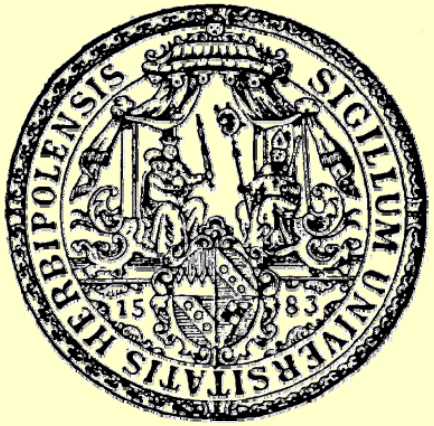
Porins of mycolic acid containing Actinomycetales

University of Würzburg
Department of Biotechnology
Benz Group



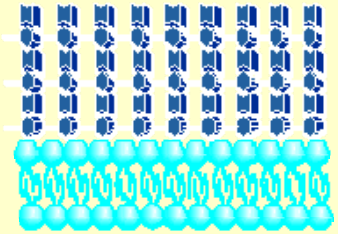
Philipp Knörzer - diploma student

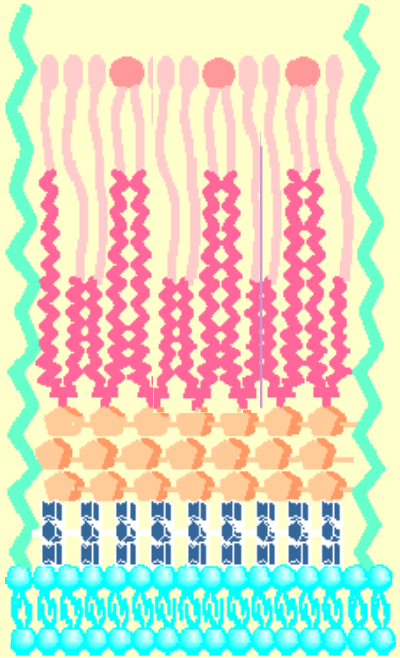
Structure of the cell envelope of cell wall containing bacteria

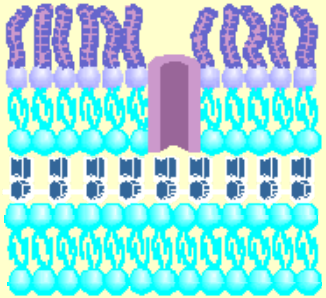
Gram- positive

Gram- positive / mycolic acids

Gram- negative







lipid bilayer

peptidoglycan

acyl lipids

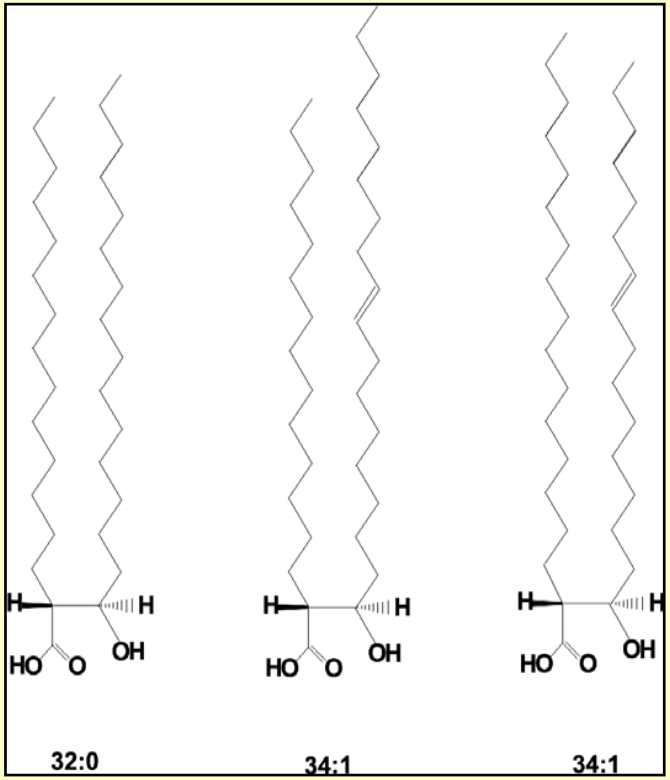
LAM

mycolic acid

arabinogalactan

LPS

porin



α-alkyl, β-hydroxy fatty acids
C32-34 corynomycolic acids

The important structural element of the cell wall of the mycolata is the mycolic acid layer. Minnikin (1982)[1]

- mycolic acids are linked through ester bonds to the arabinogalactan attached to the murein of the cell wall.
- The chain length of these 2-branched, 3-hydroxylated fatty acids varies considerably within the mycolic-acid-containing taxa.
  - short in *corynebacteria* (22 - 38 carbon atoms)
  - medium in *gordonae* (52 - 60 c-atoms) and *nocardia* (46 - 58 c-atoms)
  - long in *mycobacteria* (60 - 90 c-atoms). and *Tsukamurella* (64 - 74 c-atoms)

The cell wall of the mycolata acts as permeability barrier for hydrophylic substances.

Structure of the cell envelope of cell wall containing bacteria

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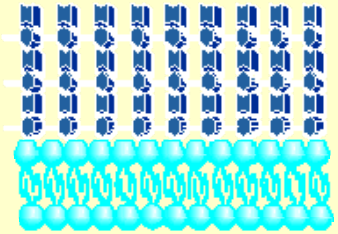
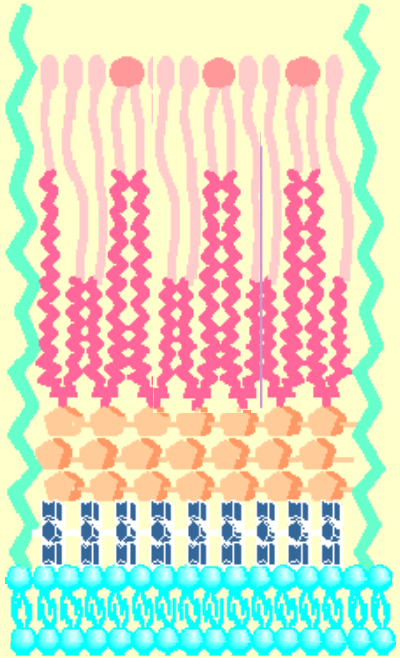
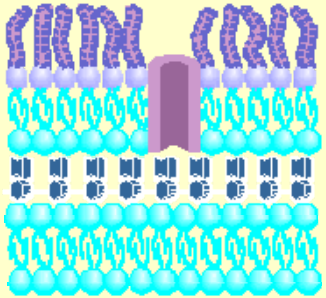


Gram- positive / mycolic acids



Gram- negative



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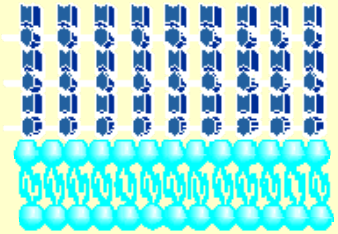
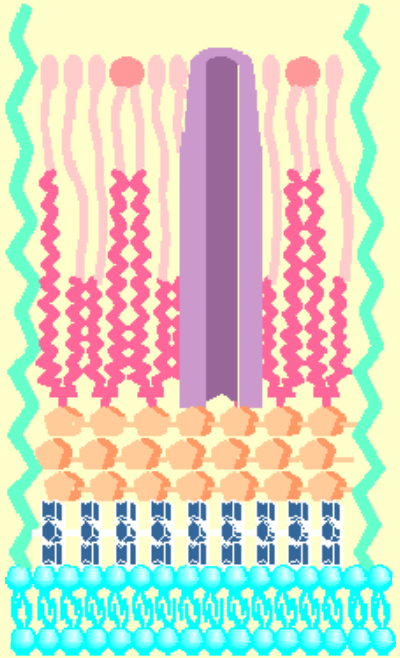
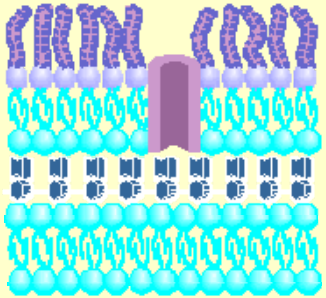


Gram- positive / mycolic acids



Gram- negative



lipid bilayer

peptidoglycan

acyl lipids

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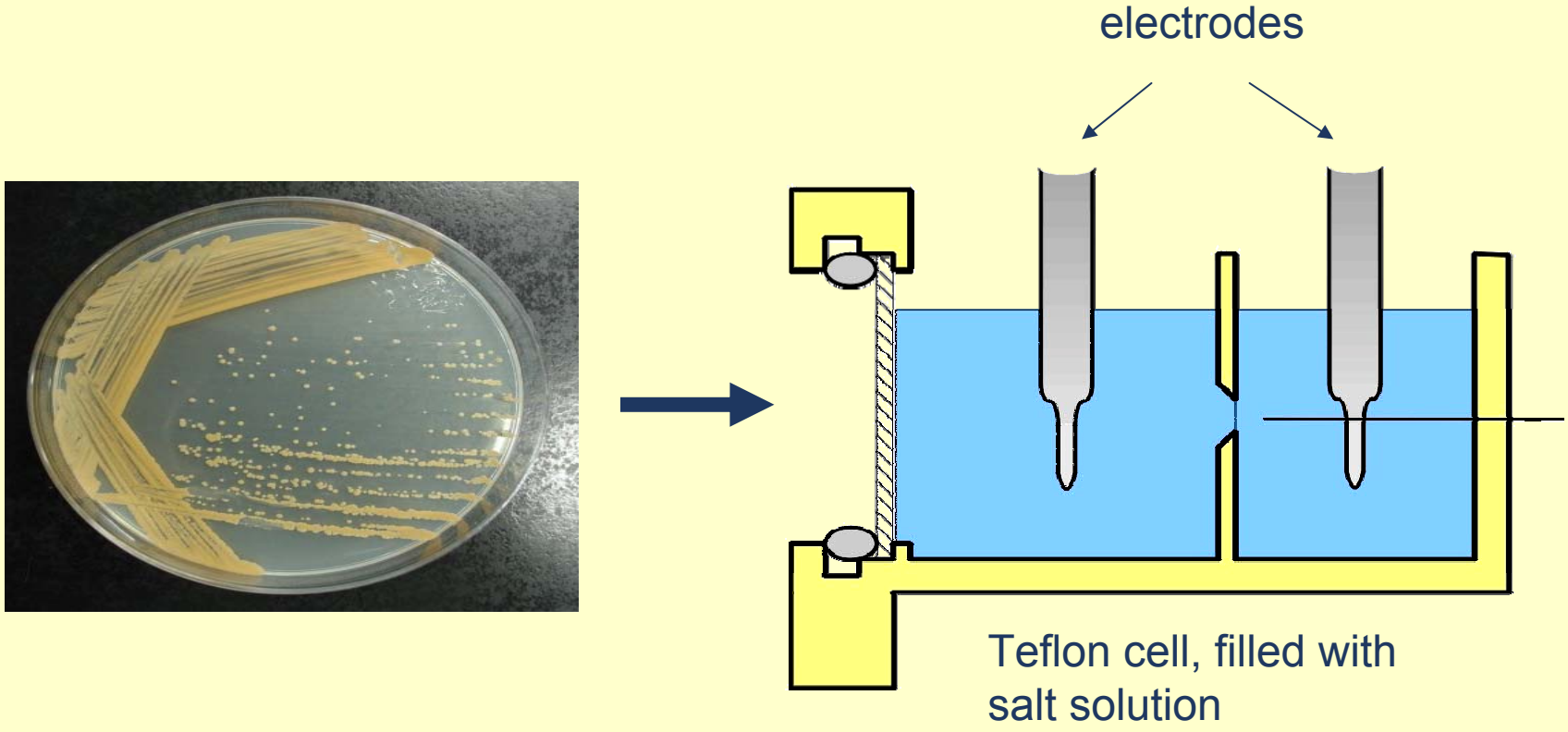
Porins in the outer Lipid-Layers of *Mycobacteria*; Trias et al $(1992)_2$, Trias and Benz $(1993)_3$, Trias ans Benz $(1994)_4$



Motivation of investigation in cell permeability of Mycobacteria

- Important human pathogens
  - *M. tuberculosis*
  - *M. leprae*
  - *M. avium intracellulare*
  - Rising incidence, associated with Immun-Suppression

Mycobacteria are naturally resistant to a wide range of antibiotics!!

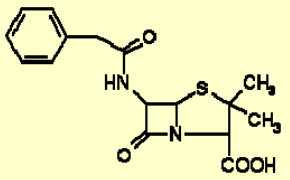
http://www.klinik-donaustauf.de/Tuberkulose%20Roentgenbild.jpg

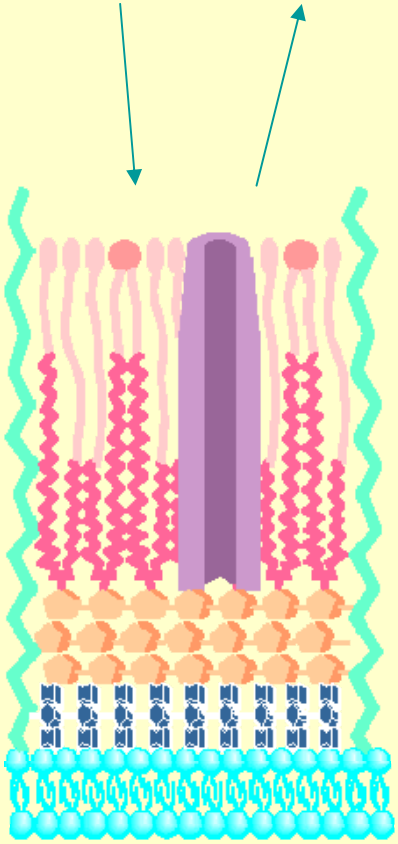
http://louletania.blogs.sapo.pt/arquivo/Lepra.jpg

Lungs of a patient with tuberculosis and hands of a patient with leprosy-infection

Organs used in common transplantations
Heart
Lungs
liver
Pancreas
Islets of Langerhans
kidney

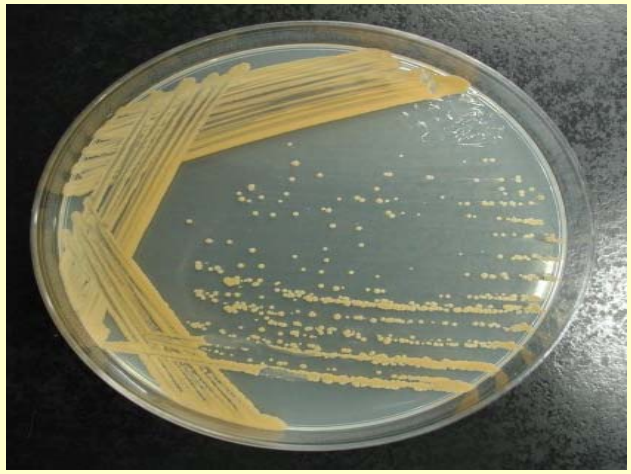
Resistance of Mycobacteria to a wide range of antibiotics

