling by the tutors of the first chromatogram (probably due to faulty mobile phase), procedure was repeated.

In accordance with usual standards, a cell wall preparation of the analyzed strain should be effectuated as well, to obtain a reliable interpretation of the chromatogram (was not done due to the limited time available). Therefore, the interpretation of chromatogram can not be considered as very reliable.



Peptidoglycan test (Di-Amino-Acid) scan of Gram-positive strain *W2-27-16*

6. In Vitro Identification System - API Test (Experiment 6)

The api-20-NE system is a standardized method combining 8 conventional tests and 12 assimilation tests for the identification of non-fastidious (pathogenic) Gram-negative rods not belonging to the *Enterobacteriaceae*. Pathogenic organisms having demanding nutritional requirements and requiring appropriate handling precautions are not included in the api20NE database.

6.1 Inoculation - Day 3: 3rd of Feb. 1999

material: marking pen
burner and igniter
autoclaver
incubator
inoculation needle
1 Pasteur pipette w/ bulb
1 test tube with stopper
5mL 0.9% NaCl solution*
1L bottle of deionized water
bottle of acetone
1 Test strip api20NE with incubation box, tray, and lid
1 ampoule of AUX medium
flask of NIT1 reagent ($CH_3COOH \cdot 5N$)
flask of NIT3 reagent (-"-)
flask of JAMES reagent (J2183)
flask of OX reagent (TMPPDA)
flask of mineral oil
few grams of Zn dust
Reference book for api20NE tests
colony: PYE plates w/ the following strains:
W1-27-28 (4-sector dish)

(*) see appendix for preparation

Purpose: Since this particular stripe api20NE is designed for G^{neg} bacteria, only the is *W1-27-28* strain will undergo testing.

Procedure: Pipette approx. 5mL of 0.9% NaCl solution into a test-tube, pop, and autoclave (sterilized) at 120°C for at least two hours.

- Disinfect workbench by pouring a squirt of acetone onto it and once evaporated, place running burner in close range.
- Using the inoculation needle, pick 1-4 well-isolated colonies of identical morphology from the 4-sector agar plate and disperse them well in the saline (sterilized) solution.
- Prepare an incubation box, tray and lid, and record the strains' ID onto the elongated flap of the tray.
- Distribute about 5mL of demineralized water into the bottom of the tray to create a humid atmosphere. Remove the strip from its packing, place it in the tray and discard the desiccant.
- Inoculate tests *NO_3* to *PNPG* by distributing the saline suspension into the tubes (and not the cupules) using the same pipette (avoid formation of bubbles by placing the pipette on the side of the cupule).
- Open an ampoule of AUX-medium and pipette 200µL (6-8 drops using Pasteur pipette) of the remaining saline suspension to the ampoule. Homogenize well with pipette, avoiding the formation of bubbles.
- Fill the tubes and cupules of tests *GLU* to *PAC* with the AUX suspension. Take care to leave a flat or slightly convex, but not concave, meniscus (Cupules under or overfilled may give incorrect results). Add mineral oil to the cupules of the 3 underlined tests (*GLU*, *ADH*, and *URE*) until a convex meniscus is formed.
- Close the incubator and incubate at 30°C for 24 hours.

Note: Keep acetone bottle away from burner's flame!
Do not touch the cupules while working with the strip and do not leave the strip exposed to air for extended period of time after incubation.

Results and Evaluation: Day 5: 5th of Feb. 1999

NO_3 test: After recording all the spontaneous reactions (see scan below) add 1 drop of NIT1 and 1 drop of NIT2 reagents to the NO_3 cupule. After 5 minutes keeping the assimilation test covered with the incubation lid, no reaction was obtained. A few grams of Zn dust added to the NO_3 cupule, yielded a reddish hue, indicating that nitrates present in the tube were reduced to nitrite by the zinc. Both the NO_2 as well as N_2 test were negative.

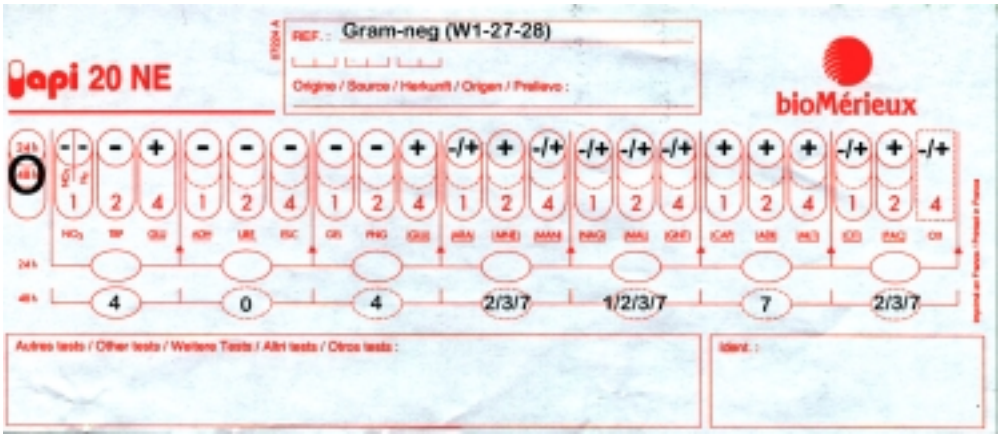
TRP test: Adding 1 drop of JAMES reagent to the TRP cupule did not result in a immediate change of color, hence negative.

