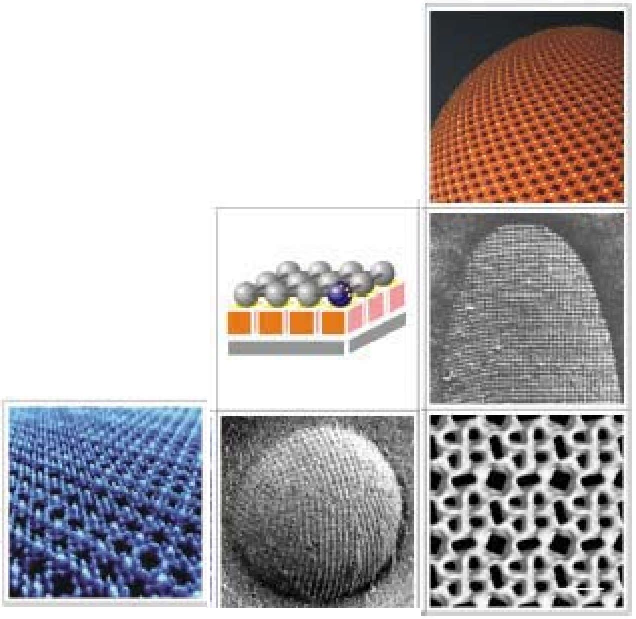
Drug delivery

Protein based
Drug delivery systems

S-layer proteins as basic building blocks in a biomolecular construction kit



Outline

- Development of a (bio)molecular construction kit
- Description of S-layer proteins (basic principles)
- S-layers as nano-scale patterning elements for life- and non- life science applications (selected exmples)

Which are the basic building blocks in a biomolecular construction kit

- Biological molecules (e.g. Proteins, Lipids, Glycans, Nucleic acids)
- Chemically or Genetically Modified Molecules
- Chemically Synthesized Molecules

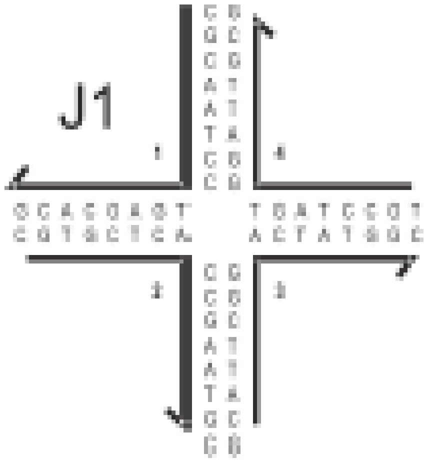
Key capabilities

- Self-assemble
- Molecular recognition
- Adaptable and evolved structure & function
- Dynamic structures

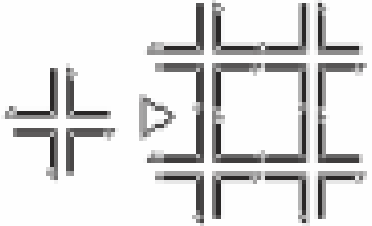
Basic Structures (Patterning Elements) for Generating Complex Supramolecular Structures

- DNA
- Monomolecular crystalline bacterial cell surface layers (S-layers)

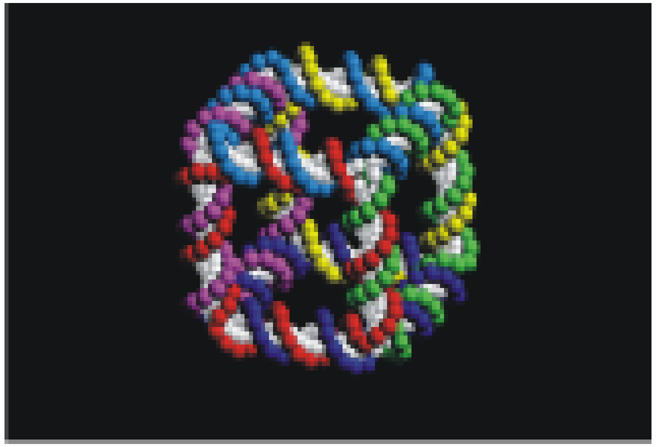
Nanoscale Assembly and Manipulation of Branched DNA (Ned Seeman, NY University)

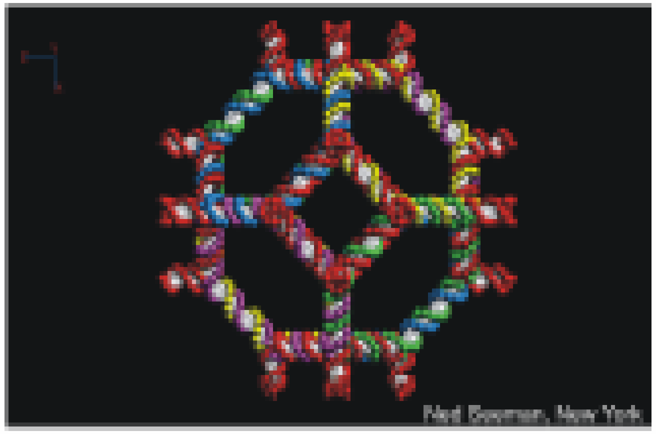


basic structure: junction J1



main features of "nanoscale assembly"