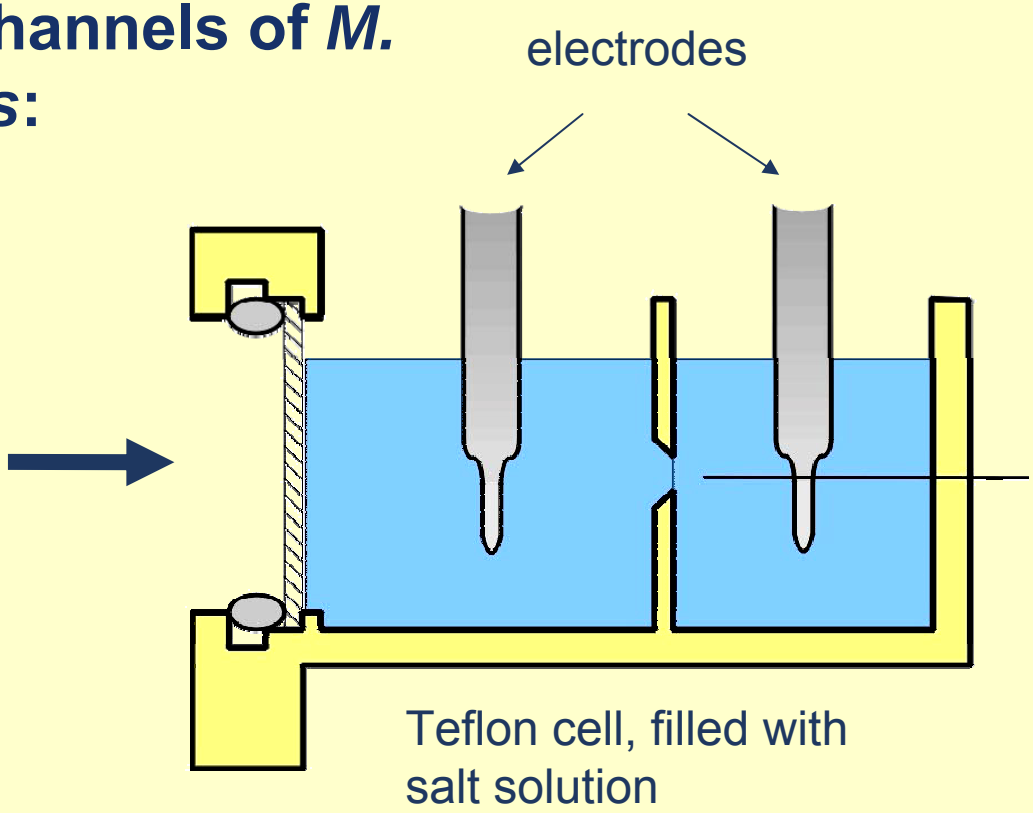




- Natural Resistance of Mycobacteria; Bloom and Murray (1992)[4],
- Mycolic acids and other lipids act as permeability barrier toward hydrophylic compounds; Hui (1977)[5], Jarlier (1991)[6]
- Pores are repelling antibiotics with negative charges

Isolation of cell wall channels of *M. smegmatis*:





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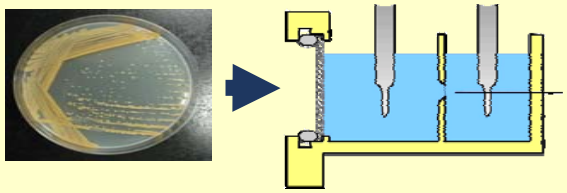
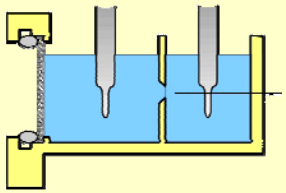


- A very efficient method is the isolation of the cell wall channel using organic solvent treatment of whole cells:

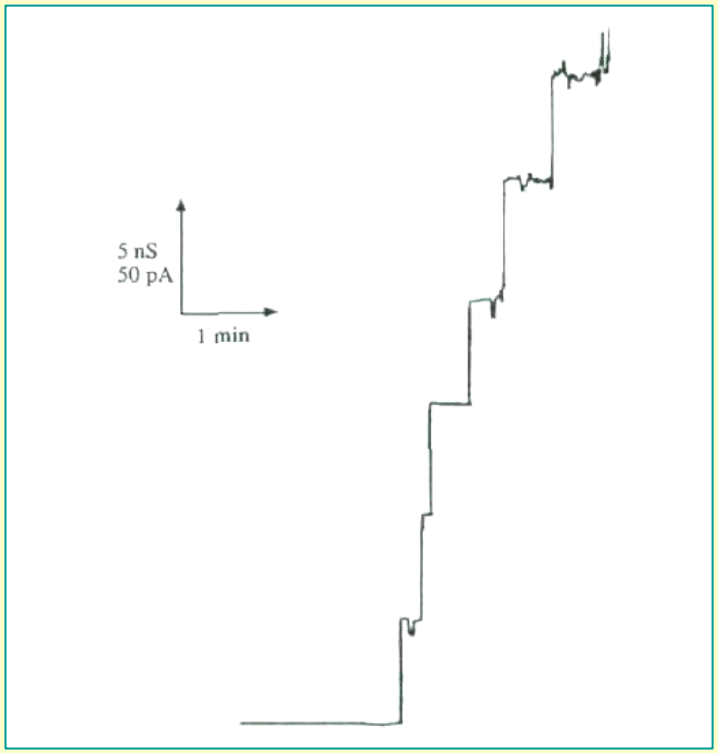
1. Extraction of the cells with chloroform-methanol (1:2) for 10 hours at room temperature.
2. Precipitation of the protein with ether in the cold.
3. Purification of the cell wall channel by chromatography across a Mono-Q column using a linear salt gradient between 0 and 1 M NaCl.

→ **Investigation of the biophysical properties of the Channel**

Biophysical properties - Single channel conductance

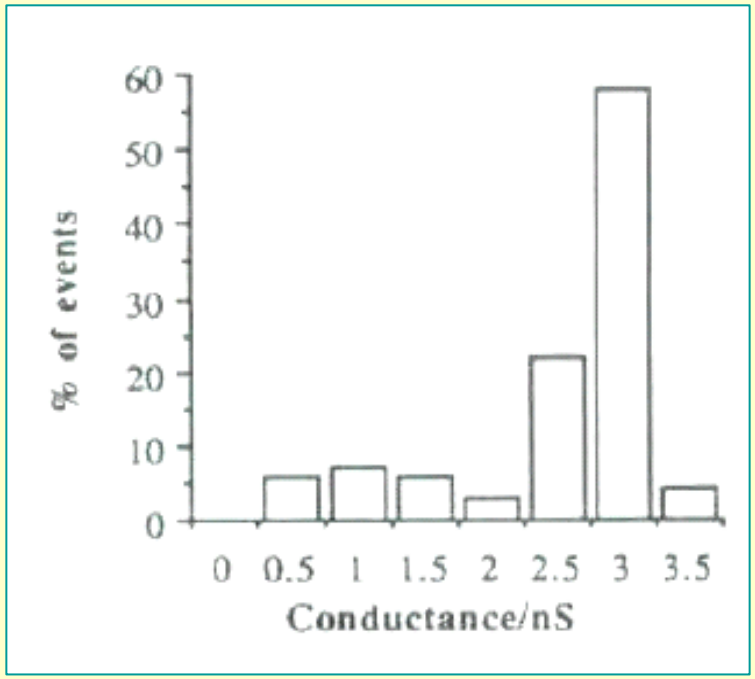


Single-channel recording of *M. smegmatis*



Benz and Trias (1994)$_3$

➔ **Average single-channel conductance is about 3 nS in 1 M NaCl (227 single channel events)**

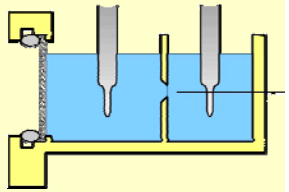


Benz and Trias (1994)$_3$

→ **The channel allows translocation over the cell wall**

→ **Open and closed states of the channel are existing**

Biophysical properties - conductance in different salts

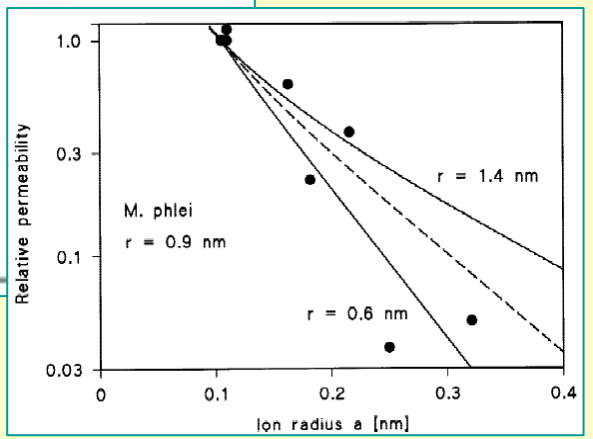


Single-channel conductance of the porin of *M. smegmatis* in different salt solutions

Salt	Dehydrated ion radius of cation (a^*/nm)	Hydrated ion radius of cation (a/nm)	G/nS	
			M. smegmatis	*M. chelonae*[a]
KCl	0.133	0.111	4.1	2.7
LiCl	0.068	0.216	2.0	0.9
NaCl	0.097	0.163	2.9	1.4
NH_4Cl	0.143	0.111	4.2	2.8
RbCl	0.147	0.105	4.4	
CsCl	0.167	0.106	4.4	
TrisCl	–[b]	0.321	0.88	
Kacetate (pH 7)	0.133	0.111	3.9	
KNO_3	0.133	0.111	3.9	
$CaCl_2$	0.099	0.275	2.3	

Benz and Trias $(1994)_3$

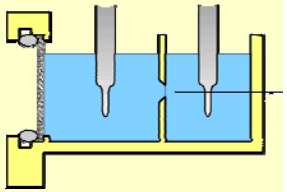
Channel radii are calculated by Renkin equation *Nikaido (1981)*$_8$ and stocks equation *Trias and Benz (1994)*$_3$



Rieß $(2001)_9$

- The channel shows selectivity for cations
- Conductance is associated with hydrated radius
- Wide and water filled channel
- Calculation of the channel radius

Biophysical properties – Negative point charges in the channel mouth



Influence of NaCl to single channel conductance

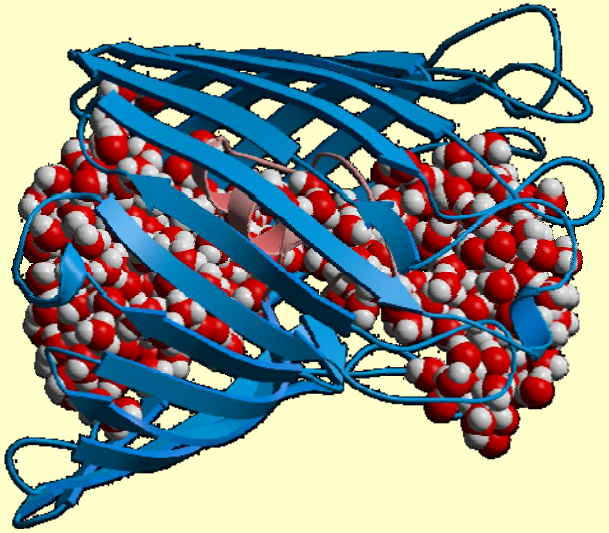
c/M	a	ϕ/mV	c_0^+/M	G/nS	G^*/nS
0.01	0.92	61	0.11	0.20	0.018
0.03	0.87	43	0.16	0.40	0.075
0.1	0.78	21	0.23	0.90	0.39
0.3	0.70	6.9	0.39	1.2	0.92
1	0.64	0.75	1.03	2.9	2.8
3	0.56	0.02	3.00	5.8	5.8

Benz and Trias (1994)[3]

- Single channel conductance was not a linear function of the bulk aqueous salt concentrations
- No saturation at high ion concentrations
  ➔ no binding site to cations

2 types of channels in the outer membrane Nikaido et al (1994)$_7$

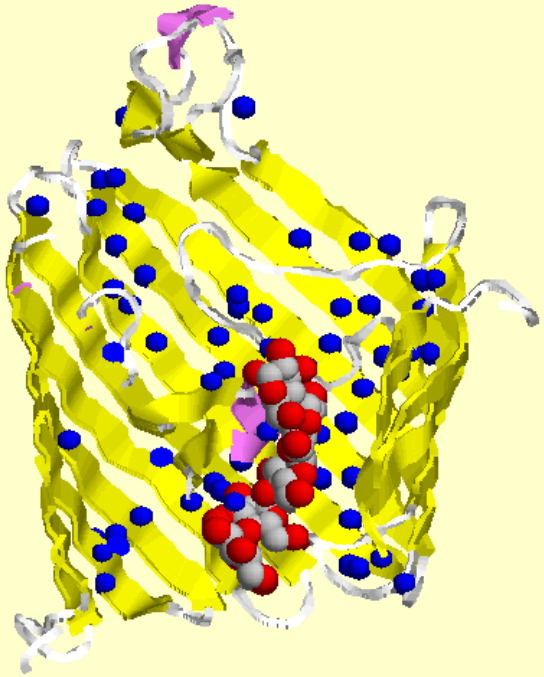
- General diffusion proteins
- non specific diffusion of hydrophilic compounds up to certain size



OmpF-channel

http://www.biologie.uni-hamburg.de/lehre/bza/kanal/1mpocutw.gif

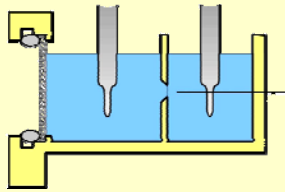
- specific porins
- binding sites allow preferential diffusion of bound molecules



PhoE-channel

http://www.rug.nl/gbb/research/researchGroups/molecularDynamics/picture6big.jpg

Biophysical properties – Negative point charges in the channel mouth



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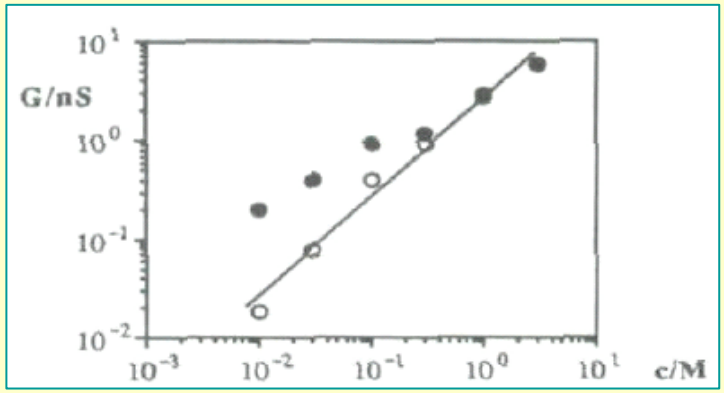

Benz and Trias (1993)[2]

→ **Negative point charges**

- Single channel conductance was not a linear function of the bulk aqueous salt concentrations
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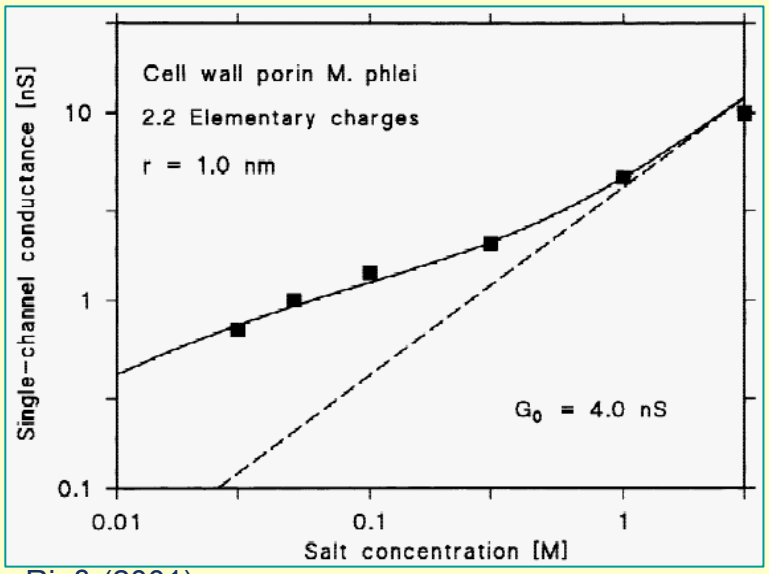
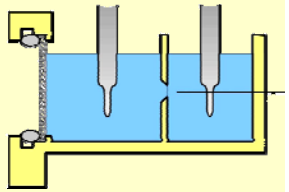