Assimilation test: Opaque cupules indicating a positive result have been detected at the following pockets: *GLU* (glucose), *MNE* (mannose), *CAP* (caprate), *ADI* (adipate), *MLT* (malat), and *PAC* (phenyl-acetate).

Weak growth (slight opacity) has been found with the following cupules: *ARA* (arabinose), *MAN* (mannitol), *NAG* (N-acetyl-glucosamine), *MAL* (maltose), *GNT* (gluconate), and *CIT* (citrate). Results have been evaluated as +/-.

OX test: Due to unavailability of OX-reagent (tetramethyl-p-phenylen-diamine), this test has not been executed; therefore evaluated as +/-.

Note: Handle reagents carefully as these are very corrosive and harmful substances. Zinc dust when in contact with water generates highly flammable gases!



Evaluation sheet of api-test strip

Identification of strain: On the result sheet, the tests are separated into groups of 3 and a number 1, 2, or 4 is indicated for each. By adding the numbers corresponding to positive reactions within each group, a 7-digit number is obtained with constitutes the numerical profile.

NO_2 / N_2	TRP	GLU	ADH	URE	ESC	GEL	PNG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OX
-	-	+	-	-	-	-	-	+	+/-	+	+/-	+/-	+/-	+/-	+	+	+	+/-	+	+/-
1	2	**4**	1	2	4	1	2	**4**	1	2	4	1	2	4	**1**	**2**	**4**	1	**2**	4
4			0			4			1,2,3, or 7			1,2,3, or 7			7			2,3, or 7		
Identification code: 404xx7y with x taking values of 1, 2, 3, or 7 and y taking 2, 3, or 7																				

Although the analytical profile index theoretically results in an exorbitantly large number of possible combinations, consulting the reference manual excluded many combinations.

The selected few combinations which are listed, overwhelmingly favor the genus *Acinetobacter* over the genus *Pseudomonas*. This lineage is even more supported by the fact that the former does have quinone (according to the quinoine analysis obtained in experiment 4) as their primary cell wall cytochrome whereas the later does not.

Introduction

A broad selection of bacterial strains has been collected from the Museum of Natural History in Vienna. Their cultivation on full-medium agar plates, and their isolation were done previously by the professor and his colleagues. During the practical exercise, the strains under investigation were Gram-negative and Gram-positive bacteria. They seem to be related to the genus of dustborne *Mycobacteria* and *Acinotebacter*.
The present laboratory exercise is a course for beginners in an attempt to classify the specimens collected by using chemotaxonomic analysis.
Chemosystematics depends upon the chemical analysis of microbial cells and most chemotaxonomic procedures involve, to varying degrees, the extraction, fractionation, purification and resolution of target compounds.
An advantage of this practice is that chemical data can be used to define relationships at many levels in the taxonomic hierarchy including subgeneric and in some cases subspecific rank, which can be carried out following extraction using thin-layer or paper chromatography, such analyses require more analytical experience and access to suitable instrumentation.

Methods (see table 1 for results)

Bacterial strains: The bacterial strains used in this practical exercise are:
W1-27-28 and *W2-27-16* were provided by the tutors on several full-medium PYE agar plates and kept at room temperature.

Analysis of Fatty Acids: Fatty acids forming the hydrophobic core of polar lipids are usually in the range C_{12} to C_{24} and have been shown to be relatively stable taxonomic characters when extracted from cells grown under carefully standardized conditions. Cellular fatty acids can be quantitatively analyzed using gas liquid chromatography following methylation. This analysis was already executed by the lecturer. Results were given as shown in the table 1. These results have been compared with other similar strains published of several microbiological publications.

Microscopical Examination: Morphological observation of live and non-stained specimen under the microscope was somewhat difficult to differentiate. However, the Gram-stained specimens revealed that specimen *W1-27-28* are coccus-like aggregations, whereas *W2-27-16* are rod-shaped. A closer look identified those rods to be cocci lined to one another like a chain.

Gram reaction: The 3% KOH-test was used first. Subsequently, a Gram-staining reaction was executed just to confirm those results.
It is important to note that the basis for this differentiation is one of rate, rather than an absolute characteristic of bacteria. For this reason, the Gram staining reaction must be performed with great care.

Polar lipids extraction and analysis: The classic method of polar lipid extraction utilizes a monophasic mixture of chloroform, methanol and water for the extraction containing cell debris of the specimens.
A modified procedure in which an initial extraction with hexane removes non-polar compounds such as isoprenoid quinones. In this way, menaquinones and polar lipids can be extracted from a single sample of biomass. Thin layer chromatography can be used to determine a simple two-dimensional patterns of polar lipids which may be characteristic features of individual taxa.

Extraction and Analysis of Quinones: Quinones are found in the cytoplasmic membranes of most prokaryotes, and play an important role in electron transport and energy generating systems. The most widely distributed types of isoprenoid quinones are the menaquinones (vitamin K) and the ubiquinones (coenzyme Q9). Both types vary in the length of the polyprenyl side-chain, with menaquinones showing marked differences in the degree of hydrogenation of the isoprene units.
Isoprenoid quinones are free lipids that can be readily extracted from bacterial cells using lipid solvents such as acetone, chloroform and hexane. It is normally achieved with any one of these solvents or with a mixture of any two of them. They are also susceptible to photo-oxidation in the presence of oxygen and strong light but it is not necessary to work in a nitrogen atmosphere or dim light. It is however, good practice to conduct extraction and subsequent purification procedures fairly rapidly.