http://seemanlab4.chem.nyu.edu/homepage.html

EXTREMOPHILES

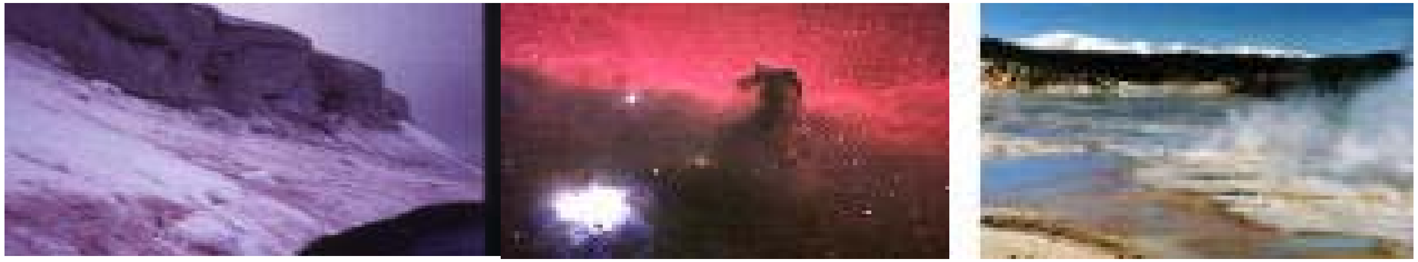
NATURE’S ULTIMATE SURVIVORS

EXTREMOPHILES

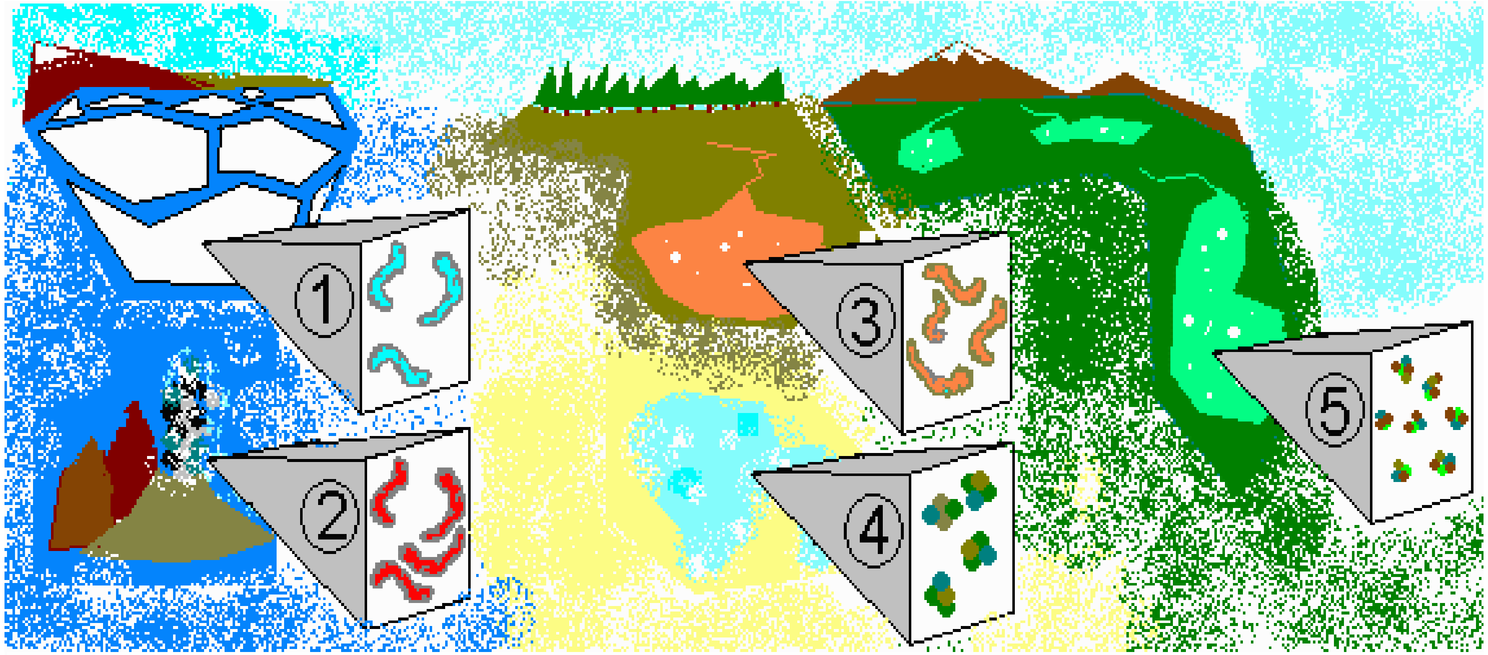
I. What are they?
II. Types of Extremophiles
III. Extreme Prokaryotes
IV. Extreme Eukaryotes
V. Extreme Viruses
VI. Evolution of Extremophiles
VII. Biotechnological Uses
VIII. Industrial Uses
IX. Extraterrestrial Extremophiles?

What are Extremophiles?

Extremophiles are microorganisms— whether viruses, prokaryotes, or eukaryotes— that survive under harsh environmental conditions that can include atypical temperature, pH, salinity, pressure, nutrient, oxic, water, and radiation levels



Types of Extremophiles



1) Psychrophiles
Microbes that live in cold environments like sea ice and the arctic and antarctic ice packs.

2) Thermophiles
Microbes that live in very hot environments like deep sea vents and volcanic lakes.

3) Alkaliphiles
Microbes that live in basic environments like soda lakes.

4) Halophiles
Microbes that live in very salty environments like salt lakes and salt mines.

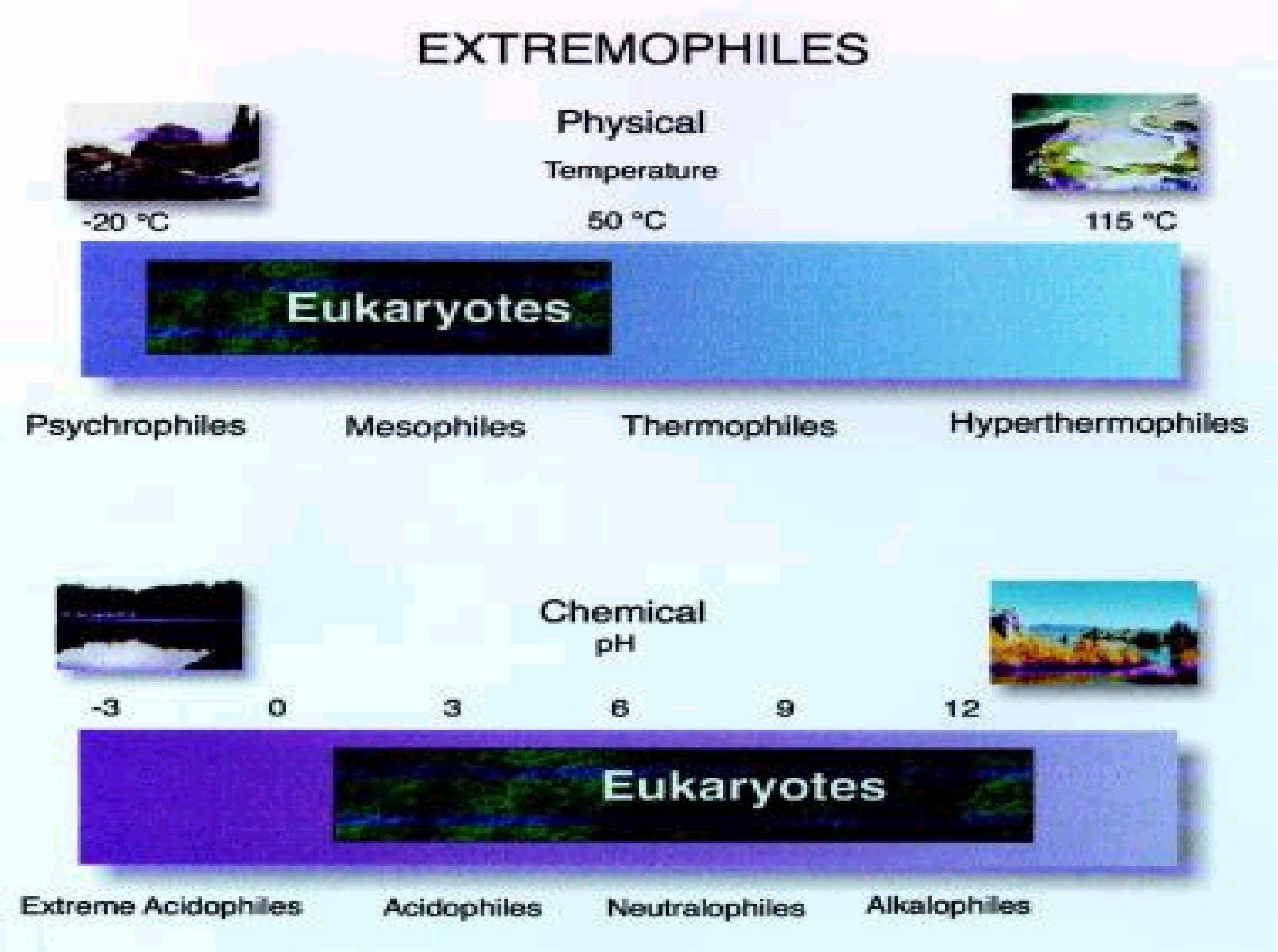
5) Acidophiles
Microbes that live in acidic environments like sulphur springs.