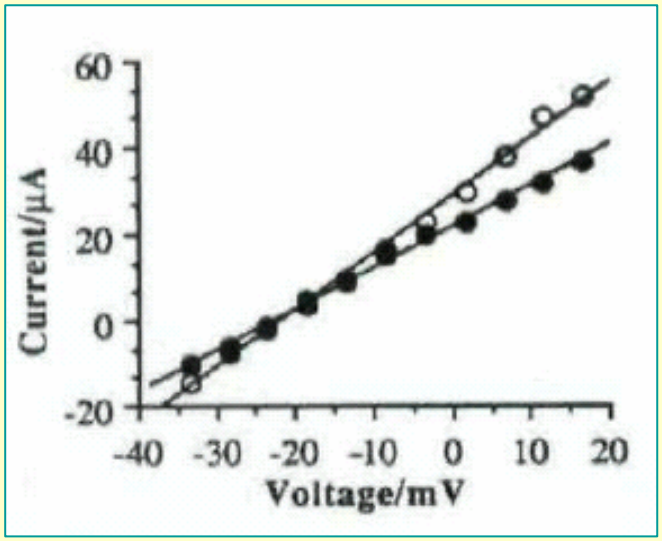
Rieß (2001)[9]

Fit for calculating of the negative point charges

Biophysical properties – Reversal potential and voltage dependence



Reverse potential of membranes containing channels of *M. smegmatis*. NaCl (empty circles), RbCl (full circles)



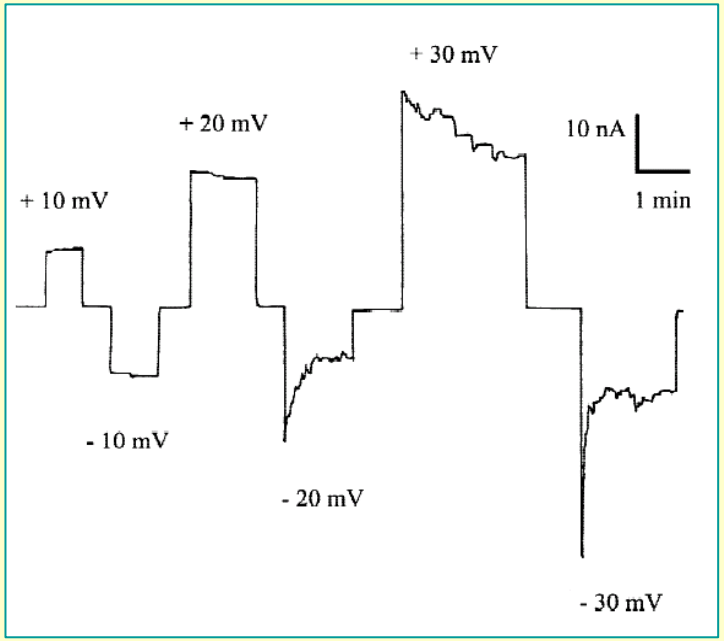
Benz and Trias (1994)[3]

- P_{cation}/P_{anion} ratios were consistent with the single channel experiments

Physiological relevance?

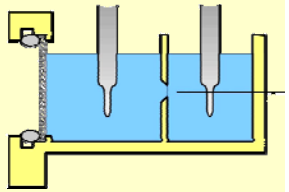
Voltage dependence of *M. phlei* Porin

- Flickering at voltages higher than 10 mV
- Asymmetric voltage-dependence of the cell wall porin



Rieß (2000)[9]

Biophysical properties – Reversal potential and voltage dependence



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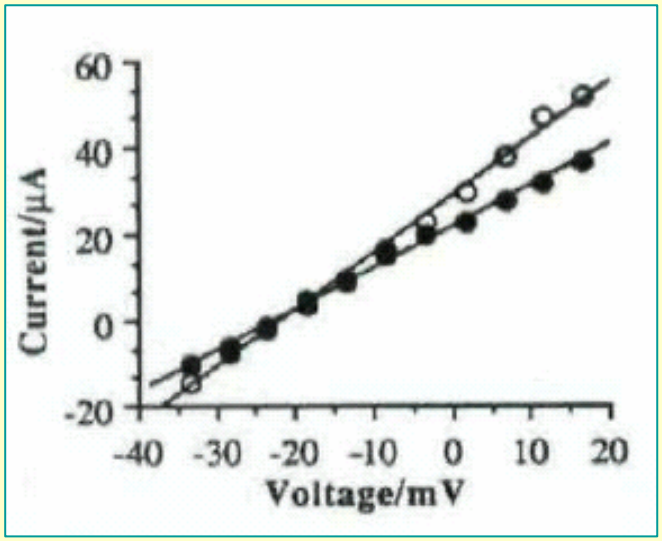

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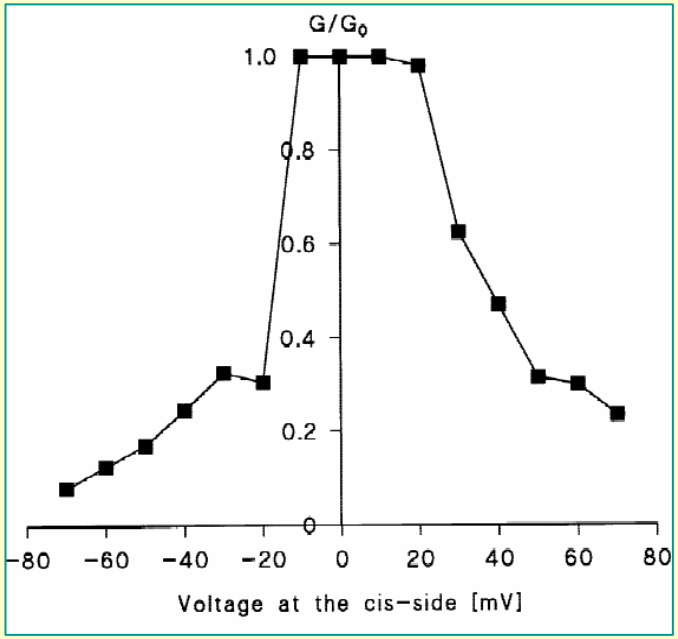


Rieß (2000)[9]

Summary

- Porins were found in many representetives of the genus *mycobacteria*
- Their ability to impove the conductance of the cell wall was shown in lipid bilayer measurements, as well as in liposome swelling experiments
- The porins of fast growing mycobacteria showed common features
- techniques were designed to calculate the single channel conductance, the pore diameter and the number of negative point charges in the channel

Table 1. Properties of mycobacterial porins.

	Λ/nS[a]	MW_O/MW_M/kDa[b]	Porin genes	Reference
M. chelonae	2.7	59[c]	1 *mspA* homologue	Trias *et al.* (1992); Trias and Benz (1993)
M. phlei	4.5	135/22	4 *mspA* homologues	Riess *et al.* (2001)
M. smegmatis	4.6	100/20	*mspA, B, C, D*[d]	Niederweis *et al.* (1999); Stahl *et al.* (2001)
M. tuberculosis	0.7[e]	38[c]	*ompATb*[e]	Senaratne *et al.* (1998)
M. tuberculosis	0.7	15[c]	ND	Kartmann *et al.* (1999)
M. tuberculosis	3	>60[c]	ND	Kartmann *et al.* (1999)
M. bovis BCG	0.8	ND	ND	Lichtinger *et al.* (1999)
M. bovis BCG	4	ND	ND	Lichtinger *et al.* (1999)

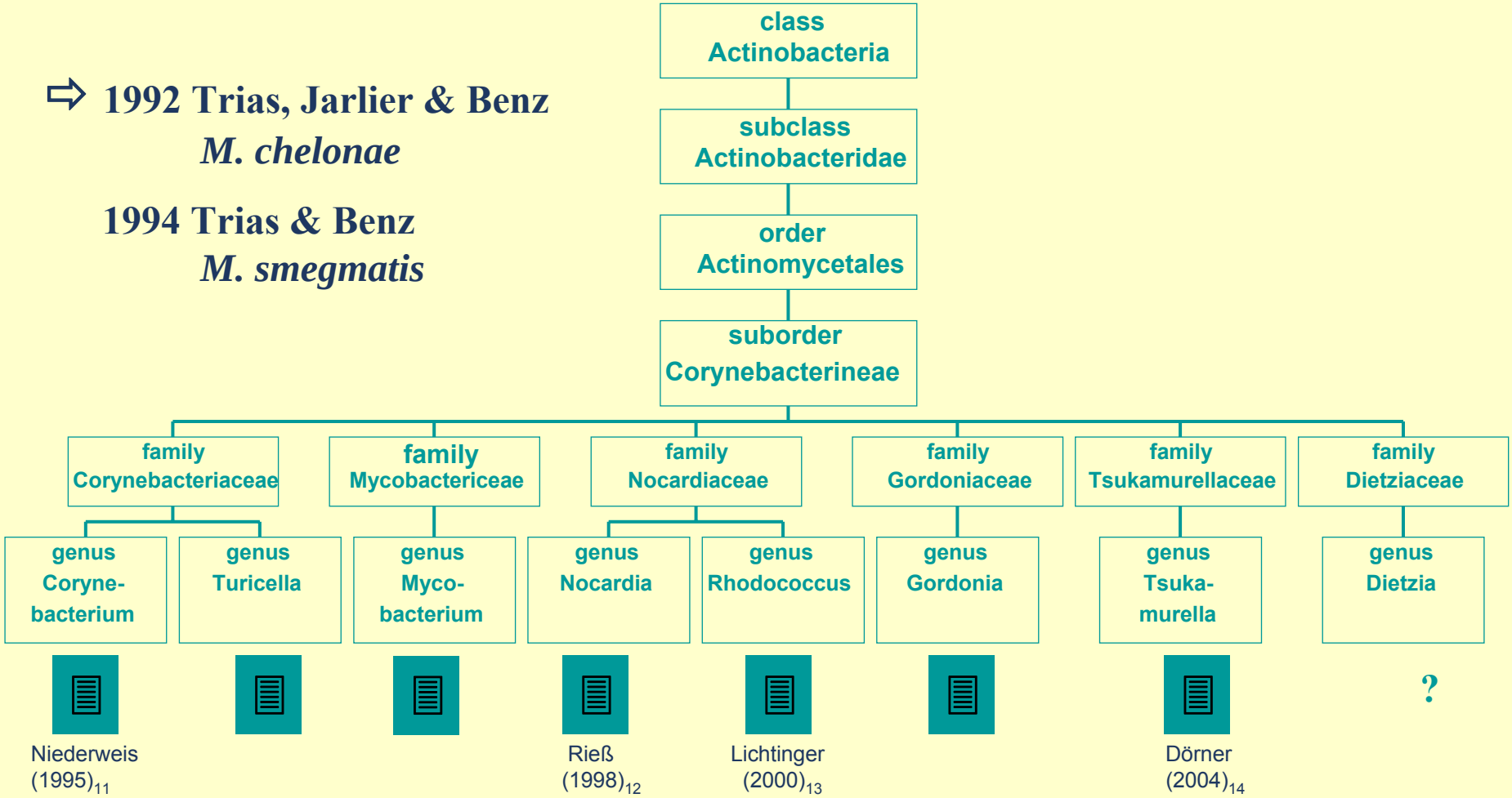
Niederweis $(2003)_{10}$

Are porins existing in other gram$^+$ bacteria as well?

Phylogenetic tree according to Stackebrandt et al. 1997

⇨ **1992 Trias, Jarlier & Benz**
M. chelonae

1994 Trias & Benz
M. smegmatis



Exist channel-forming proteins in all Corynebacterineae?

Porins in different members of the Corynebacterineae

Cell wall porin	*G* in 1 M KCl (nS)	Selectivity P_c/P_a in KCl	Negative point charges at the channel mouth	Channel diameter nm	Reference
M. phlei	4.5	14.9	2.2	1.8[b]; 2.0[c]	This study
M. smegmatis	4.1	9.7	4.0	1.8[b]; 3.0[c]	Trias & Benz, 1994
C. glutamicum	5.5	8.1	2.0	2.2[b,c]	Lichtinger et al., 1998
R. erythropolis	6.0	11.8	2.7	2.0[c]	Lichtinger et al., 2000
N. farcinica	3.0	8.2	1.3	1.4[b]; 1.6[c]	Rieß et al., 1998

Rieß (2001)[9]

- All are containing negative point charges
- Some of them are voltage-dependent, (most of them close when the side of the addition of the protein has negative polarity)

Table 3
Comparison of the cell wall channel properties of different actinomycetes

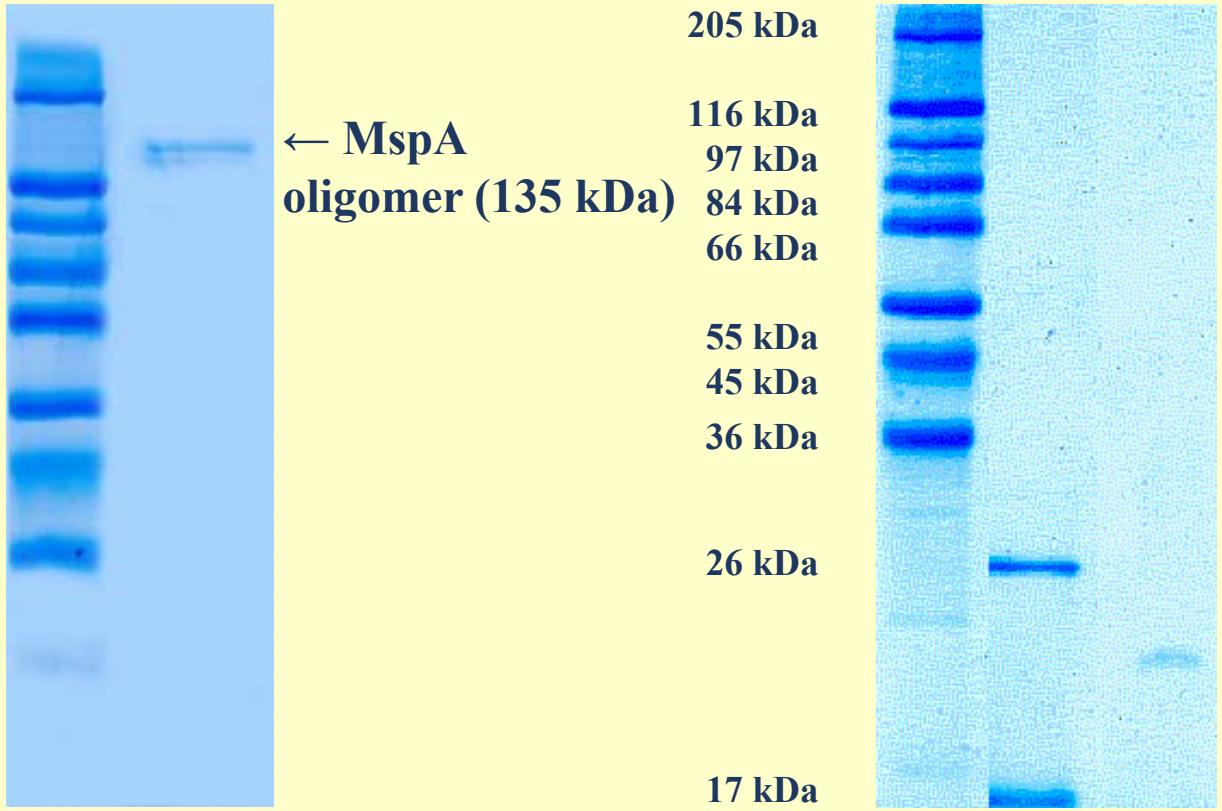
Cell wall protein of	*G* (nS) in 1 M KCl	Channel diameter (nS)
T. inchonensis	4.5	2.0[a,b]
M. smegmatis	4.1	1.8[a], 3.0[b]
M. phlei	4.5	1.8[a], 2.0[b]
N. farcinica	3.0	1.4[a], 1.6[b]
C. glutamicum	5.5	2.2[a,b]