Extraction and Analysis of Di-Amino Acids: The value of peptidoglycan structure of Gram-positive bacteria in taxonomy has been widely recognized. Although there is considerable inter-species variation in the detailed structure of peptidoglycan, its chemical architecture remains constant.
β-1,4-linked disaccharides of N-acetylglucosamine and N-acetylmuramic acid form glycan chains up to 100 units long, which are covalently cross-linked in three dimensions by oligopeptides interconnecting the 3-O-lactoyl groups of muramic acid residues in different glycan chains, this structure is very stable. To hydrolyze the glycosidic links in the glycan chains efficiently, 6M HCl at 100°C for 16h under the same conditions is needed to degrade the oligopeptides to their constituent amino acids; by that time, considerable degradation of the aminosugars of the glycan remains insoluble and intact under a variety of conditions that can be used to solubilize and remove other cellular constituents. DAP isomers run slower than other amino acids and give characteristic green spots.

Fermentation test: The ability to attack different substrates consisting of carbon, hydrogen and oxygen varies with the battery of enzymes present in the bacteria being identified. Complex carbohydrates are first hydrolyzed by extracellular enzymes to molecules of sufficiently small size to enter readily into the cell. Further dissimilation results in end-products that can be detected by various methods. The carbohydrate decomposition spectrum determined by production of acid from substrates is now regarded as a secondary tool in classification. However, for the characterization of lower taxa and for epidemiological purposes it is still a valuable feature.

- **Phenol Red Broth Base** and the complete carbohydrate media (i.e. carbohydrates, polyhydric alcohols, glycosides and other fermentable compounds) are used in fermentation studies for the cultural identification of pure cultures of microorganisms. A satisfactory basic medium for determining the fermentation reactions of microorganisms must be capable of supporting growth of the organisms under study, and free from fermentable carbohydrates which could give erroneous interpretations. It must be stable, uniform in composition, give distinct reactions and yield accurate results. The phenol red is an excellent indicator of changes of reactions from red to yellow. The addition of some carbohydrate may result in an acid reaction.
- **Mineral Base**, ammonium sulfate is the sole source of nitrogen, and the various types of sugars added later on serve as a source of carbon in this medium. A positive reaction is indicated by a precipitate after several days of incubation at room temperature.

API-Test Strip: The api-20-NE system is a standardized method combining 8 conventional tests and 12 assimilation tests for the identification of non-fastidious (pathogenic) Gram-negative rods. Pathogenic organisms having demanding nutritional requirements and requiring appropriate handling precautions are not included in the api-20-NE database.

Table 1 - Summarized Results:

Strain	***W1-27-28***		***W2-27-16***	
GRAM-Reaction (KOH)	coagulation - G^{Neg}		no coagulation -G^{Pos}	
GRAM-Staining	reddish - G^{Neg}		purple - G^{Pos}	
Colony shape	circular, slightly elevated		rather a dense continuos carpet	
Colony color	white/yellow		yellow/orange	
Cell shape	cocci		coccoidal-chains	
Fatty Acid (saturated)	12:0	5.5%		
	14:0	0.8%	14:0	5%
	a-15:0	1.2%	15:0	4%
	16:0	13.2%	16:0	24%
	16:1	44.5%	a-17:0	0.6%
Fatty Acid (unsatureted)	18:1cis9	24%	18:1cis9	14.5%
	18:1trans9 cis11	5.7%	24:184 TBSA	14.7%
Hydroxy Acid	12:0-3-OH	3.4%	none	
	14:0-3-OH	0.4%		
Quinone	Ubiquinone Q8	40%	Menaq. Mk10	*
	Ubiqionone Q9	60%	Menaq. Mk9-H_2	*
Polar Lipids	PE, PI, PIM		PE, PI, PIM	
Di-Amino Acid (Peptidoglycan)	not performed		Dap	
API-test strip	indicates acinetobacterial relationship		not performed	
Fermentation test	Phenol Red	Mineral	Phenol Red	Mineral
D-Glucose	+	+	+	+
Gluconat	-	+	-	+
Lactose	-	+	-	+
Inulin	n.p.**	n.p.**	n.p.**	n.p.**
D-Manitol	-	+	-	+
Mannose	n.p.**	n.p.**	n.p.**	n.p.**
Raffinose	-	-	+	-
D-Ribose	+	+	+	+
Trehalose	-	+	-	+
D-Xylose	+	+	+	+
Amber		-		+
Citric Acid		+		+
D-Sorbitol		+		+
Fumarin		-		+

(*) No results obtained. However, we made observations with fellow colleagues working on another strain and by comparison, we found surprising similarities with our sample. Therefore, their strain provided us with a clue of what the menaquinone analysis could have been. According to professional advice provided by Dr. Busse, the Mk-type present is likely to be Mk-9H_2.
Highly saturated menaquinones are easily destroyed during preparation and storage. Our specimens were not properly protected against light and oxygen; consequently, they did not yield the expected results.

(**) Not performed due to non-sterile preparation of sugar broth.