Types of Extremophiles

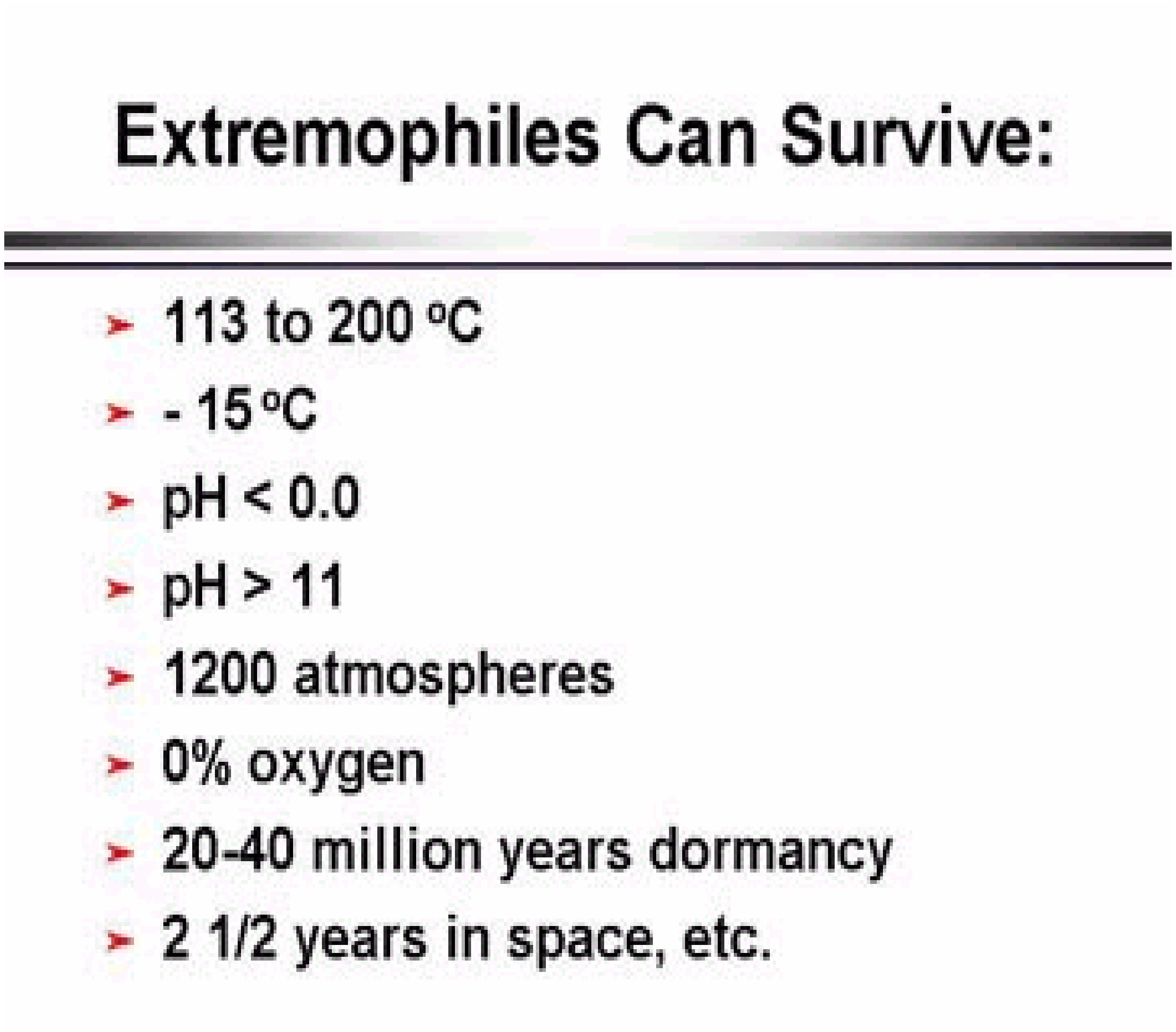
Other types include:

- **Barophiles** -survive under high pressure levels, especially in deep sea vents
- **Osmophiles** –survive in high sugar environments
- **Xerophiles** -survive in hot deserts where water is scarce
- **Anaerobes** -survive in habitats lacking oxygen
- **Microaerophiles** -survive under low-oxygen conditions only
- **Endoliths** –dwell in rocks and caves
- **Toxitolerants** -organisms able to withstand high levels of damaging agents. For example, living in water saturated with benzene, or in the water-core of a nuclear reactor

Environmental Requirements



Surviving the Extremes

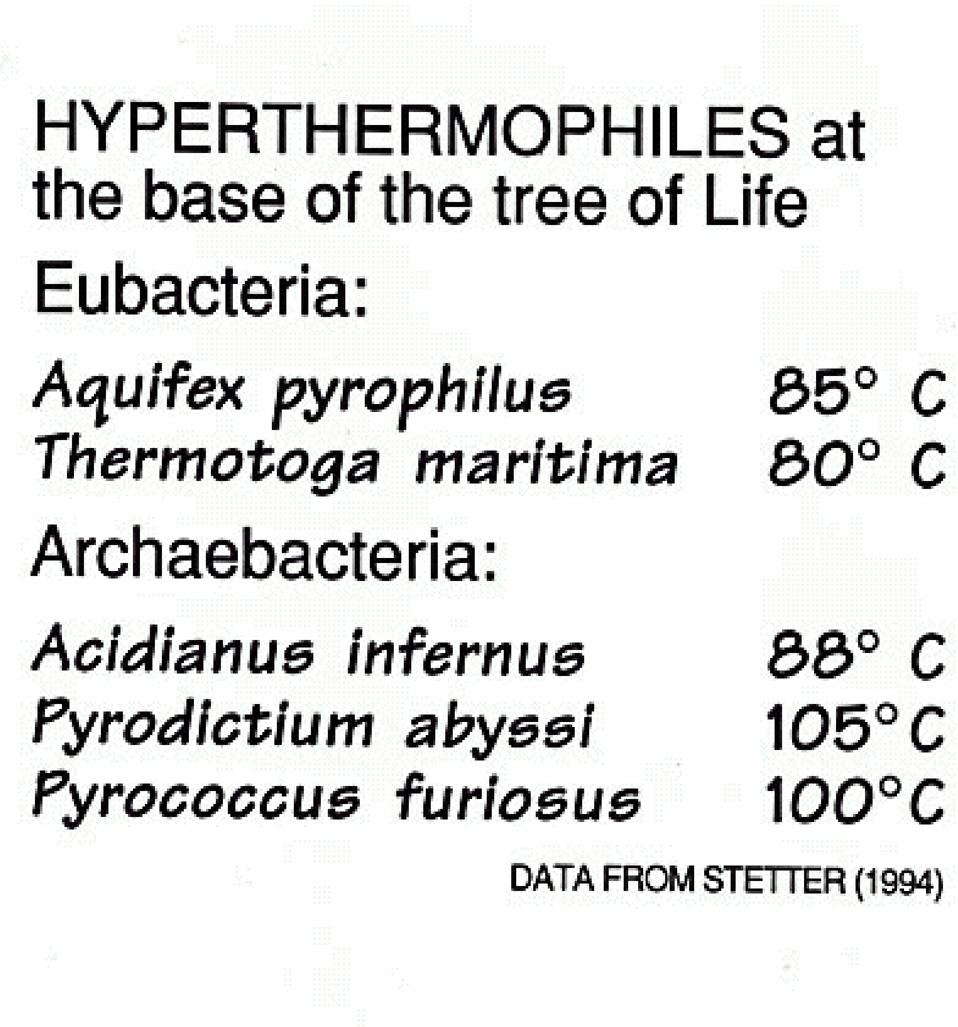


Extremophiles Can Survive:

- 113 to 200 °C
- - 15 °C
- $\text{pH} < 0.0$
- $\text{pH} > 11$
- 1200 atmospheres
- 0% oxygen
- 20-40 million years dormancy
- 2 1/2 years in space, etc.