Dörner (2004)[14]

→ **Investigation of the porins at molecular levels**

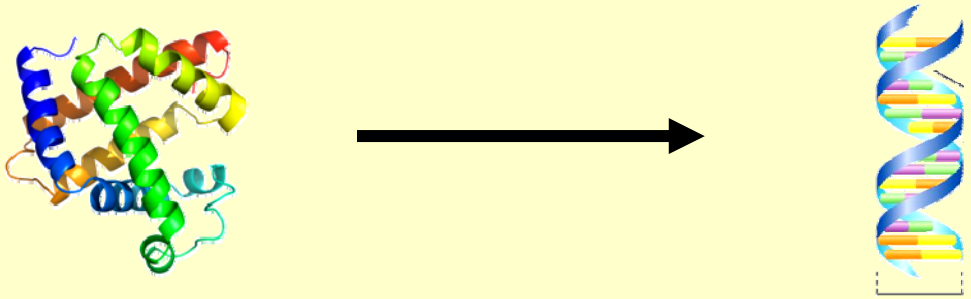
Further purification and investigation of the protein

SDS-PAGE of purified MspA



Solubilization at 30°C

Solubilization at 100°C



Cloning of the *mspA* gene encoding a porin from *Mycobacterium smegmatis*

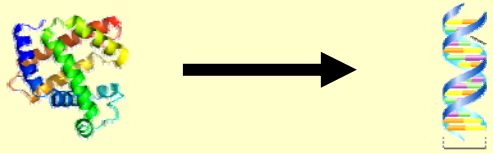
Michael Niederweis,[1] Sabine Ehrt,[2] Christian Heinz,[1] Uta Klöcker,[3†] Stefanie Karosi,[1‡] Kristine M. Swiderek,[4] Lee W. Riley[2] and Roland Benz[3*]

- First cloned gene encoding a porin from gram+ bacteria
- Niederweis (1999)$_{15}$

```
Sequence 700 BP; 116 A; 240 C; 215 G; 129 T; 0 other;
ggggccgccg gcgatacagt tagggagaac atgaaggcaa tcagtcgggt gctgatcgcg
atggttgcag ccatcgcggc gcttttcacg agcacaggca cctctcacgc aggcctggac
aacgagctga gcctcgttga tggccaggac cgcaccctca ccgtgcagca gtgggacacc
ttcctcaatg gtgtgttccc cctggaccgc aaccgtctta cccgtgagtg gttccactcc
ggtcgcgcca agtacatcgt ggccggcccc ggtgccgacg agttcgaggg cacgctggaa
ctcggctacc agatcggctt cccgtggtcg ctgggtgtgg gcatcaactt cagctacacc
accccgaaca tcctgatcga cgacggtgac atcaccgctc cgccgttcgg cctgaactcg
gtcatcaccc cgaacctgtt ccccggtgtg tcgatctcgg cagatctggg caacggcccc
ggcatccagg aagtcgcaac gttctcggtc gacgtctccg gcgccgaggg tggcgtggcc
gtgtcgaacg cccacggcac cgtgaccggt gcggccggcg gtgtgctgct gcgtccgttc
gcccgcctga tcgcctcgac cggtgactcg gtcaccacct acggcgaacc ctggaacatg
aactgattcc tggaccgccg ttcggtcgct gagaccgctt
```

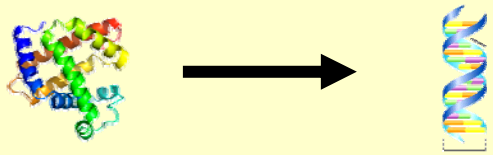
mspa gene, EMBL Nucleotide Sequence Database ID AJ001442

Cloning of the *mspA*-gen

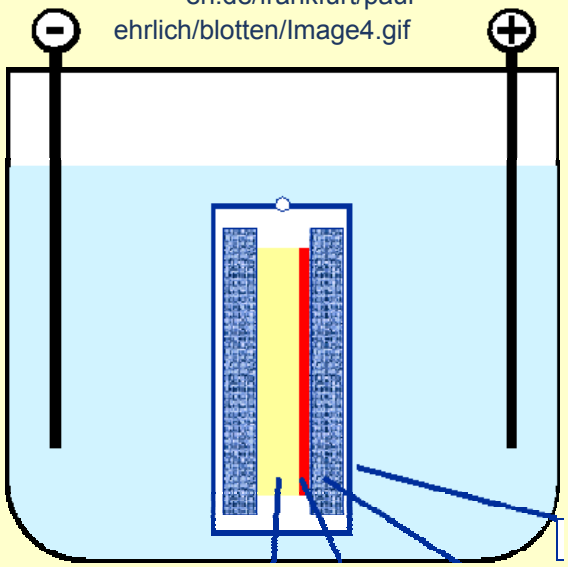


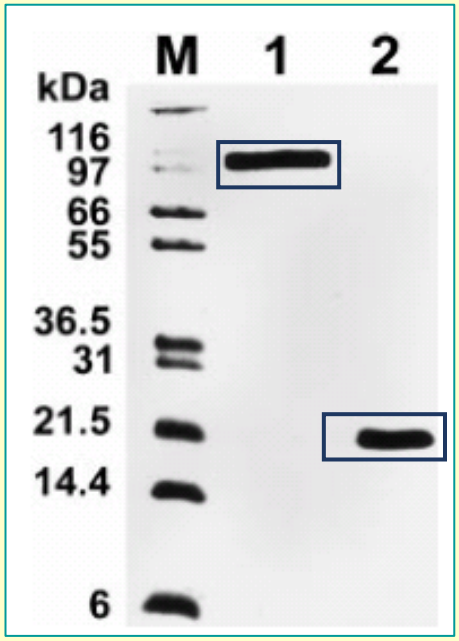
- Purification of the porin
  - Detergents for solubilisation
  - Concentration by two phase-precipitation
  - Analysis by SDS-Page
- Cloning and sequence analysis of the *mspA* gene
  - Transfer from the SDS-gel to a PVDF-membrane and excision of interesting bands
  - Edman degradation and Primer-Modelling
  - Amplification of a genomic library of *M. smegmatis*
- Biochemical analysis of MspA-Porin
- Expression of the *mspA* gene in *E. coli*
- Channel properties of the MspA porin
- Occurrence of the *mspA* gene in mycobacteria

Transfer to a PVDF-Membran and excision of bands



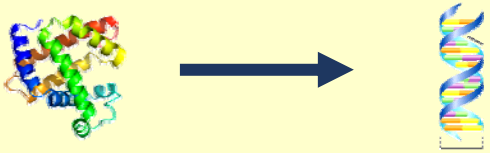
http://www.schulserver.hessen.de/frankfurt/paulehrlich/blotten/Image4.gif

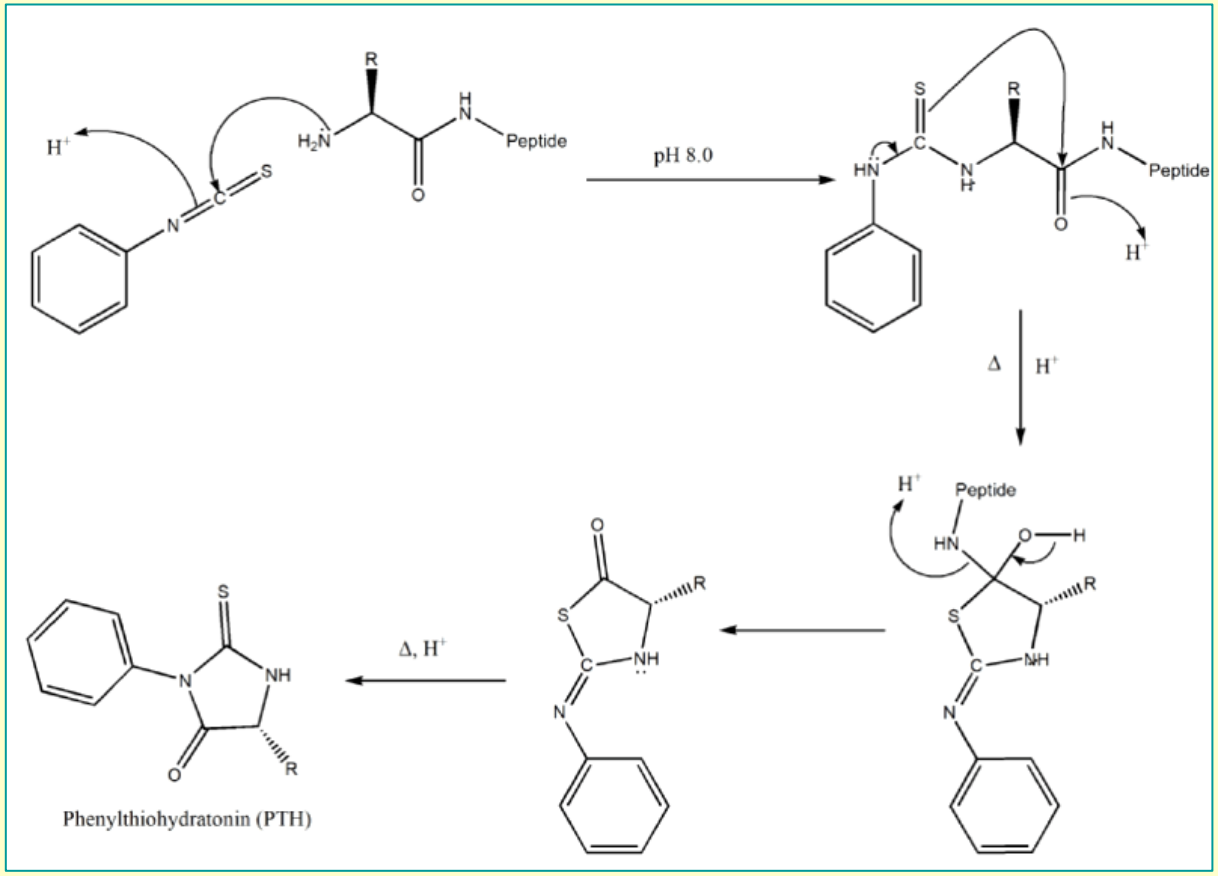




Niederweis (1999)[15]

Edman degradation



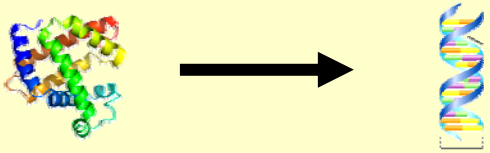


Sequencing amino acids in a peptide

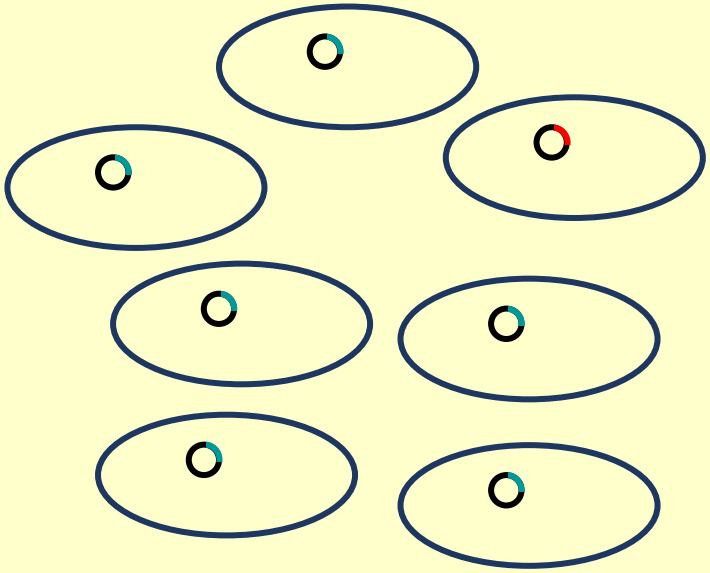
- Phenylisocyanate reacts with uncharged terminal amino group
- cleavage of the terminal amino group as thiazolinone under acidic conditions
- Identification of the Amino acid by chromatography or electrophoresis

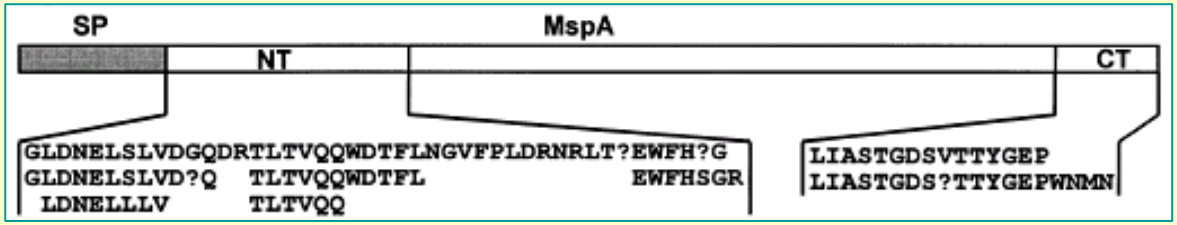
http://upload.wikimedia.org/wikipedia/commons/thumb/1/1f/Edman.gif/600px-Edman.gif

Primer Modelling and amplification of a genomic library of *M. smegmatis*



- Translation of the amino sequence into DNA-Sequence according the codon usage of Mycobacterium
- Modelling one primer
- PCR with primer and a primer binding to the plasmid in an E. coli library of *M. smegmatis*
- Sequencing of the positive clones

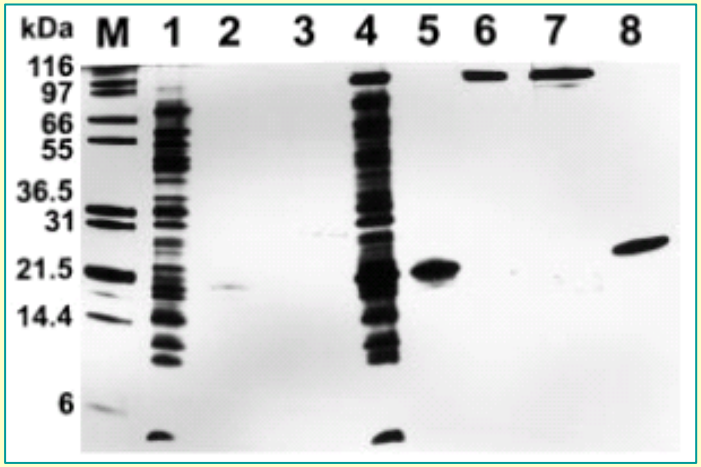




Niederweis (1999)[15]