Results and Discussion

The present laboratory exercise is a course for beginners to classify the specimens collected by using chemotaxonomic analysis which it is a modern tool for characterizing bacteria. In this protocol we attempted to classify two strains of bacteria (***W1-27-28*** and ***W2-27-16***).

Strain *W1-27-28*: The results of these analysis revealed that the strain is likely to belong to the class of *Proteobacteria*, in the δ-subclass of the (super-) family *Neisseriaceae*.
The characteristics of the isolates fit into the description of **aerobic**, rapidly growing at room temperature (≤ 5 days for growth), slightly pigmented (**white-yellow**) which **stain reddish** indicating that they are **Gram-negative** bacteria favoring humid over dry conditions. The microscopic analysis of the stained sample show that cocci tend to aggregate moderately; fig.1.
Colonies are extremely mucoid and adherent to the substrate agar, and produce a mild disagreeable odor.
Typical acinetobacterial **saturated fatty acids** with a fairly large percentage involve the **16:0** chain length.
The characteristical **non-saturated fatty acid** component of acinetobacter in the form of **18:1-cis9** is also present. Further evidence was given by the occurrence of both the **12:0-3-OH** and **14:0-3-OH** hydroxy acids.
Quinones found in Gram-negative bacteria, have been isolated as ubiquinones (coenzyme Q) with the following ratio: **Q8** (40%) and **Q9** (60%); see fig.2.
Although most strains display no growth factor requirements, some bacteria attack glucose more vigorously; these are found amongst the genus *Acinetobacter*; they contain an aldose dehydrogenase and are able to acidify glucose media, containing other sugars such as D-xylose, L-arabinose, D-galactose, and D-mannose.
*Acinetobacter*s produce a strong acid reaction at the surface, which spreads downwards on further incubation. Citrate and many other organic compounds may be utilized as well.
Most strains of *Acinetobacter* can grow in a simple mineral medium containing a single carbon and energy source such as acetate.
Analysis of **polar lipids** via TLC, display a suspicious similarity of posphatidyl-ethanolamine (**PE**), phosphatidyl-inositol (**PI**), and phosphatidyl-inositol-mannoside (**PIM**), with the mycobacterial strain (described later on). Consulting numerous articles and publications, we could not identify a distinct lipid-pattern, characteristic for *Acinetobacter*; fig. 3.
Similarly, neither the presence of phosphatidyl-glycerol (**PG**) could be used to show that it should be an essential component of an *acinetobacterial* cell wall; fig. 4.
The strongest evidence convincing us that this strain could belong to the acinetobacterial taxon, is given by the evaluation of the identification code of the api-test. Since most *Acinetobacters* are oxidase-negative, (lack of cytochrome c), additional support can not be provided because the oxidase test-solution was not available for experimentation.

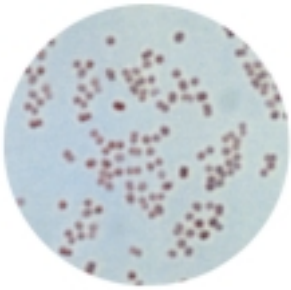


Fig.1 - purple stain of ***W1-27-28***

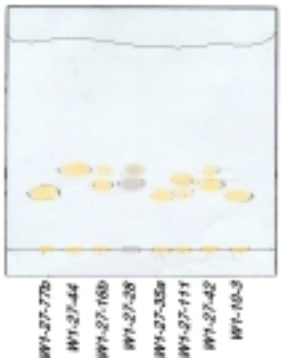


Fig.2 - ubiquinone separation

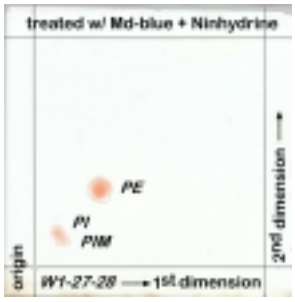
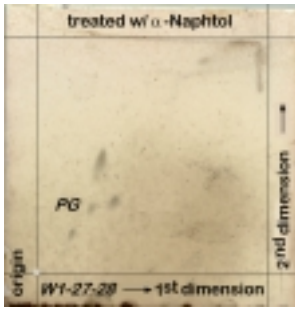


Fig.3,4 - 2D-TLC of polar lipids



Strain *W2-27-16*: The results of these analysis showed that the strain is related to the **mycobacteria** which are members of the order Actinomycetales and family Mycobacteriacea.

The characteristics of the isolates fit into the description of **mesophilic**, **aerobic**, rapidly growing (≤ 5 days for growth), **yellow-orange** pigmented mycobacteria which **stain purple** indicating that they are **Gram-positive** bacteria. The microscopic analysis of the stained sample show that these cocci tend to cluster together more or less into chains of four; fig. 5.

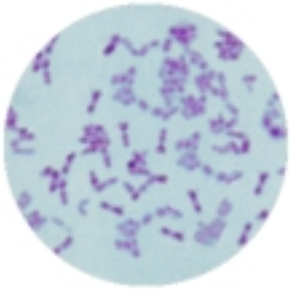


Fig.5 - purple stain of ***W2-27-16***

The outer membranes of mycobacteria and related organisms contain substantial amounts of long-chain fatty acids (**a-17:0**), and mycolic acids, which are considered to be esterified to an arabino-galactan linked to the peptidoglycan.

Oleic acid derivatives such as (**cis-9**) is present in large amounts and is a typical feature in actinomycetes. Fatty acids found exclusively in this group are those with a methyl-group in position 10 (**10-Me-18:0**) which is commonly known as tuberculostearic acid (**TBSA**).