EXTREME PROKARYOTES

Hyperthermophiles



HYPERTHERMOPHILES at the base of the tree of Life

Eubacteria:	
Aquifex pyrophilus	85° C
Thermotoga maritima	80° C
Archaebacteria:	
Acidianus infernus	88° C
Pyrodictium abyssi	105° C
Pyrococcus furiosus	100° C

DATA FROM STETTER (1994)

- -Members of domains Bacteria and Archaea
- -Held by many scientists to have been the earliest organisms
- -Early earth was excessively hot, so these organisms would have been able to survive

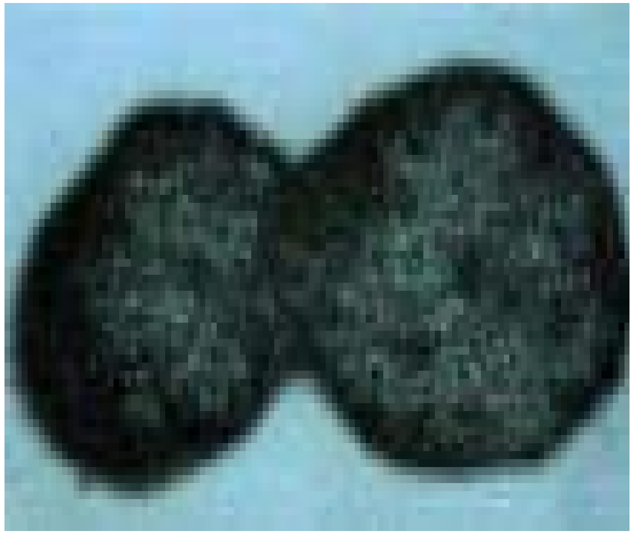
Morphology of Hyperthermophiles

- -Heat stable proteins that have more hydrophobic interiors, which prevents unfolding or denaturation at higher temperatures
- -Have chaperonin proteins that maintain folding
- -Monolayer membranes of dibiphytanyl tetraethers, consisting of saturated fatty acids which confer rigidity, preventing them from being degraded in high temperatures
- -Have a variety of DNA-preserving substances that reduce mutations and damage to nucleic acids, such as reverse DNA gyrase and Sac7d
- -They can live without sunlight or organic carbon as food, and instead survive on sulfur, hydrogen, and other materials that other organisms cannot metabolize

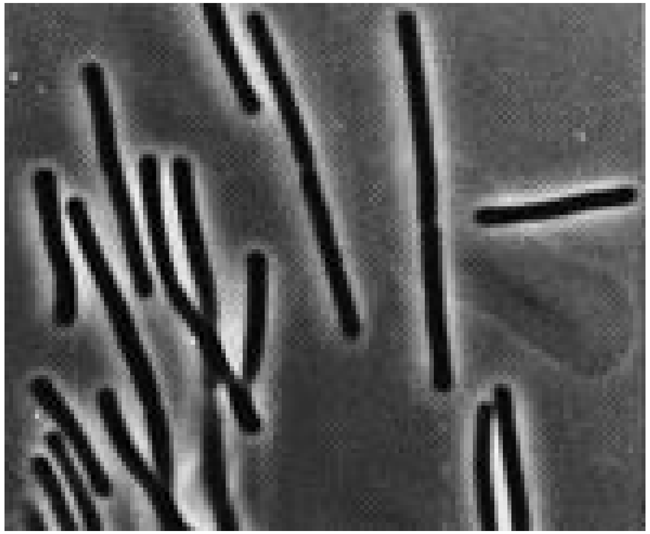
The red on these rocks is produced by *Sulfolobus solfataricus,* near Naples, Italy

Some Hyperthermophiles

Frequent habitats include volcanic vents and hot springs, as in the image to the left



Pyrococcus abyssi 1μm



Thermus aquaticus 1μm

Deep Sea Extremophiles

A **black smoker, a** submarine hot spring, which can reach 518- 716°F (270-380°C)

The **deep-sea floor** and **hydrothermal vents** involve the following conditions:

low temperatures (2-3º C) – where only **psychrophiles** are present

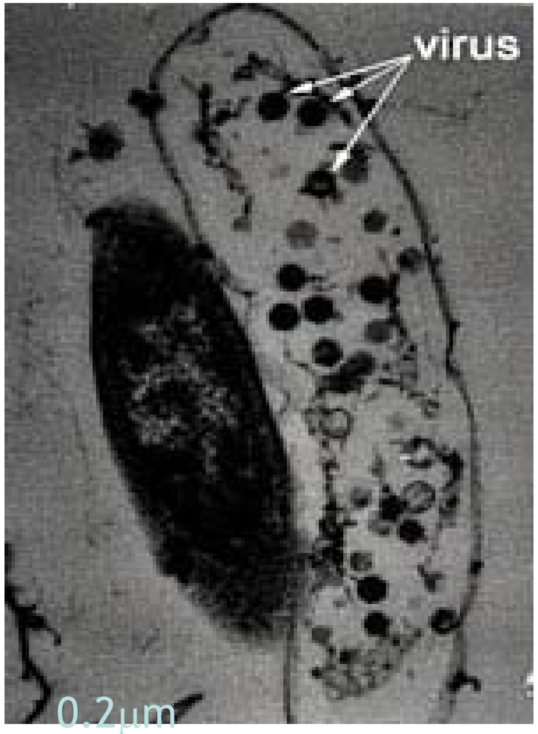
low nutrient levels – where only **oligotrophs** present

high pressures – which increase at the rate of 1 atm for every 10 meters in depth (as we have learned, increased pressure leads to decreased enzyme-substrate binding)

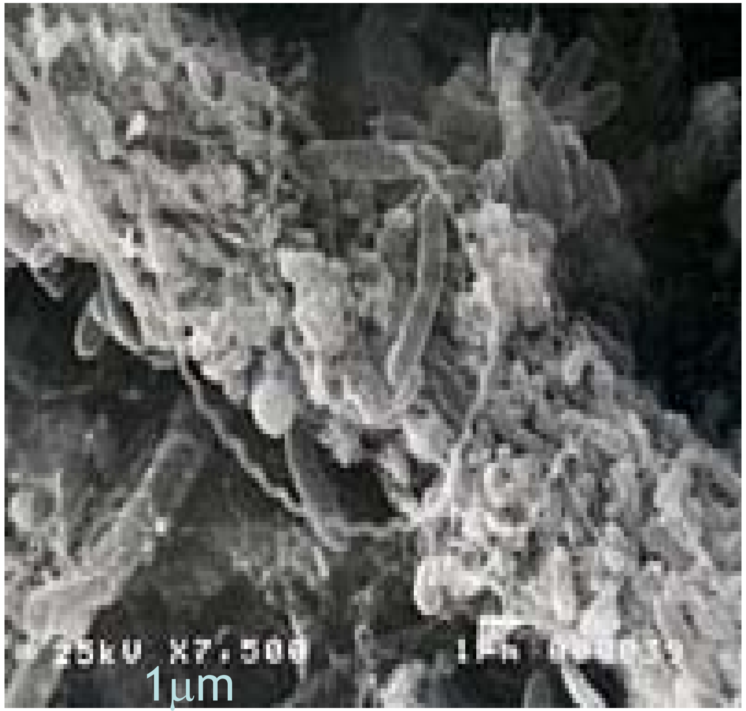
barotolerant microorganisms live at 1000-4000 meters

barophilic microorganisms live at depths greater than 4000 meters

Extremophiles of Hydrothermal Vents



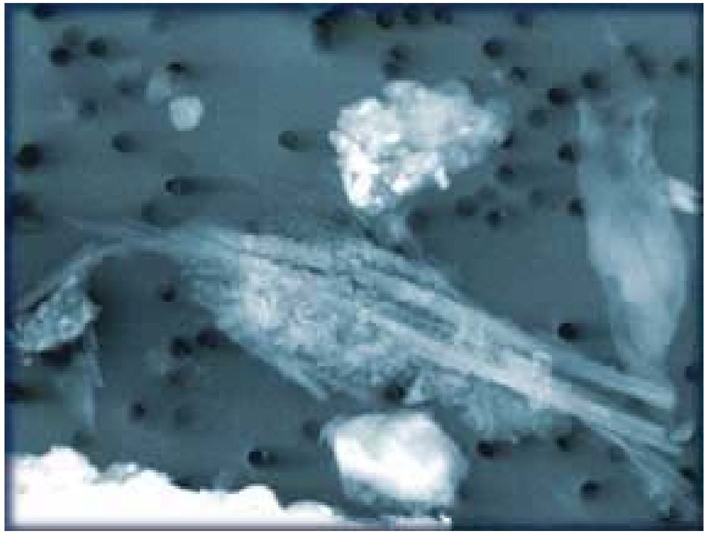
A cross-section of a bacterium isolated from a vent. Often such bacteria are filled with viral particles which are abundant in hydrothermal vents