Expression of the *mspA* gene in *E. coli*

- Ligation of the *mspA* gene in an expression vector (T7-promotor) => Plasmid pMN501
- Transformation in *E. coli Bl21 (DE3)*
- Purification of the cell wall proteins by boiling in water
- Analysing of the proteins by SDS-page
- Analysing of the protein by Western-Blotting and sequencing by Edman degradation

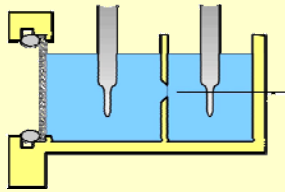


Niederweis (1999)[15]

Line 1 till 3; control-peptid, line 4; 1 µg of total protein, line 5 till 8 elutions of line 4 at specific molecular masses

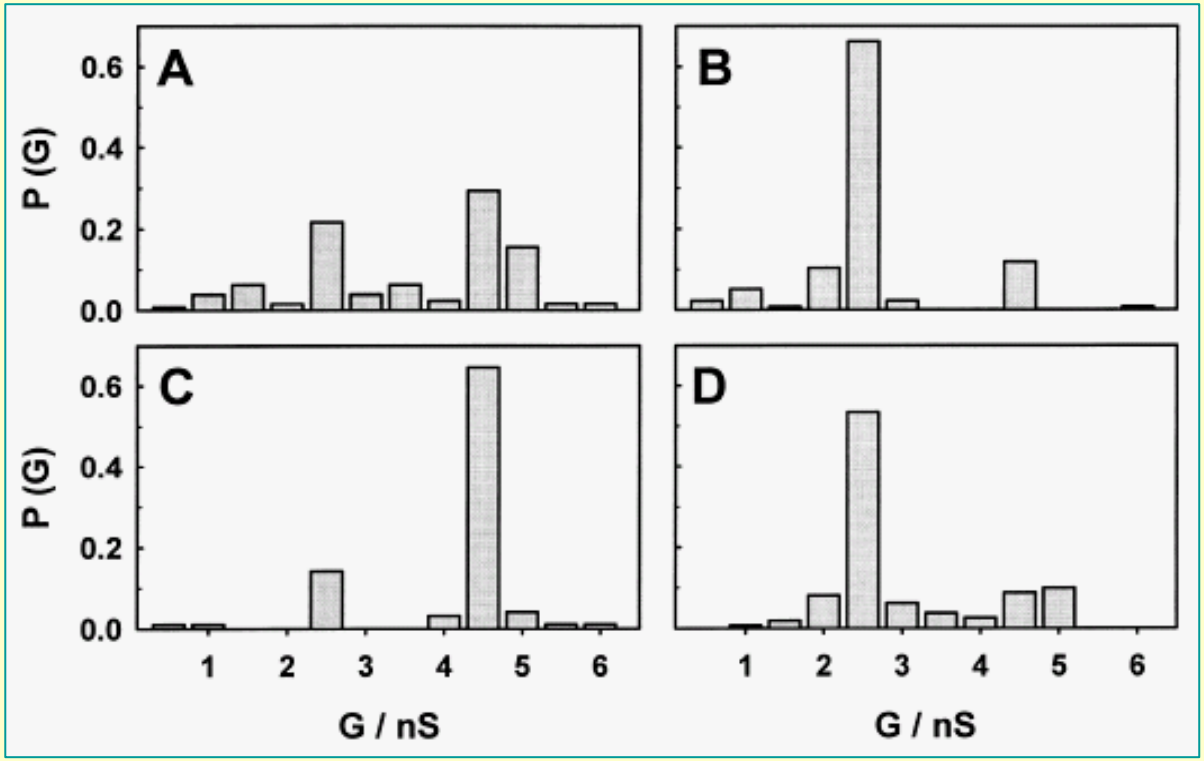
Investigation of the recombinant MspA in bilayer experiments

Investigation of recombinant MspA-protein in bilayer experiments



Extract of *M. smegmatis* with 1 % Genapol

Porin purified from *M. smegmatis*



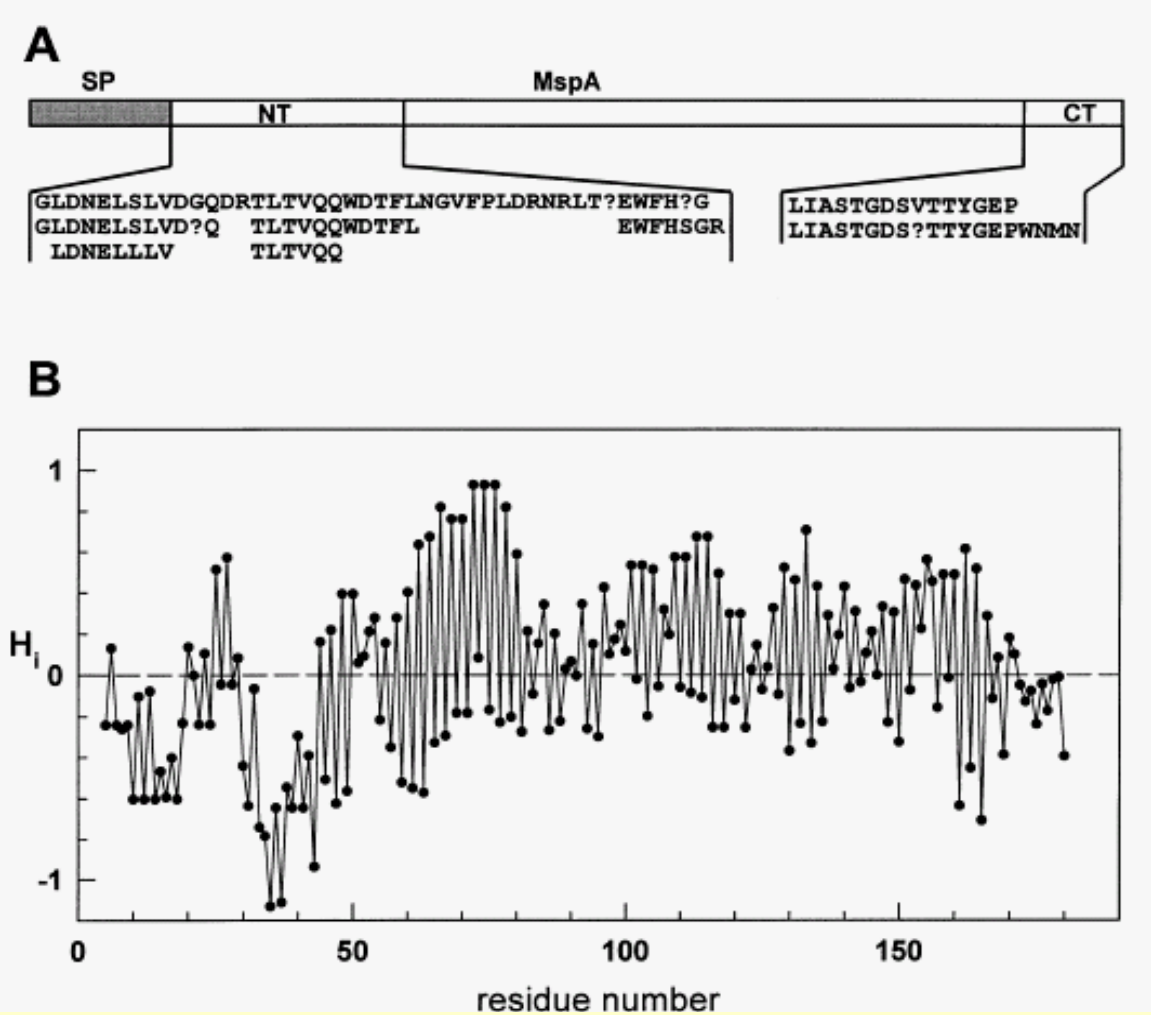
Niederweis (1999)[15]

Extracts of *M. smegmatis* with methylenechloride/methanol

Recombinant MspA purified as a 100 kDa protein complex after exression in *E. coli*

Different MspA confirmations?

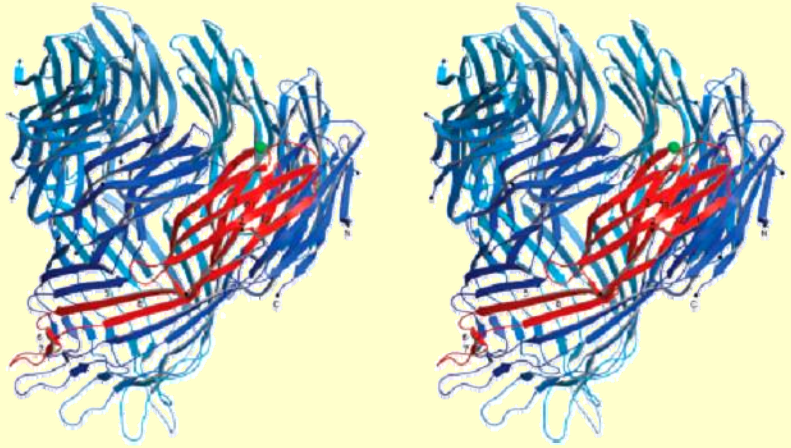
Structure of MspA

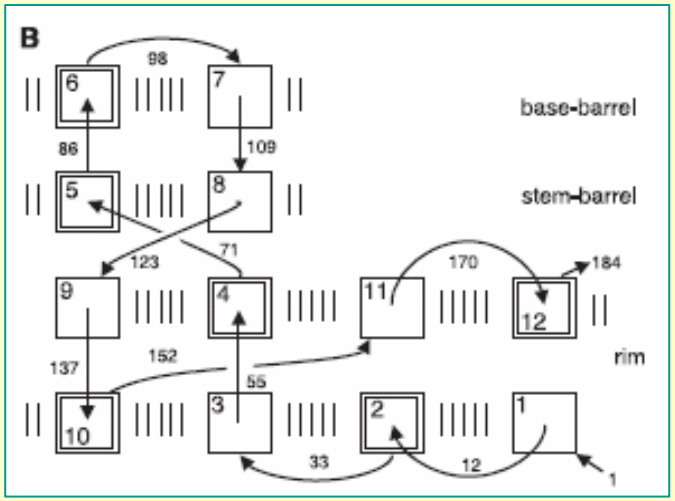


Niederweis (1999)[15]

- no transmembrane helices
- β-strands of porins line a water filled channel => they must be amphiphatic (alternating hydrophobic and hydrophilic residues)
- With 184 amino acids surprisingly small (half the number of amino-acids for a membrane twice as thick compared to porins of E. coli)
- Forms different Oligomers up to 220 kDa

Structure of MspA

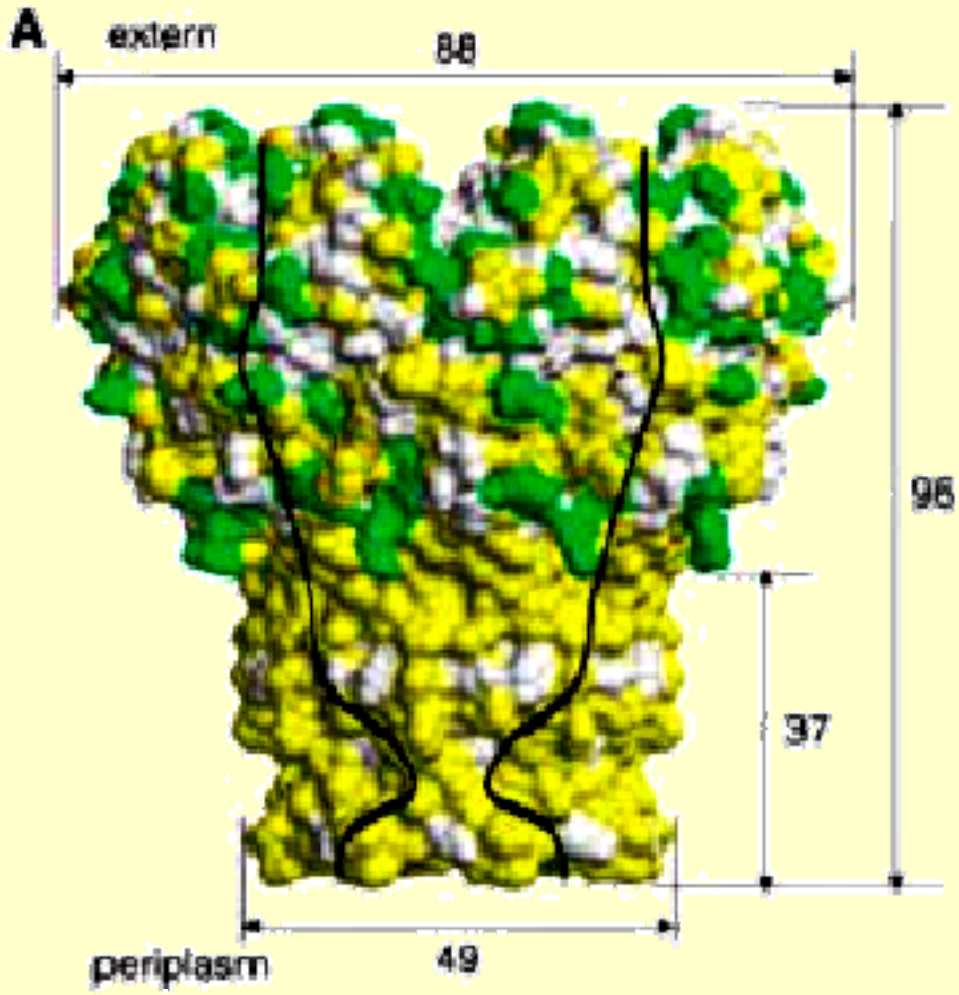




Faller (2004)[16]

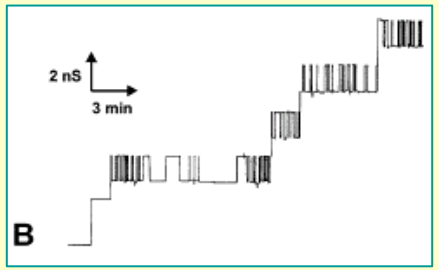
- structure cleared by X-Ray Analysis
- homoacetomeric goblet-like conformation with a single central channel
- octamer with eightfold rotation symmetry
- two consecutive β–barrels with nonpolar outer surfaces, that form a ribbon around the porin
- too narrow to fit the thickness of the mycobacterial outer membrane in contemporary models
- 134-residue-domain forms the thick rim of the goblet
  - a sandwich of two four-stranded completely antiparallel β-sheets
- a 50-residue loop forms the stem and the base
  - two 16-stranded conventional barrels
- pore eyelet ➔ reducing of β–barrel diameter without changing the number of strands

Structure of MspA



Faller (2004)[16]

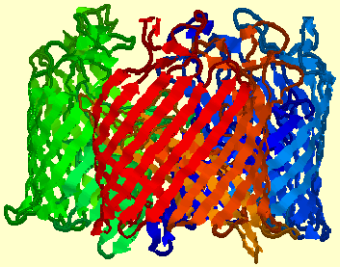
- the outer surface of the goblet shows a clear subdivision
  - polar surface of the globular rim domains
  - nonpolar surface of the goblet´s stem and base
- channel diameter between 48 Å and 10 Å at the pore eyelet
- the pore eyelet may fold back into the channel interior ➔ restricts the area accessible to diffusion



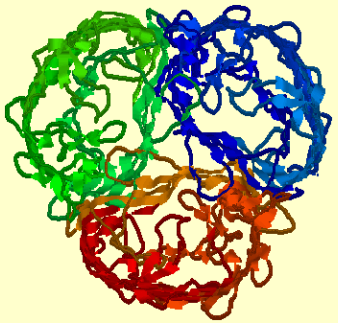
Niederweis (1999)[15]