The first extraction and separation of **quinones** via TLC-analysis reveal that this strain did not contain ubiquinone, but an aggregation at the **menaquinone** position which is typical for Gram-positive bacteria. A second TLC-analysis did not give us the expected results because of improper laboratory handling of the specimens (no protection against light and oxygen). Observations made with fellow colleagues working on another strain, we found surprising similarities with our sample; therefore, we suspect that menaquinone analysis of our sample could have been either Mk-10 or Mk-9-H_2; fig. 6.

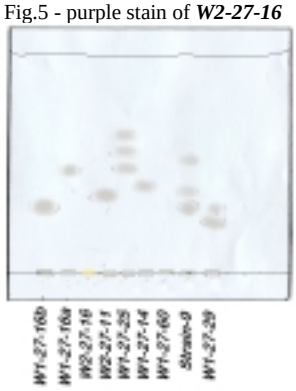


Fig.6 - menaquinone separation

Di- and tetra-hydrogenated menaquinones are quite widespread amongst actinomycetes. The position of saturation of the dihydrogenated menaquinones of members of the Gram-positive taxa invariably occurs in the second unit from the ring system, e.g. MK-9-H_2 of *Mycobacterium* species.

The TLC analysis of **polar lipids** display a characteristic distribution of posphatidyl-ethanolamine (**PE**), phosphatidyl-inositol (**PI**), and phosphatidyl-inositol-mannoside (**PIM**); those are typically present in many mycobacteria; fig. 7.

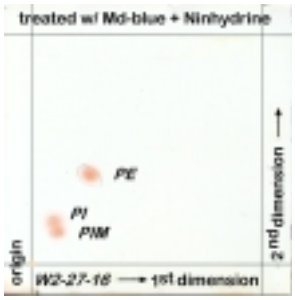
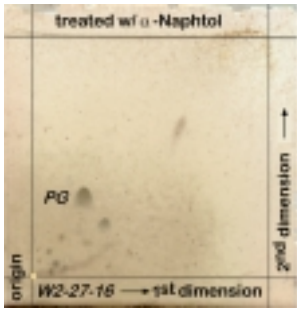


Fig.7,8 - 2D-TLC of polar lipids

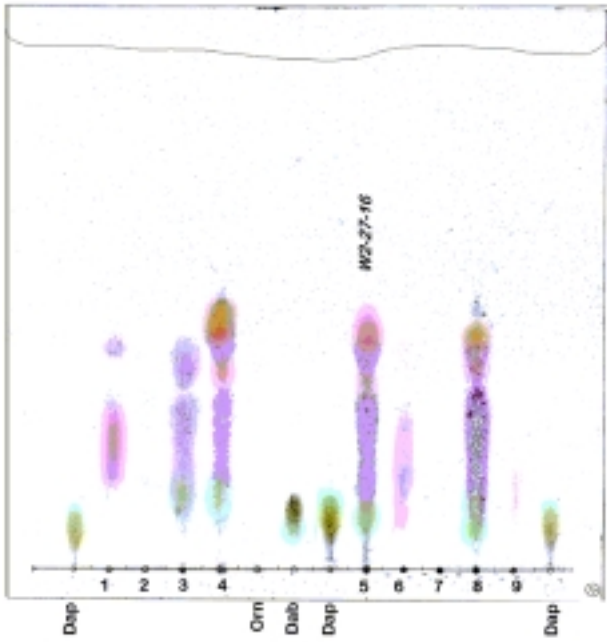
The **peptidoglycan** test confirms the presence of diaminopimelic acid (**Dap**) which is an essential component of Gram-positive bacteria, including actinomycetes; fig. 9.



As previously stated, there is little evidence that this strain does not belong to the family Mycobacteriacea - genus *Mycobacterium*. This can be emphasized by the results of the carbon substrate utilization test which reveal a wide range of sugar- and mineral-utilization to synthesize the fatty acid via the aerobic pathway.

Therefore, according to *Bergey's Manual*, growth at room temperature (≈22°C) and fermentation patterns (D-glucose, D-xylose, and D-ribose as well as iron uptake), indicate close relationship with the following species: *M.gordonae, M.szulgai, M.simiae, M.scrofulaceum, M.intracellulare, M.phlei, M.aurum, M.komossense, M.parafortuitum, M.vaccae.*

Fig. 9 - Peptidoglycan analysis
(Di-Amino-Acid)
of Gram-positive strain



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- *Chemical Methods in Bacterial Systematics*, Michael Goodfellow & David E. Minnikin, Academic Press, Orland, Fla, 1985 - USA
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- *Biology of Microorganisms* 8th ed., Brock T.D., Madigan M.T., Martinko M.T., Parker J., Prentice Hall, New Jersey 1997 - USA
- *An Introduction to Genetic Analysis*, 6th ed. Griffiths A.J.F., Miller J.H., Suzuki D.T., Lewontin R.C., Gelbart W.M., Freeman and Company, New York 1996 - USA
- *The Nature of Life*, 3rd ed. Postlethwait J.H., Hopson J.L., McGraw Hill, New York 1995 - USA
- *Bacterial Systematics*, N.A.Logan, Blackwell Science, Oxford 1994 - UK
- *Bergey's Manual of Systematic Bactereology*, Vol. I and II, Baltimore 1986 - USA
- *API 20NE* - Analytical profile index, 4th ed., Marcy-l'Etoile 1990 - FRA