A bacterial community from a deep-sea hydrothermal vent near the Azores

Natural springs which vent warm or hot water on the sea floor near mid-ocean ridges. Associated with the spreading of the earth's crust. High temperatures and pressures

Psychrophiles



Some microorganisms thrive in temperatures well below the freezing point of water, such as in Antarctica

Some researchers believe that psychrophiles live in conditions mirroring those found on Mars

Psychrophiles possess:

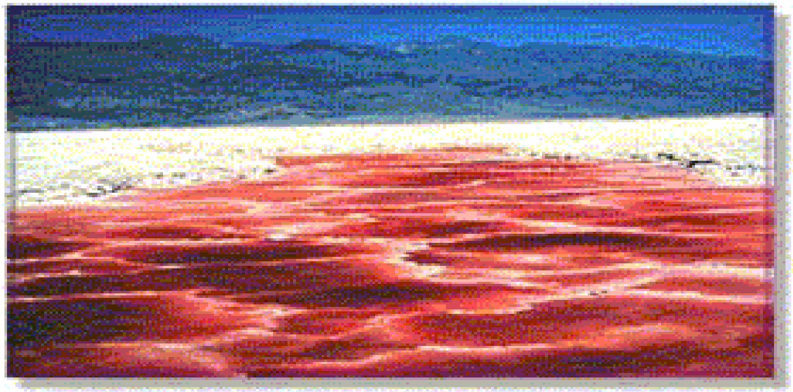
-proteins rich in α-helices and polar groups which allow for greater flexibility

-"antifreeze proteins" that maintain liquid intracellular conditions by lowering freezing points of other biomolecules

-membranes that are more fluid, containing unsaturated *cis*-fatty acids which help to prevent freezing

-active transport at lower temperatures

Halophiles



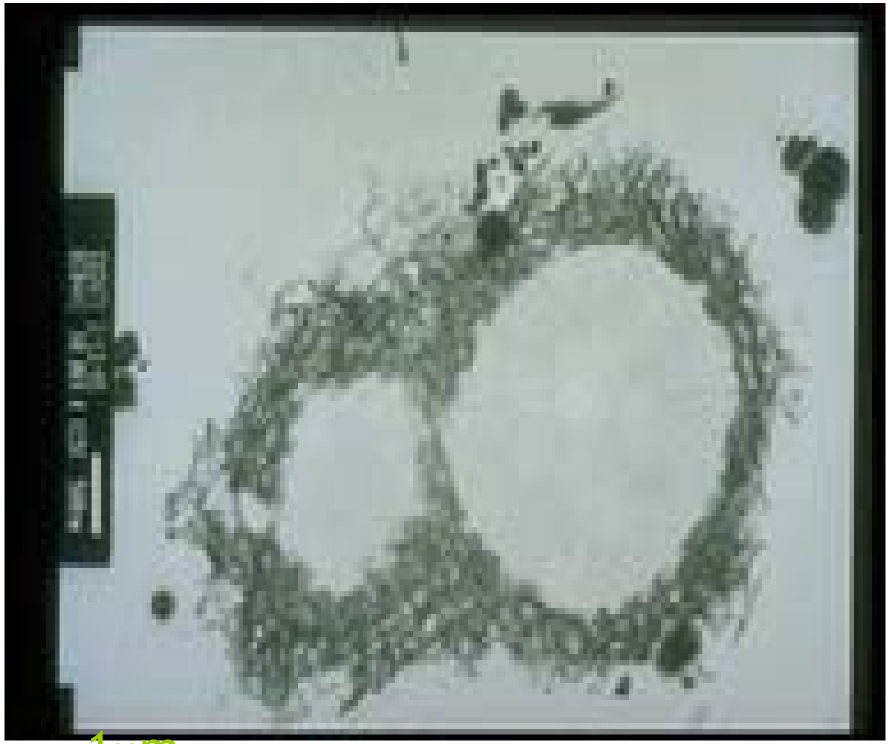
The vivid red brine (teaming with halophilic archaebacteria) of Owens Lake contrasts sharply with the gleaming white deposits of soda ash (sodium carbonate). The picturesque Inyo Range can be seen in the distance.

-Divided into mild (1-6%NaCl), moderate (6-15%NaCl), and extreme (15-30%NaCl)

-Halophiles are mostly obligate aerobic archaea

How do halophiles survive high salt concentrations?
-by interacting more strongly with water such as using more negatively charged amino acids in key structures
-by making many small proteins inside the cell, and these, then, compete for the water
-and by accumulating high levels of salt in the cell in order to outweigh the salt outside

Barophiles



A sample of barophilic bacteria from the earth's interior

-Survive under levels of pressure that are otherwise lethal to other organisms

-Usually found deep in the earth, in the deep sea, hydrothermal vents, etc

-scientists believe that barophiles may be able to survive on the Moon and other places in space

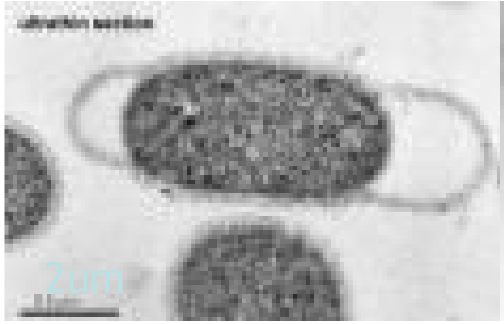
Xerophiles

Extremophiles which live in water-scarce habitats, such as deserts

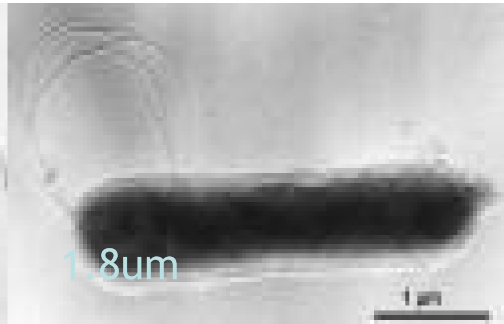
Produce **desert varnish** as seen in the image to the left

Desert varnish is a thin coating of Mn, Fe, and clay on the surface of desert rocks, formed by colonies of bacteria living on the rock surface for thousands of years