Comparison of MspA with Porins of gram negative Bacteria

Side view of the LamB trimer of *E. coli*



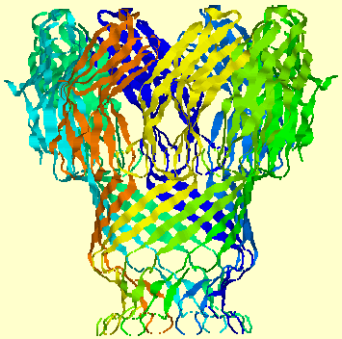
View from the external side



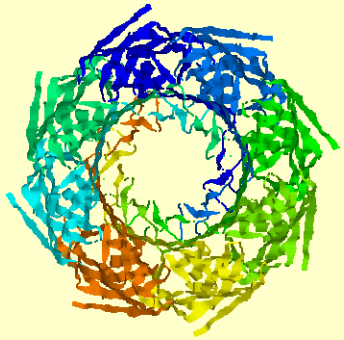
presentation of Prof. R. Benz

- No sequence similarities to porins of gram$^-$ bacteria
- B-structure differs completely from its counterparts in gram$^-$ bacteria
- The oligomer forms a single channel, compared to trimeric channels of gram$^-$ bacteria where each monomer forms a channel
- Length of 9,6 nm compared with 4 nm pores in gram$^-$ bacteria
- a 45-fold lower number of MspA-channels exists in the cell wall compared to gram$^-$ bacteria

Side view of MspA of *Mycobacterium smegmatis*

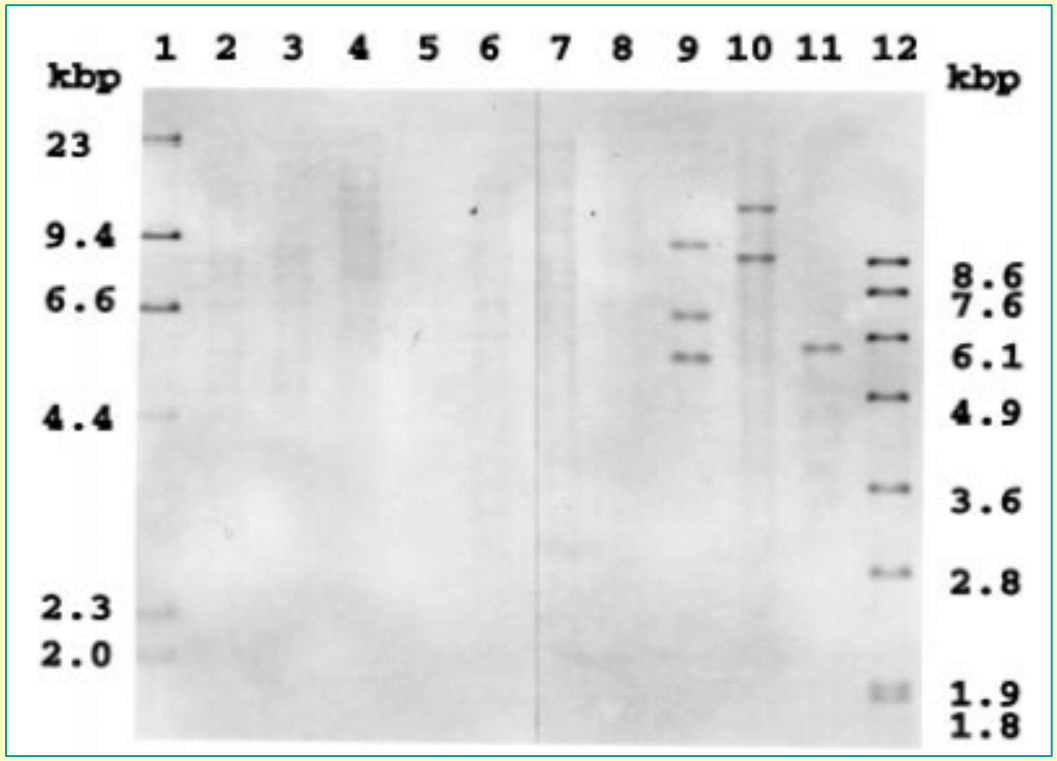


View from the external side



presentation of Prof. R. Benz

Occurance of the *mspA*-Gene in Mycobacteria



Niederweis (1999)[15]

Lane 1+12: Marker Lane 2: M. tuberculosis, lane 3: M. bovis, lane 4: M. africanum, lane 5: M. microti, lane 6: M. avium, lane 7: M. intracellulare, lane 8: M. kansasii, lane 9: M. smegmatis, lane 10: M. fortuitum, lane 11: M. chelonae

- Chromosomal DNA was digested and analysed by Southern blotting with a digoxigenin-labelled probe of the *mspA*-gene
- None of the slow-growing mycobacterial strains hybridized => specific to fast growing Mycobacteria
- *M. smegmatis* chromosome contains several copies of the porin gene

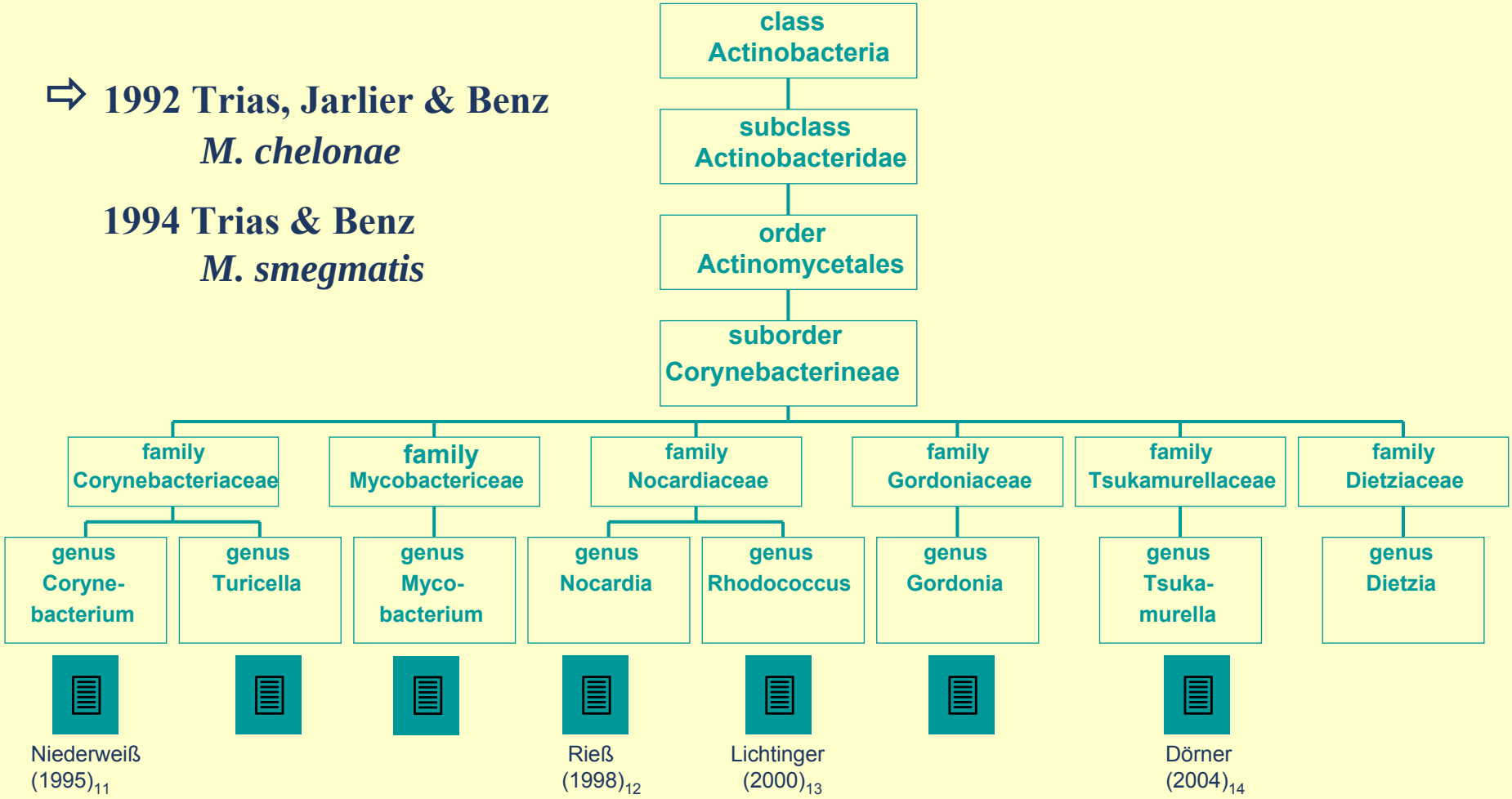
Identification of *mspB, mspC*, *mspD* in *M. smegmatis* Stahl et al (2001)[17]

Occurrence of *mspA* in other Corynebacterineae?

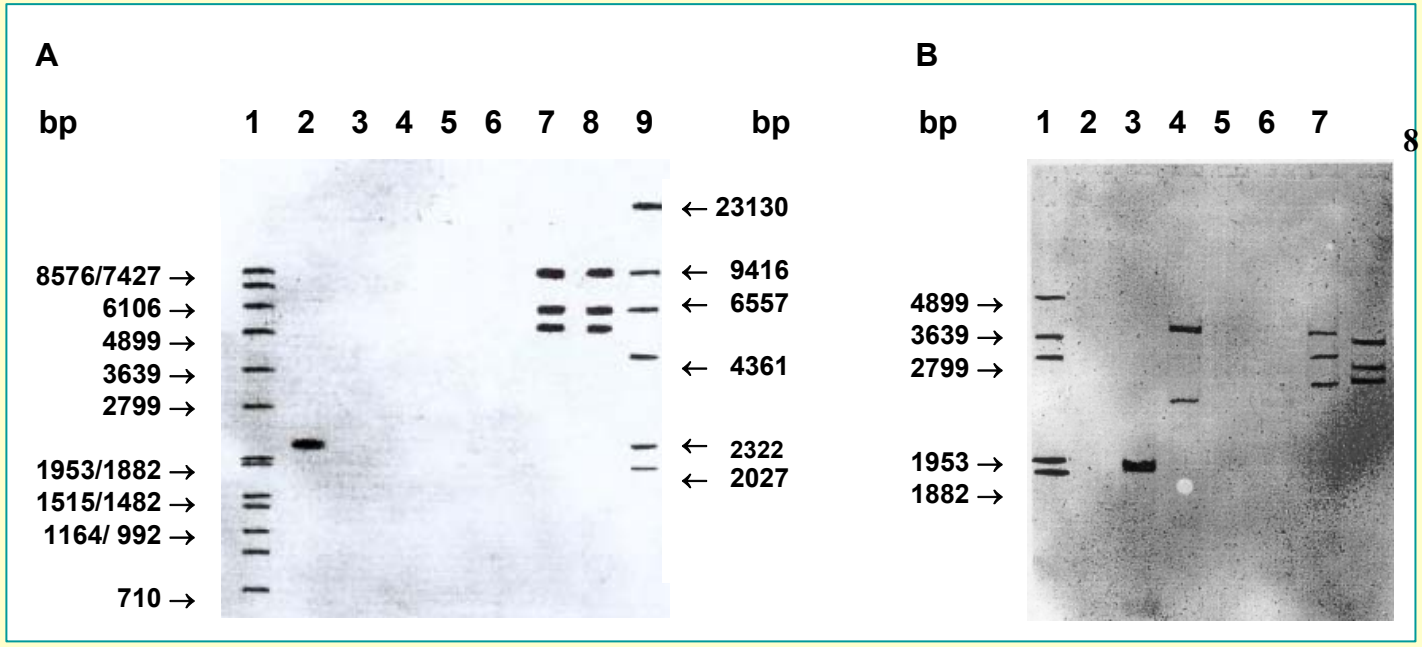
Phylogenetic tree according to Stackebrandt et al. 1997

⇨ **1992 Trias, Jarlier & Benz**
M. chelonae

1994 Trias & Benz
M. smegmatis



Occurance of the *mspA-Gene* in Corynebacterineae



Rieß (2001)[9]

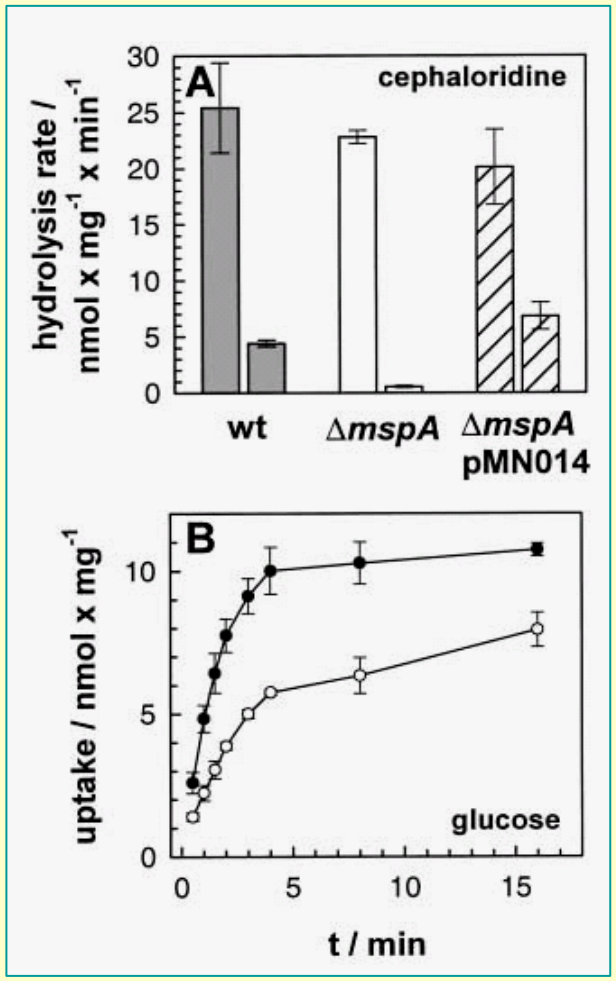
A: High stringency (60°C):
2 *T. inchonensis*; 3 *N. farcinica*; 4,5 free; 6 *R. equi*; 7 *M. phlei*; 8 *M. smegmatis*

B: Low stringency (40°C):
2 free; 3 *T. inchonensis*; 4 *N. farcinica*; 5,6 free;7 *R. equi*; 8 *M. phlei*

- **Southern blot analysis concerning the presence of the *msp*A gene in different species of *Corinebacterineae***

- **Chromosomal DNA of each strain was cut with *Bam*H1, separated on a 0.7% TAE agarose gel and Southern blotting was performed with a primer derived from *mspA***

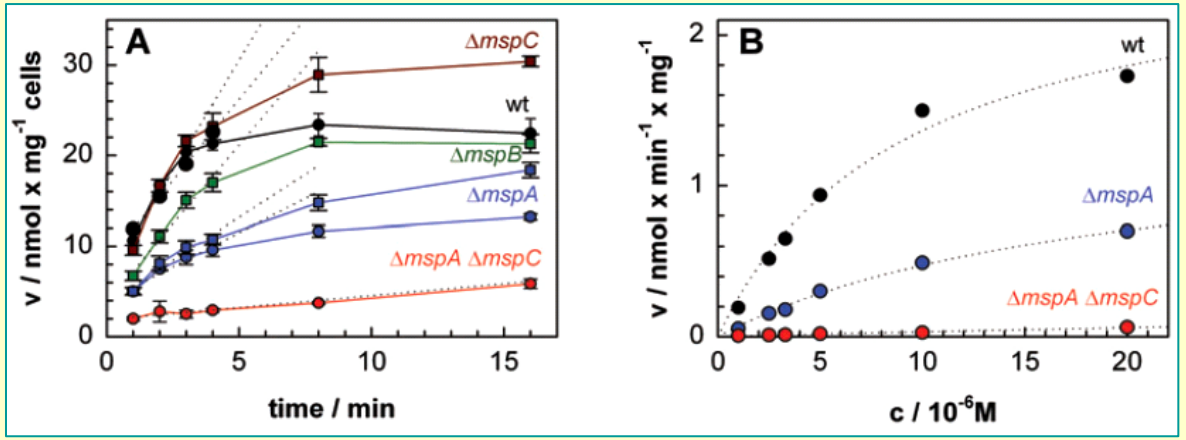
MspA as transporter for hydrophilic compounds and its role to the growth rate Stahl $(2001)_{17}$, Stephan $(2005)_{18}$, Wolschendorf $(2007)_{21}$



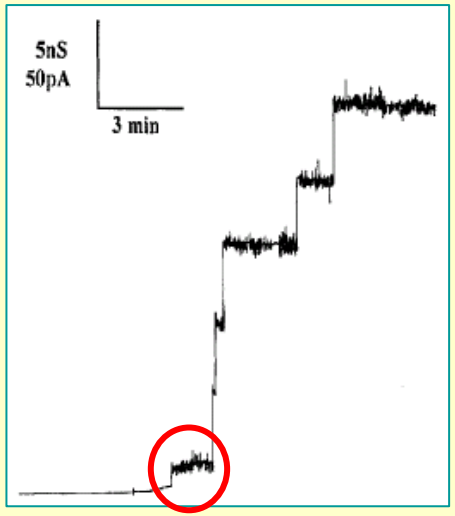
Stahl $(2001)_{17}$

- MspA represents the major porin of *M. smegmatis*
- MspA is important for the transport of glucose, serine and β–lactam antibiotics
- deletion of one porin activates other silent porins in the genome
- seems to be important for phosphate uptake but adverse properties like negative point charges

does a channel with selectivity for anions exist?