Selected articles of:

- *International Journal of Systematic Bacteriology*, Vol. 49, part I/IV London 1999 - UK.
- *International Journal of Systematics Bacteriology, pp. 25-35, 1999 - UK*
- FEMS - Microbiology Letters (ELSEVIER)
- Archives of Microbiology (Springer Verlag)

1. Preparation of NaCl solution:

Purpose: Liquefying an appropriate amount of solid NaCl to obtain a NaCl solution with the desired mass-percentage rate.

material: marker pen (water proof)
digital flat-pan balance
4 100mL volumetric Florence flask
1L bottle of deionized water
chemicals: crystalline, pure sodium chloride (NaCl)

Procedure: For every experiment, weigh the listed amount (according to the results obtained from formula 1.1), place it in separate volumetric Florence flask, fill up to graduation mark with deionized water, and mark flask properly.

Note: Never pour back any residual NaCl extracted from the container of origin back into it to avoid contamination of pure raw material.

Results and Evaluation:

Formula 1.1:

$m_{NaCl} = w \cdot 10 \cdot V_{flask}$	w, mass percentage V_{Flask}, volume of Florence flask	[%] [L]

Results (indicated in gray): equals to(mass concentration)

Used in experiment	w [%]	V_{Flask} [L]	m_{NaCl} [g]	β [g/L]
1.2 + 2.2	0.5	0.1	0.5	5
3.1	0.3	0.1	0.3	3
4.2	0.3	0.1	0.3	3
6.1	0.9	0.1	0.9	9

2. Preparation of KOH solution:

material: marker pen (water proof)
1000µL pipette w/ blue tips
50mL volumetric Florence flask
1L bottle of deionized water
chemicals: MERCK master base
≈ 1mL (w = 35%) KOH

Purpose: Preparation of a *standardized base* (w= 3%) out of the master base provided from MERCK Industries.

Procedure: According to formula 2.1 a desired mass percentage of 3% would consist of 30g KOH and 970g of H_2O. 30g of KOH equal to a concentration of 0.535mol/L (formula 2.2). Finally formula 2.3 and 2.4 provides the volume of concentrated base needed to be pipetted into the a 50mL volumetric flask, filled up with deionized water (till graduation mark) to obtain the desired base.

Note: To avoid contamination of pure raw material, never pour back any residual KOH extracted from the container of origin.

Results and Evaluation:

Formula 2.1:

$w_{KOH} = \frac{m_{KOH} \cdot 100}{m_{KOH} + m_{KOH}}$	w, mass percentage m, mass of substance	[%] [g]

Formula 2.2: ($c = n/V$; $n = m/M$)

$c_{diluted} = \frac{m_{KOH}}{M_{KOH} \cdot V_{ciluted}}$	m, mass of dissolved KOH M_{KOH}, molar mass of KOH $V_{diluted}$, volume of 3% sln	[g] [g/mol] [L]

Formula 2.3:

$c_{concentrated} = \frac{w_{base} \cdot \rho_{w\text{-}35\%}}{100 \cdot M_{KOH}}$	w, mass percentage ρ, density M, molar mass	[%] [g/L] [g/mol]

Formula 2.4: ($c_1 \cdot V_1 = c_2 \cdot V_2$)

$V_{concentrated} = \frac{c_{diluted} \cdot V_{diluted}}{c_{concentrated}}$	c, concentration V, volume	[mol/L] [L]

Results (indicated in gray):

Used in experiment	w [%]	ρ_{KOH}* [g/L]	m_{KOH} [g]	c [mol/L]	$V_{diluted}$ [L]	V_{Flask} [L]	$V_{concentrated}$ [µL]
1.1	3	1410	30	0.535	1	0.05	608

(*) density values obtained from provided data sheets

3. Preparation of 6M/10M acid:

Purpose: Preparation of a *standardized acid* (c = 6mol/L, 10mol/L respectively) out of the master acid provided from MERCK Industries.

material: marker pen (water proof)
Peleus rubber-bulb with 25mL volumetric pipette AS-class
100mL beaker
2 100mL volumetric Florence flask
protection glasses
paper towels
deionized water
chemicals: MERCK Master acid in total ≈ 200mL (w=32%) HCl

Procedure: Wash and rinse all utensils with deionized water. Mount the burette onto the stand w/ clamps. Preparation of 6M standardized acid:

- Calculate the volume of MASTER acid needed to prepare the acid with the desired concentration (see formula 3.1 and 3.2);
- Pour a slightly larger than calculated volume into a 100mL graduated cylinder;
- transfer out of this container the calculated amount of MASTER acid into a 100mL volumetric flask and fill up the rest with deionized water;
- seal and shake flask vigorously; store properly; mark w/ date, concentration, etc.; needed in experiment 5;
- repeat procedure with MASTER acid to obtain a 10M standardized acid.

Note: Never pour back any residual acid extracted from the MASTER tank back into it; use protection glasses at any time when handling acids.