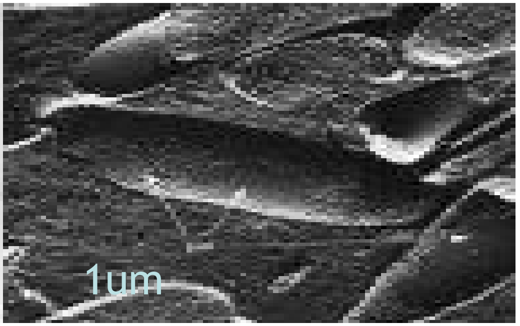
SOME COMMON GENERA OF PROKARYOTE EXTREMOPHILES



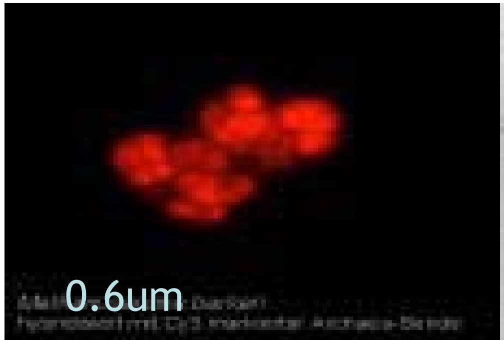
Thermotoga



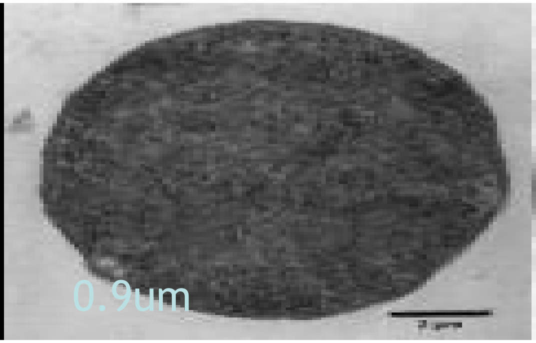
Aquifex



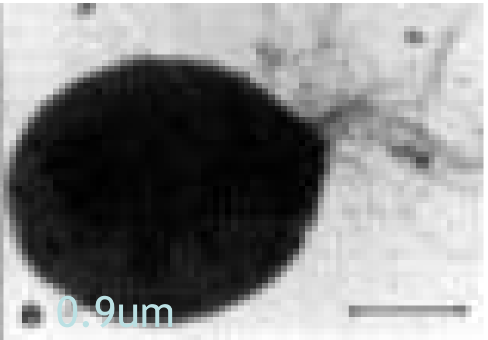
Halobacterium



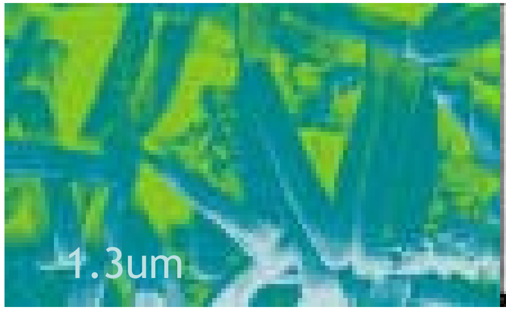
Methanosarcina



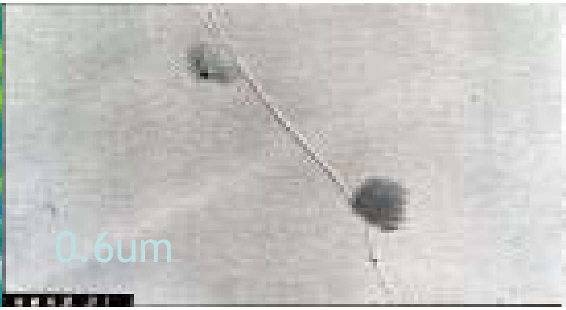
Thermoplasma



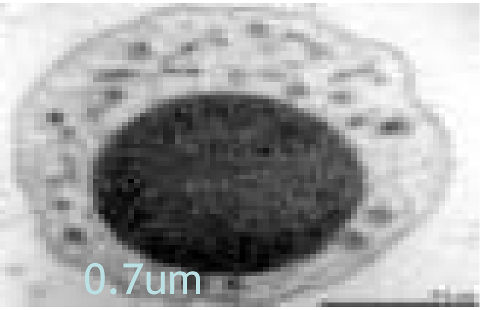
Thermococcus



Thermoproteus



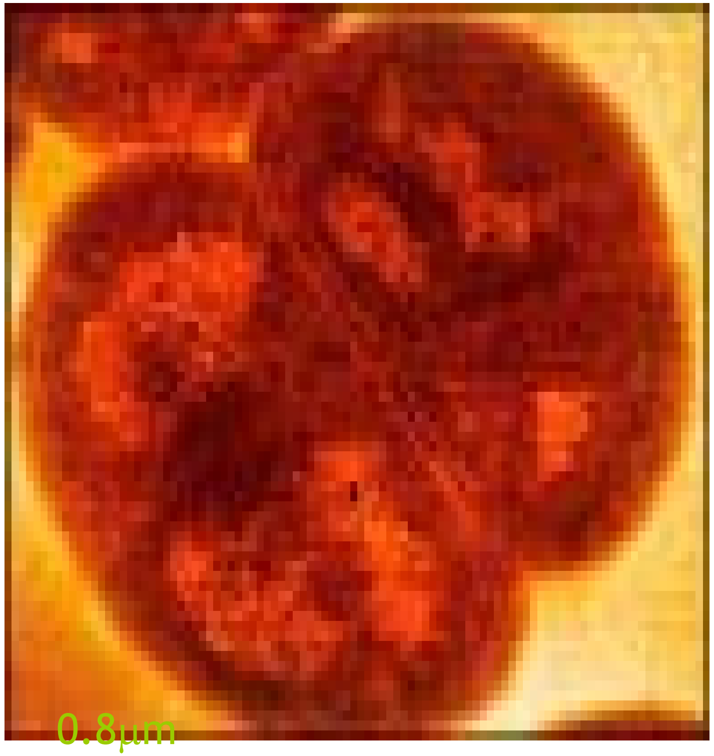
Pyrodictium



Ignicoccus

Deinococcus radiodurans

The Radiation Resistor



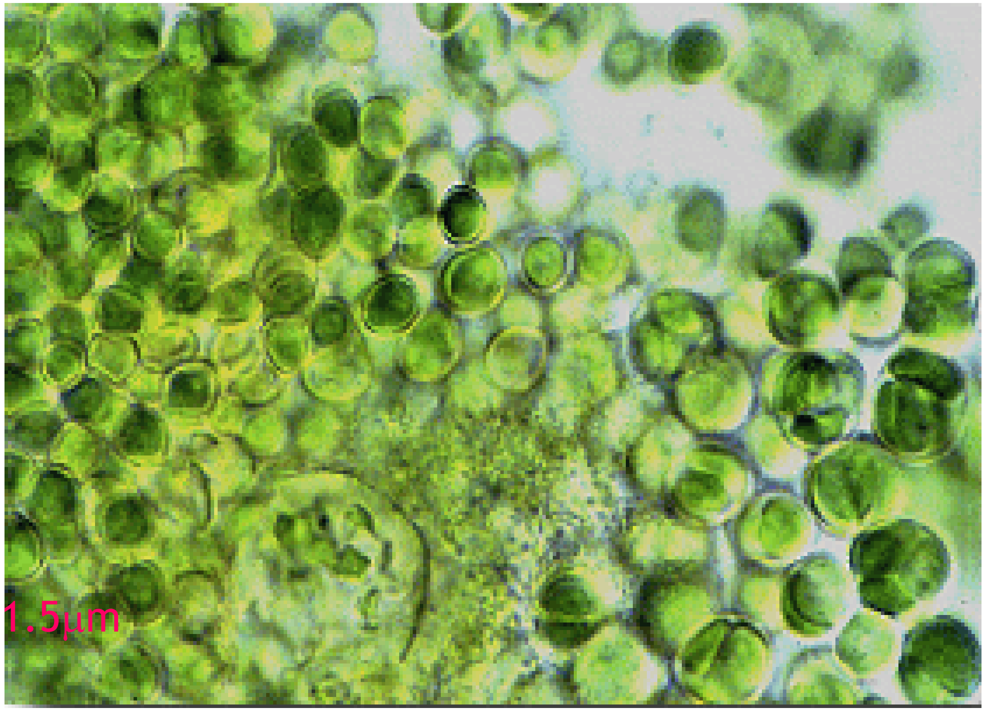
-Possesses extreme resistance to up to 4 million rad of radiation, genotoxic chemicals (those that harm DNA), oxidative damage from peroxides/superoxides, high levels of ionizing and ultraviolet radiation, and dehydration

-It has from four to ten DNA molecules compared to only one for most other bacteria