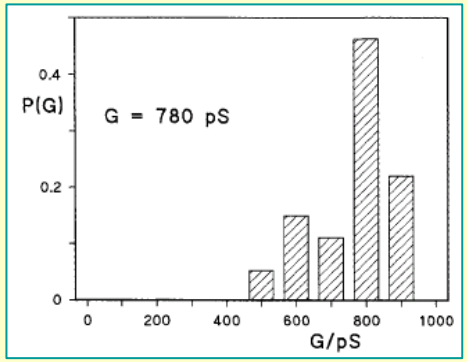


Stephan $(2005)_{18}$



Evidence for a small anion-selective channel in *Mycobacterium bovis* Lichtinger et al (1999)[19]

- two channel-forming components were found
- single channel conductance of about 800 pS in 1 M KCl
- Conductance was smaller in 1 M potassium acetate than in 1 M LiCl ➔ anions-selective
- it was not possible to relate the channel-forming activity to a defined molecular mass
- no voltage dependence



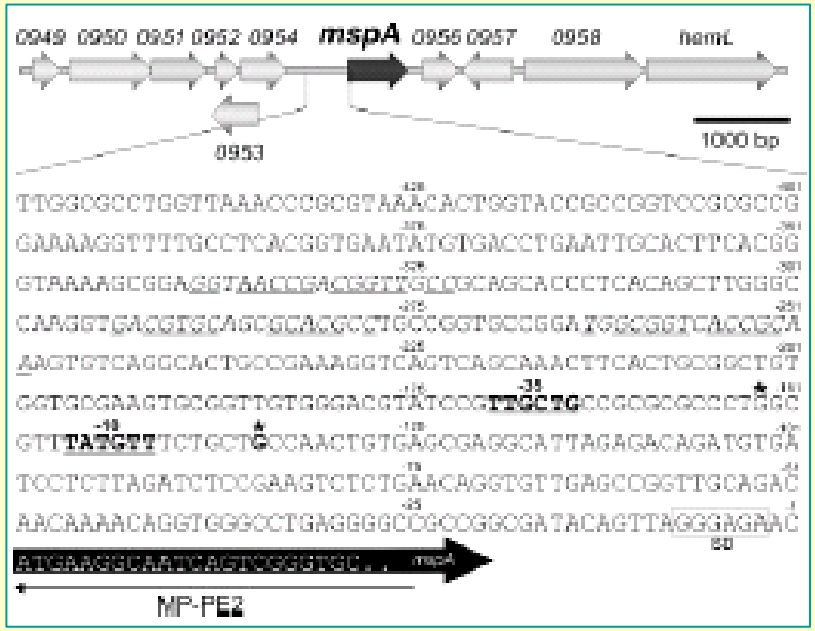
← Pores after incubating 20 h at 50 °C

Table 2
Average single-channel conductance, *G*, of the channel derived from detergent extracts of whole *M. bovis* BCG cells in different salt solutions[a]

Salt	Salt concentration (M)	Single-channel conductance *G* (pS)
LiCl	1.0	650
KCl	0.10	85
	0.30	250
	1.0	780
	3.0	1800
KCH_3COO (pH 7)	1.0	500

Lichtinger (1999)[19]

Regulation of *mspA* Hillmann $(2007)_{20}$



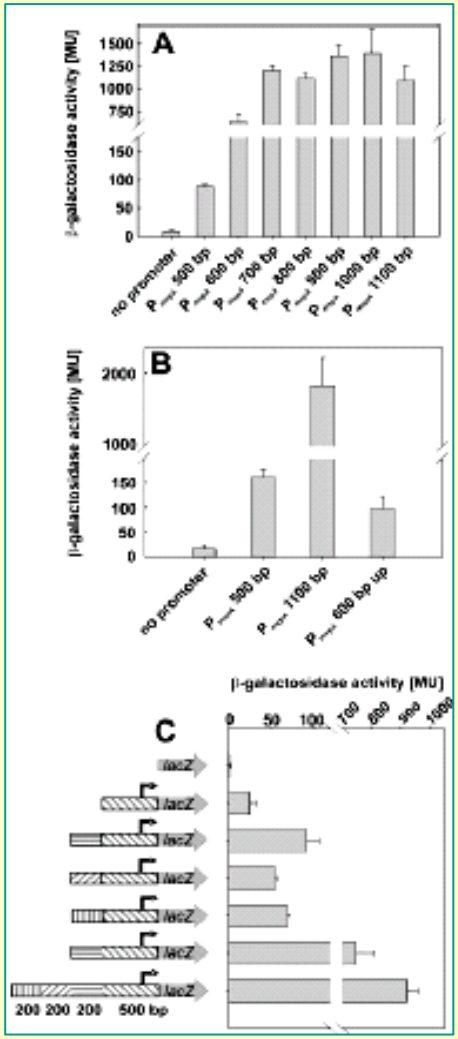
Hillmann $(2007)_{20}$

Experiments

- primer extension experiments
- β-galactosidase was used as reporter-gene
- Constitutive mycobacterial promotors P_{imyc} and P_{smyc} were used

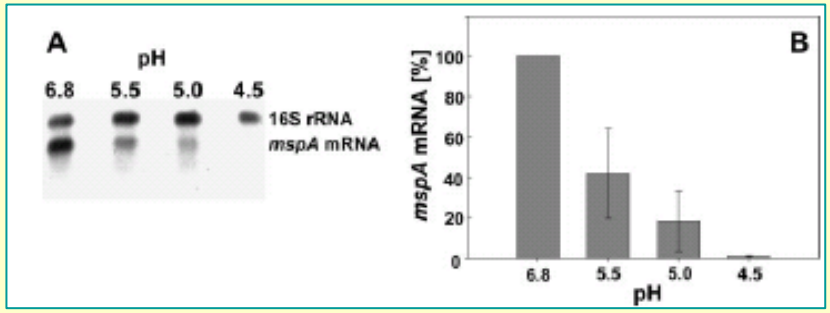
Results

- Identification of promotor; -10 and -35 region, -135 bp upstream of the *mspA-gene*
- Identification of a very long upstream activating region which is depending on the phasing of the DNA helix
- A 5‘ UTR contributes to the stability of the *mspA* transcript ➔ *mspA* is transcribed independently



Hillmann $(2007)_{20}$

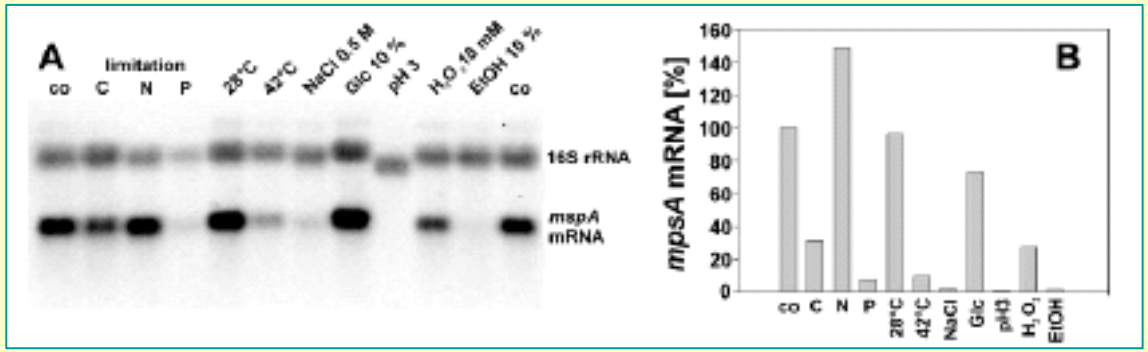
Which signals are regulating *mspA*?



Hillmann (2007)$_{20}$

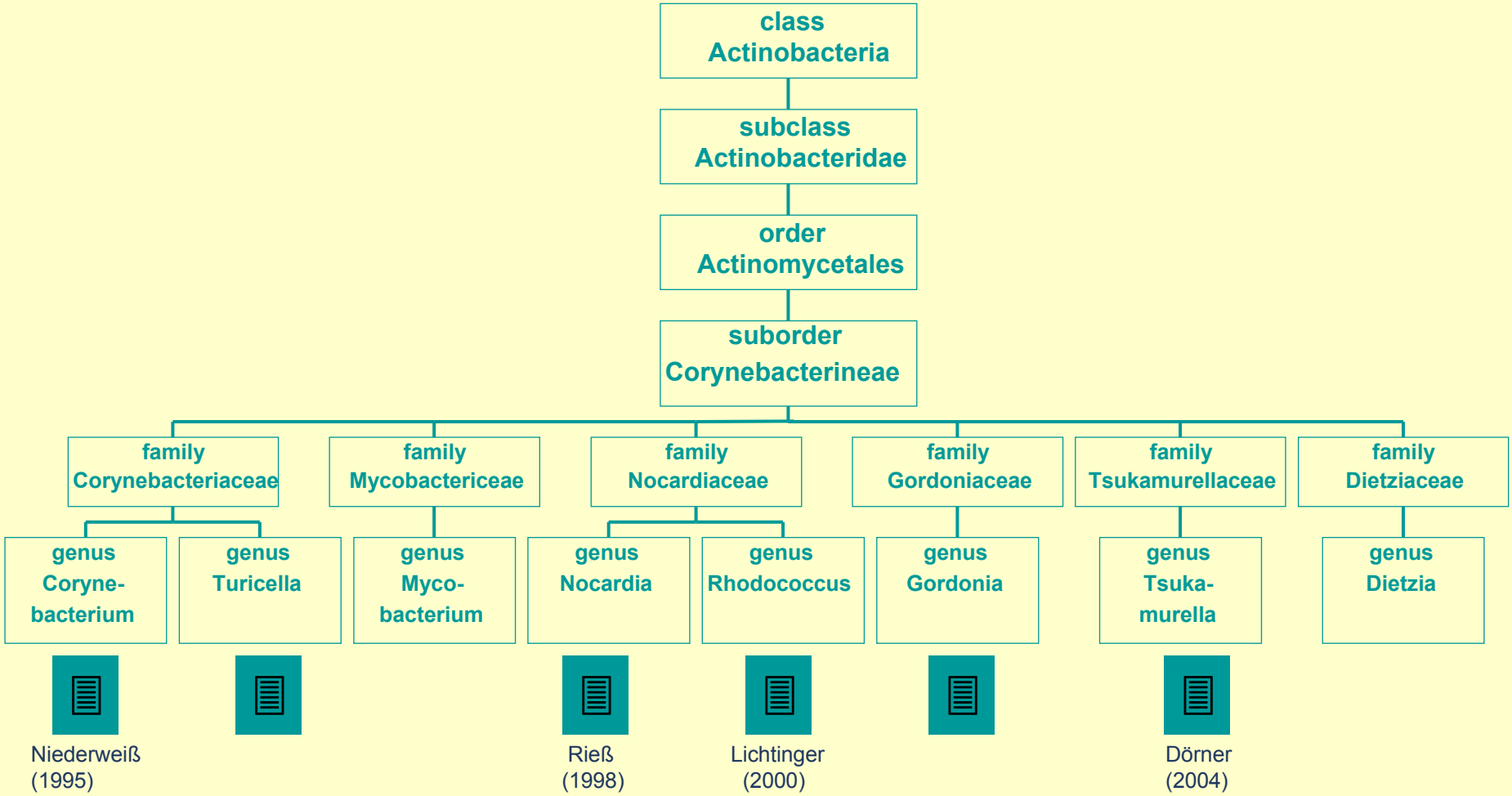
- amount of *mspA* mRNA is 25-fold decreased in stationary phase
- expression is decreased under carbon and phosphate limitation but elevated at nitrogen limitation
- high temperature, increased osmolarity, hydrogen peroxide, ethanol and low pH decreased expression of *mspA*
- *the* decrease under low pH is a specific regulatory event