Results and Evaluation:

Volumes of MASTER acid needed to be extracted from the MERCK storage bottles:

Formula 3.1:

$c_{HCl} = \frac{W_{acid} \cdot \rho_{w\text{-}32\%}}{100 \cdot M_{HCl}}$	w, mass percentage ρ, density M, molar mass	[%] [g/L] [g/mol]

Formula 3.2: ($c_1 \cdot V_1 = c_2 \cdot V_2$)

$V_{concentrated} = \frac{c_{diluted} \cdot V_{diluted}}{c_{concentrated}}$	c, concentration V, volume	[mol/L] [L]

Results (indicated in gray):

Standardized solution	w_{HCl} [%]	ρ_{HCl}* [g/L]	M_{HCl} [g/moL]	$c_{concentrated}$ [mol/L]	$c_{diluted}$ [mol/L]	$V_{diluted}$ [L]	$V_{concentrated}$ [mL]
6M HCl	32	1160	36.45	10.18	6	0.1	58.92
10M HCl	32	-"-	-"-	-"-	10	0.1	98.20

(*) density values obtained from provided data sheets

4. Medium used for agar plates:

PYE-medium: with the following compoosition
0.3% yeast extract
0.3% peptone (of meat, casein or soy-origin)
1.5% agar
Procedure: provided by the tutors

5. Disposal of used Material:

After use, all utensils which are not meant to be reused (inoculated agar plates, Pasteur pipettes, mono-use syringes, Greiner containers, ampoules, strips, and incubation boxes of the api-test) should be autoclaved, incinerated, or immersed in a disinfectant for decontamination prior to disposal.

6. Used references

- *Chemical Methods in Prokaryotic Systematics*, M. Goodfellow & A.G. O'Donnell, John Wiley & Sons, Chichester, 1994 - UK
- *Chemical Methods in Bacterial Systematics*, Michael Goodfellow & David E. Minnikin, Academic Press, Orland, Fla, 1985 - USA.
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- *API 20NE* - Analytical profile index, 4th ed., Marcy-l'Etoile 1990 - F
- Selected articles of:
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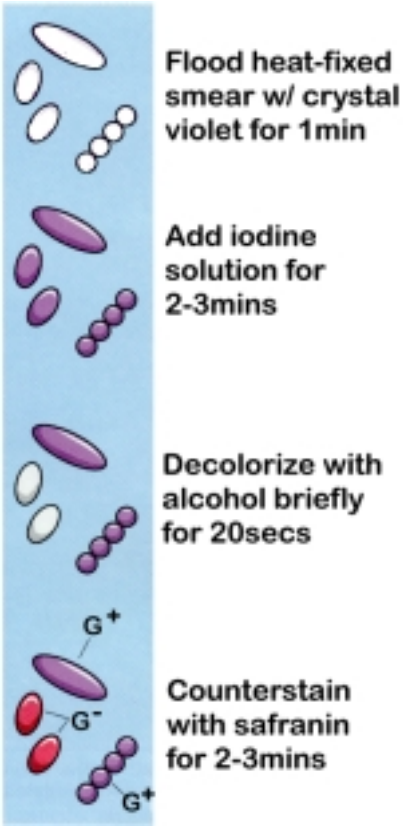
  Archives of Microbiology (Springer Verlag)

Technical Procedures and Handling

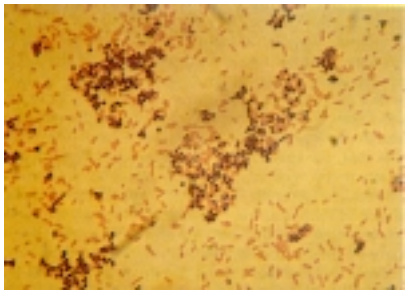
1. Gram Staining Reaction

An important differential staining procedure widely used in bacteriology.
The gram differentiation is based upon the color reaction exhibited by bacteria when they are treated with crystal violet dye followed by an iodine-potassium-iodide solution. Certain organisms lose the violet color rapidly when ethyl alcohol is applied, while others lose their color more slowly. After the decolorization step, a counterstain (safranin) is used. The **Gram-positive** (G^{Pos}) will retain a **blue** or **purple** color and will not take the counterstain safranin. The **Gram-negative** (G^{Neg}) microorganisms that unable to retain the crystal violet stain will take the counterstain and will exhibit a **pink** or **red** color. It is important to note that the basis for this differentiation is one of rate, rather than an absolute characteristic of bacteria. For this reason, the procedure must be performed with great care.

Procedure: A slide containing dried suspension of microorganisms is flooded on a vertical rack suspended in a flat pan for a minute or two with a dilute solution of dye (*crystal violet*). Rinse with an iodone containing solution (*Lugol*) and drip enough iodine solution onto the slide to cover the dried suspension - everything turns purple (in case of GRAM-Pos strains, iodine will react with the thick peptidoglycan layer to form a protective coating). Flush briefly with ethanol to decolorize unprotected spots (until no dye will come off). Rinse quickly with deionized water. Counterstain by covering the dried suspension for 2 to 3 minutes with safranin solution. (G^{Pos} cells stain purple, G^{Neg} cells pink to red)
Finally rinse with deionized water. Let slide dry and observe under microscope.
Note: Use latex gloves and protective clothing for the entire staining procedure.