-Contains many DNA repair enzymes, such as RecA, which matches the shattered pieces of DNA and splices them back together. During these repairs, cell-building activities are shut off and the broken DNA pieces are kept in place

Chroococcidiopsis

The Cosmopolitan Extremophile

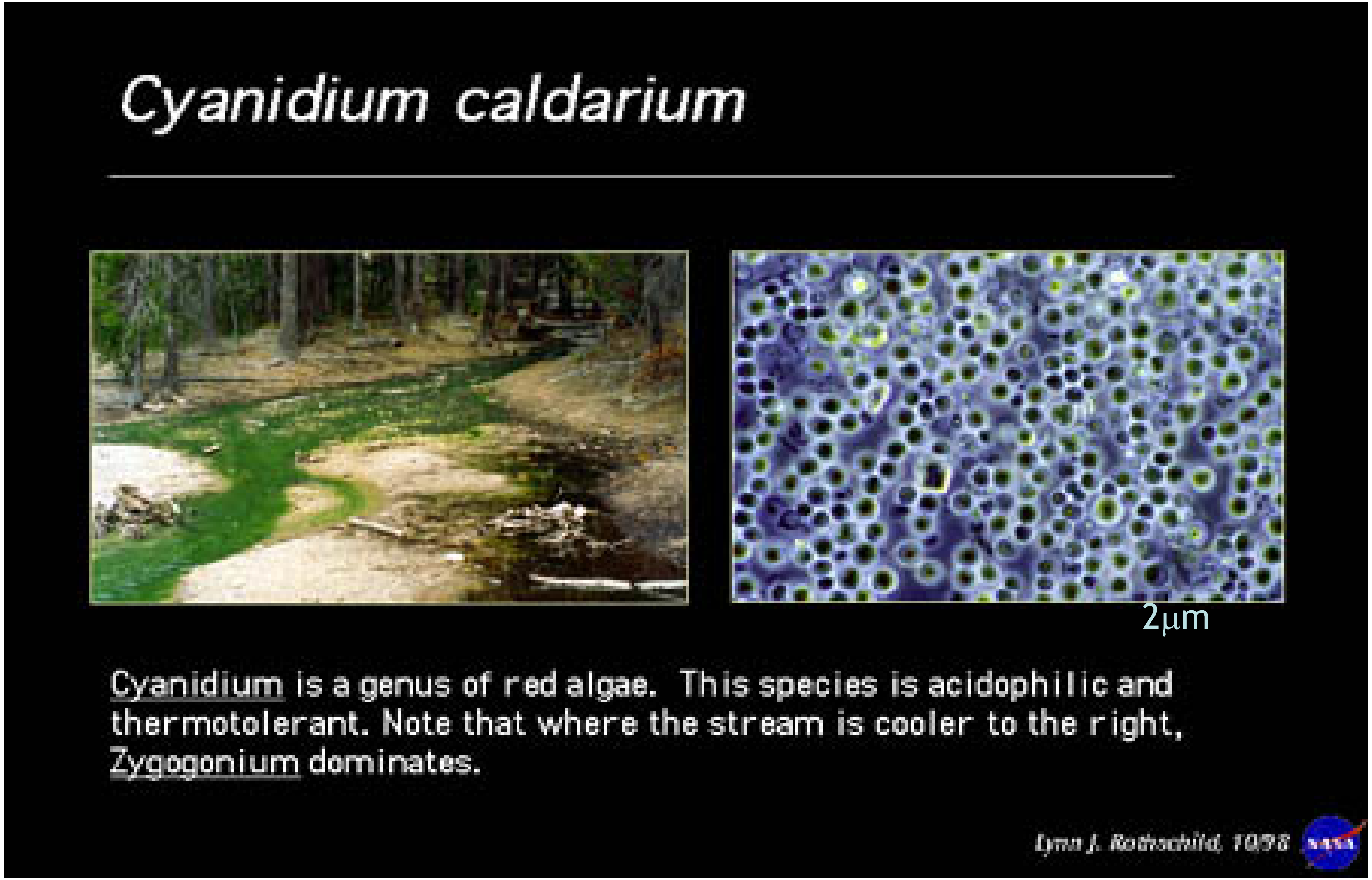


-A cyanobacteria which can survive in a variety of harsh environments, such as hot springs, hypersaline habitats, hot, arid deserts throughout the world, and in the frigid Ross Desert in Antarctica

-Possesses a variety of enzymes which assist in such adaptation

EXTREME EUKARYOTES

THERMOPHILES/ACIDOPHILES



Cyanidium caldarium

Cyanidium is a genus of red algae. This species is acidophilic and thermotolerant. Note that where the stream is cooler to the right, Zygogonium dominates.

Lynn J. Rothschild, 10/98

EXTREME EUKARYOTES

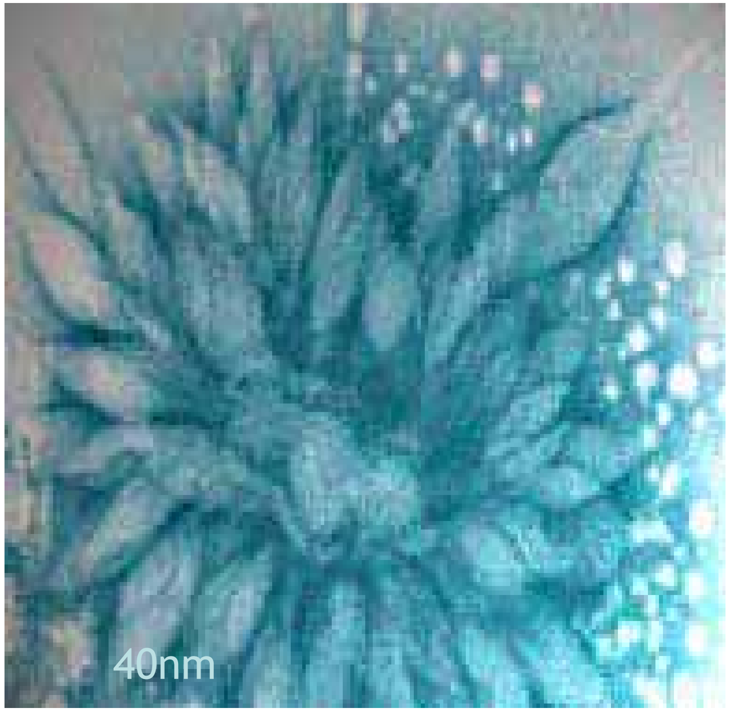
ENDOLITHS

Quartzite from Johnson Canyon, California. Sample shows green bands of endolithic algae. Rock is 9.5 cm wide

-Endoliths (also called hypoliths) are usually algae, but can also be prokaryotic cyanobacteria, that exist within rocks and caves