http://www.von-stackelberg.de/bilder/burgtor.jpg

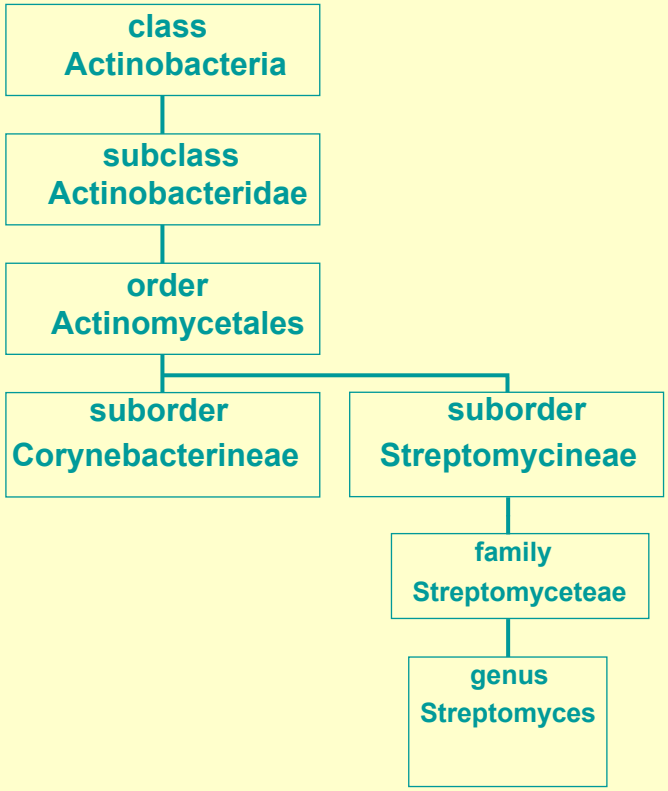


Hillmann (2007)$_{20}$

Phylogenetic tree according to Stackebrandt et al. 1997

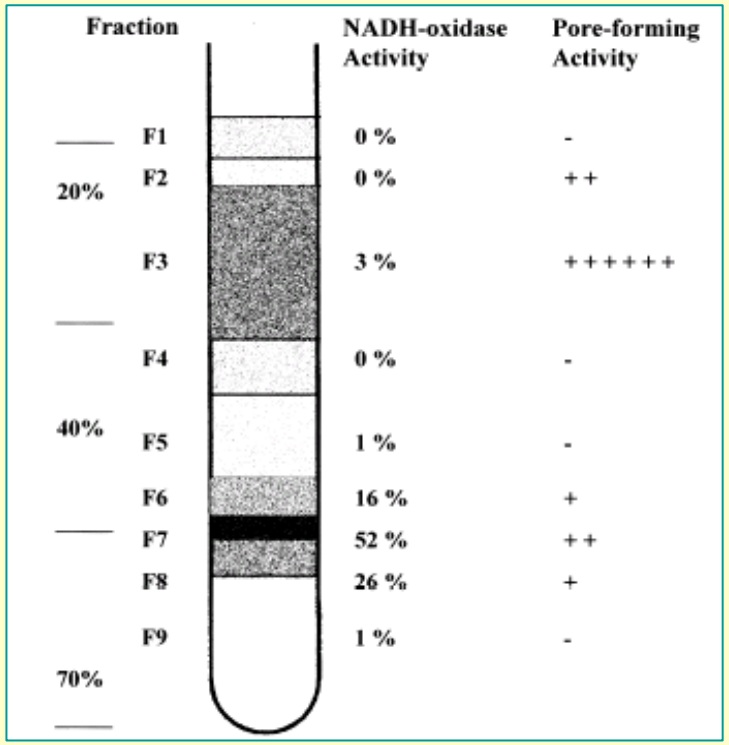


Phylogenetic tree according to Stackebrandt et al. 1997

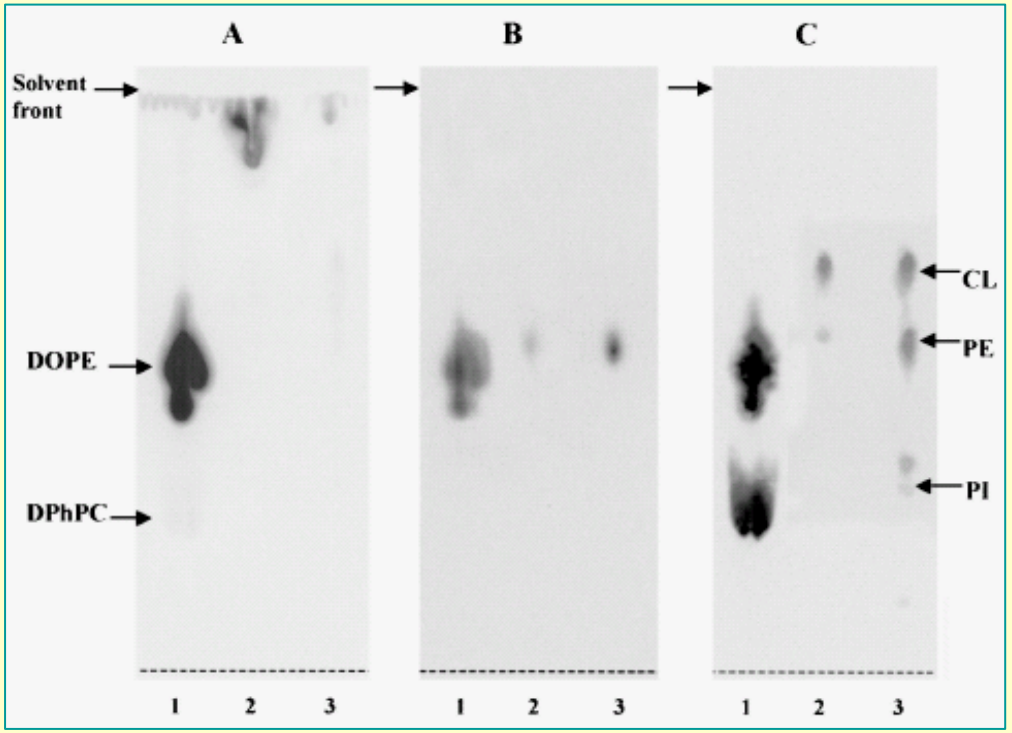


Streptomyces griseus has a second permeability barrier.

- no mycolic acids in the cell wall
- *the cell wall was analysed by sucrose-density gradient centrifugation*
- *lipids were found by one dimensional thin-layer chromatograms*
- *lysozyme was not able to lyse cells*



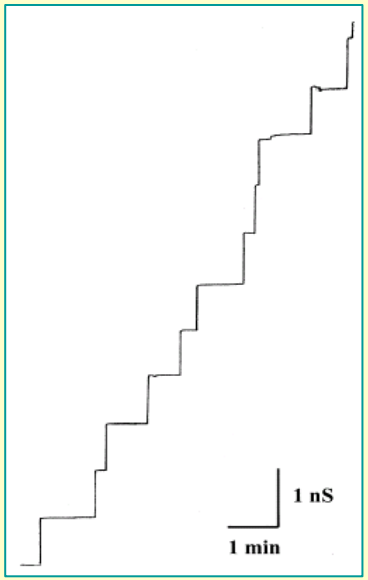
Bong Hui Kim (2001)$_{22}$



Bong Hui Kim (2001)$_{22}$

Identification of a cell wall channel of *Streptomyces griseus* Bong Hui Kim et all (2001)[22]

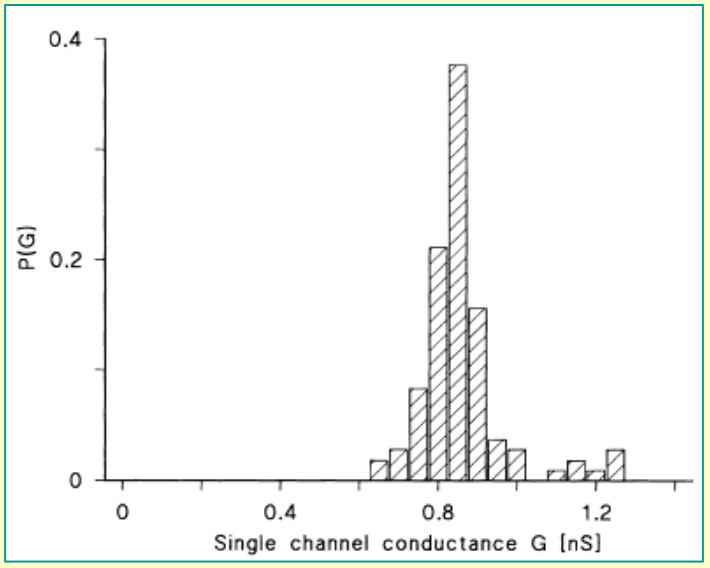
- Cell wall has a smaller density than the cytoplasmic membrane
- the current increased in a stepwise fashion similar to that observed for cell wall channels of the mycolata
- the channel is only moderatly selective to anions and cations ➔ contains probably positively and negatively charged groups
- wide and water filled channel



Bong Hui Kim (2001)[22]

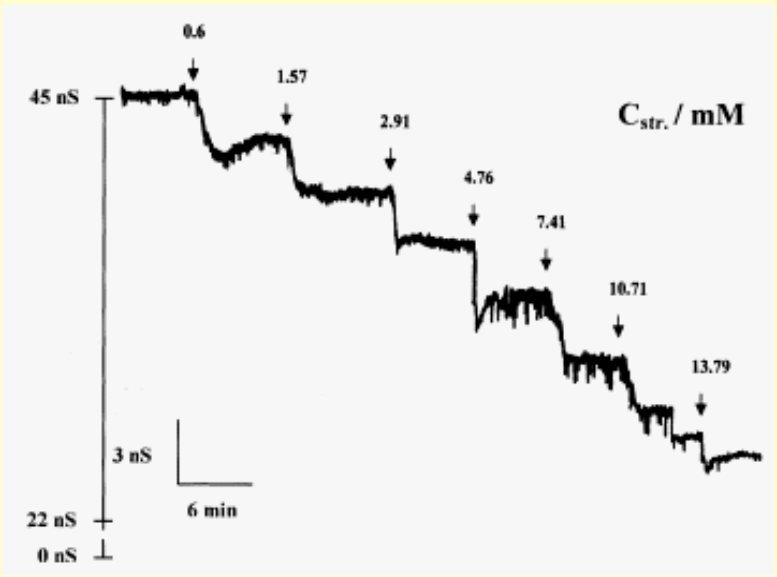
Single channel conductance of *S. griseus*
850 pS in 1 M KCl
530 pS in 1 M LiCl and
580 pS in KAc

Permeability p_{cation}/p_{anion} =1.0 and 1.5



Bong Hui Kim (2001)[22]

Streptomyces griseus cell wall channel binds Streptomycin



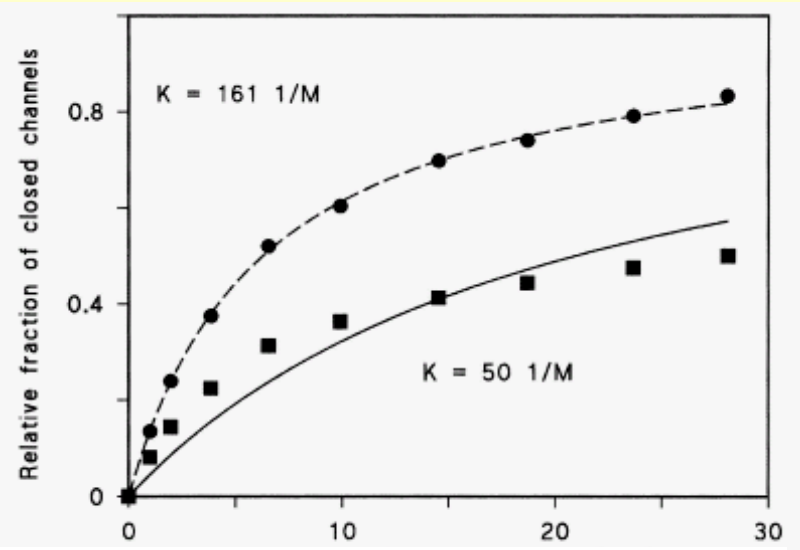
Bong Hui Kim (2001)[22]

Titration with streptomycine

➔ **Suggested a binding site for streptomycin**

- **K of 161 1/M**
- **binding decreases conductance to 40 % compared to open state**
- **binding based on ion-ion interaction**

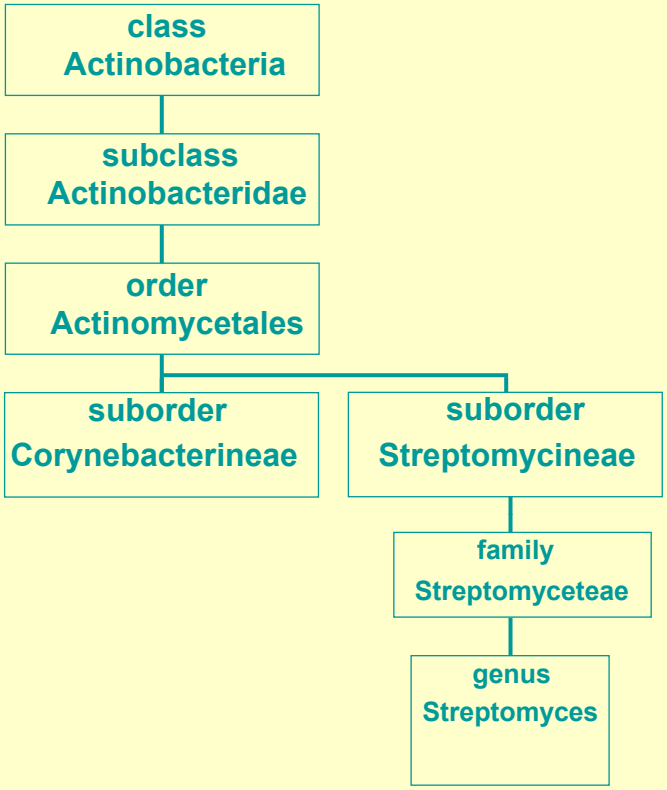
- ***Streptomyces griseus* produces the antibiotic Streptomycin**



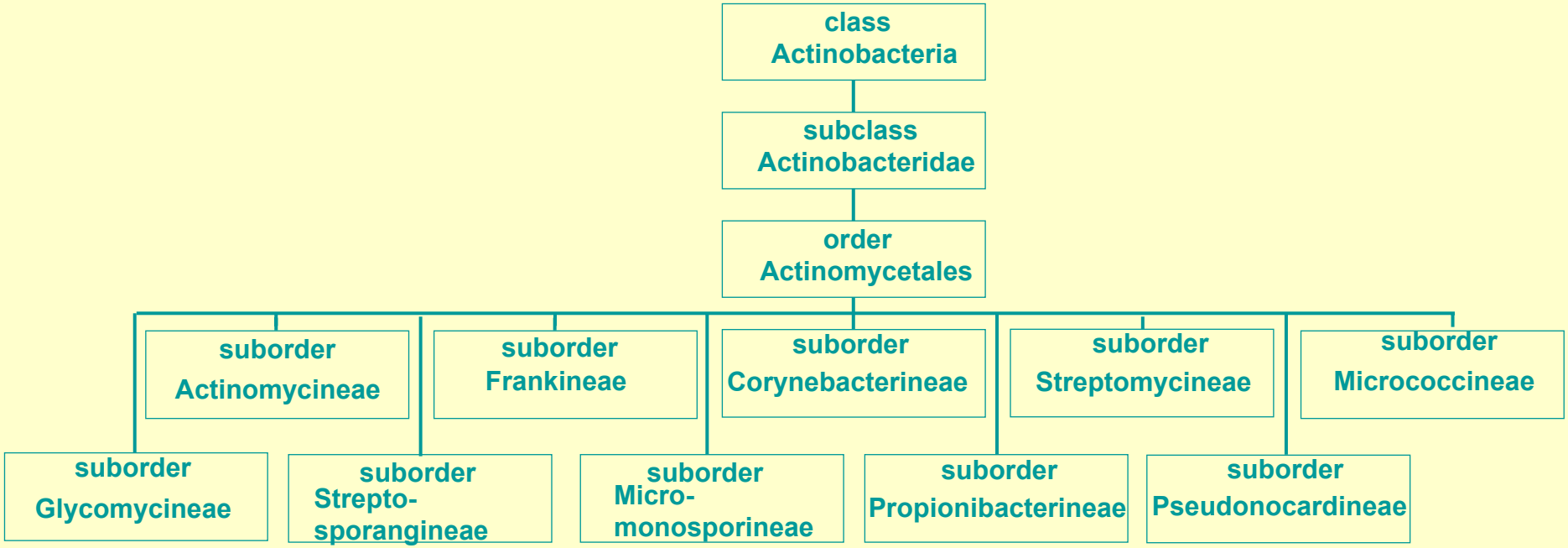
Streptomycin concentration (mM)	G/pS
0	850
1	770
2.5	670
5	640
10	530
30	450

Bong Hui Kim (2001)[22]

Phylogenetic tree according to Stackebrandt et al. 1997



Phylogenetic tree according to Stackebrandt et al. 1997



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