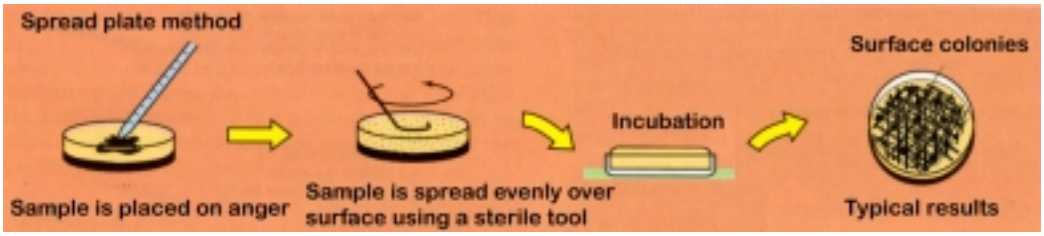
Photomicrograph of bacteria that are gram-positive (blue-purple) and gram-negative (pink-red)



2. Spread Plate Method

Purpose: Enhancing the microbiological material required for further experimentation; i.e. increasing its biomass. An isolated mother colony is required to obtain a large amount of that particular strain by self-replication.

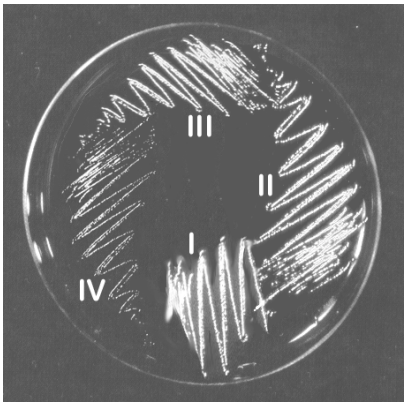
Procedure: Use a sterile inoculation loop to extract the sample and distribute it evenly over the agar. To sterilize the loop, dip it into flame of a burner until it turns glowing red, remove it carefully and place it at the edge somewhere on the agar of your collecting plate where no colonies are present. Allow a few seconds for cooling, collect your sample and inoculate it onto the center of a suitable culture plate. The sample is then evenly distributed across the agar.
Note: Make sure that the burner is turned off after use.



3. Streak Plate Technique (Four-Sector Streaking Method)

Purpose: A tiny sample of an isolated mother colony is required to separate individual cells from each other to obtain a single colony, avoiding a clustered arrangement.

Procedure: Use a sterile inoculation loop to extract a tiny sample. To make it sterile, dip it into flame of a burner until it turns glowing red, remove it carefully and place it at the edge somewhere on the agar of your collecting plate where no colonies are present. Allow a few seconds for cooling, collect your sample and inoculate it onto the edge of a suitable culture plate. Then gently drag the needle away from the inoculated site in a zigzag manner until roughly a quarter of the plate is covered. Skim the inoculation loop over the surface of the original set of streak once, and make a second set of streaks. Be careful not to cross any of the original set of streaks again. Keep doing so until all four sectors of the plate have been used. The final result should show the different streaked regions similar in appearance to the figure below.
Note: Make sure that the burner is turned off after use.



Streaks I to IV of a stroked culture dish

4. Autoclaves

Purpose: A sterilization device such as an autoclave must be available for any bacteriological investigation. Apart from periodic maintenance, each load should be monitored with temperature tape, thermographs, or other means. Minimum sterilization exposure periods are 60mins at 121°C and 1bar for regular laboratory waste.

Note: Heat-resistant gloves must be available for loading and unloading autoclaves.
An autoclave must be operated only by personnel who have been properly trained in its use.

5. Centrifuges

Purpose: To accelerate separation of suspended particles in solution, according to their molecular mass. Aerosol-free (sealed) centrifuge cups are required for processing mycobacteriology, mycology, and virology specimens. Cups should be kept clean and free of broken glass. A germicidal solution is added to the trunnion cup not only serves as a disinfectant in case of breakage but also provides a cushion effect for the tube during centrifugation.

Note: Tubes should be inspected for cracks and chipping.
A centrifuge must be operated only by personnel who have been properly trained in its use.

6. Thin Layer Chromatography (TLC)

Purpose: A sensitive technique for separating and identifying components of a mixture; it relies on the different abilities of substances to stick to surfaces (stationary phase) while the carrier medium (mobile phase) pushes the components along the sticky surface.

- **TLC** (**T**hin **L**ayer **C**.): A silicagel-coated glass-plate (stationary phase) placed in a standing position of a sealable container slightly dipped into the mixture (mobile phase) containing the dissolved components. According to the adherence of the components, certain elements will migrate faster upwards along the plate as others.
- **2D-TLC**: A probe is placed as a single dot of origin onto the plate (usually at the bottom left corner); separation of the components results in two steps: a one dimensional separation, followed by a second in which the stationary phase is turned by 90°.
- **HPTLC** (**H**igh **P**erformance **T**hin **L**ayer **C**.): Uses a finer substrate (stationary phase) than in conventional TLC.

Procedure: Assign the starting positions of the mixture under investigation with a soft pencil.
Mix running agent (mobile phase). Apply analyzing agent to the stationary phase (plate).
Shield inner wall of chromatographic chamber with absorbing paper (facilitates saturation of internal airspace with solvent).
Pour a moderate amount of running agent into the chamber (about 0.5cm high, clearly covering the bottom; mobile phase should soak the absorbent wall paper as well). Place plate vertically into the chamber and close lid.
Check from time to time until mobile phase reaches the upper limit.
Note: Avoid inhalation of the running agent, since these can be quite harmful.

For laboratory safety see *Laboratory Procedures in Clinical Microbiology*, 2nd ed., Appendix A