-Often are exposed to anoxic (no oxygen) and anhydric (no water) environments

EXTREME VIRUSES



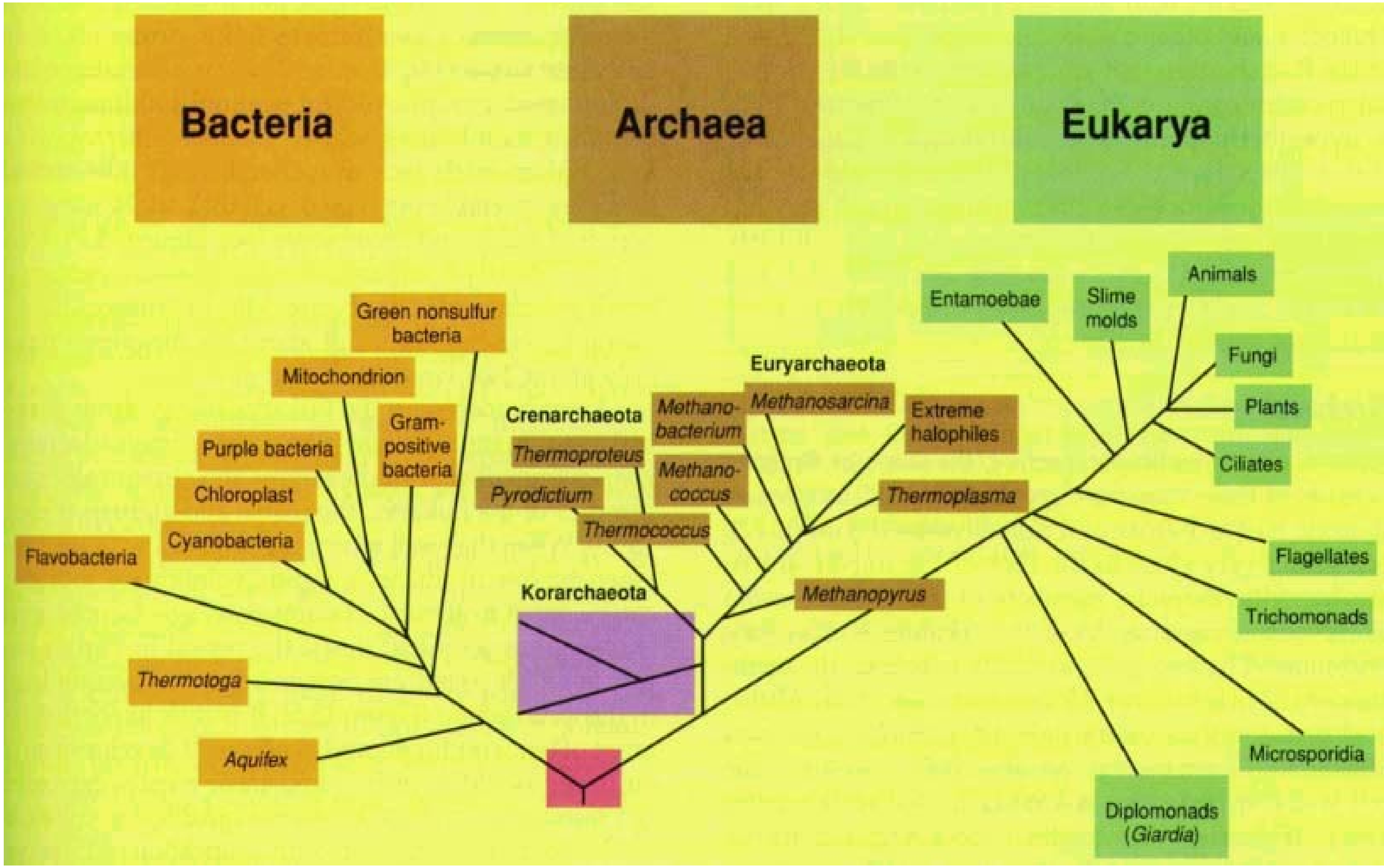
Virus-like particles isolated from the extreme environment of Yellowstone National Park hot springs

Viruses are currently being isolated from habitats where temperatures exceed 90°C

Instead of the usual icosahedral or rod-shaped capsids that known viruses possess, researchers have found viruses with novel propeller-like structures

These extreme viruses often live in hyperthermophile prokaryotes such as *Sulfolobus*

CLASSIFICATION OF EXTREMOPHILES



Extremophiles are present among Bacteria, form the majority of Archaea, and also a few among the Eukarya

PHYLOGENETIC RELATIONSHIPS

-Members of Domain Bacteria (such as *Aquifex* and *Thermotoga*) that are closer to the root of the "tree of life" tend to be hyperthermophilic extremophiles

-The Domain Archaea contain a multitude of extremophilic species:

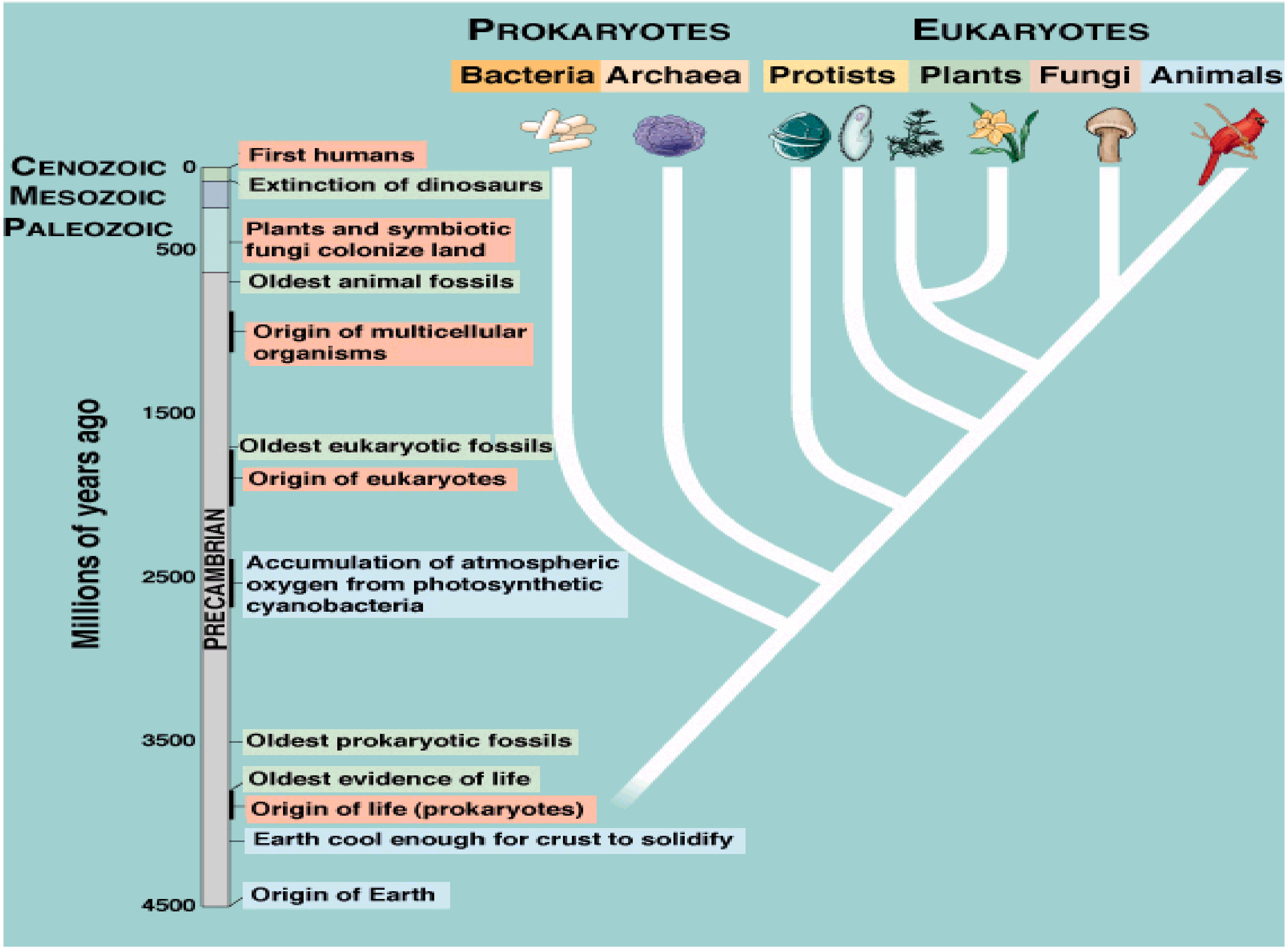
Phylum Euryarchaeota-consists of methanogens and extreme halophiles

Phylum Crenarchaeota-consists of thermoacidophiles, which are extremophiles that live in hot, sulfur-rich, and acidic solfatara springs

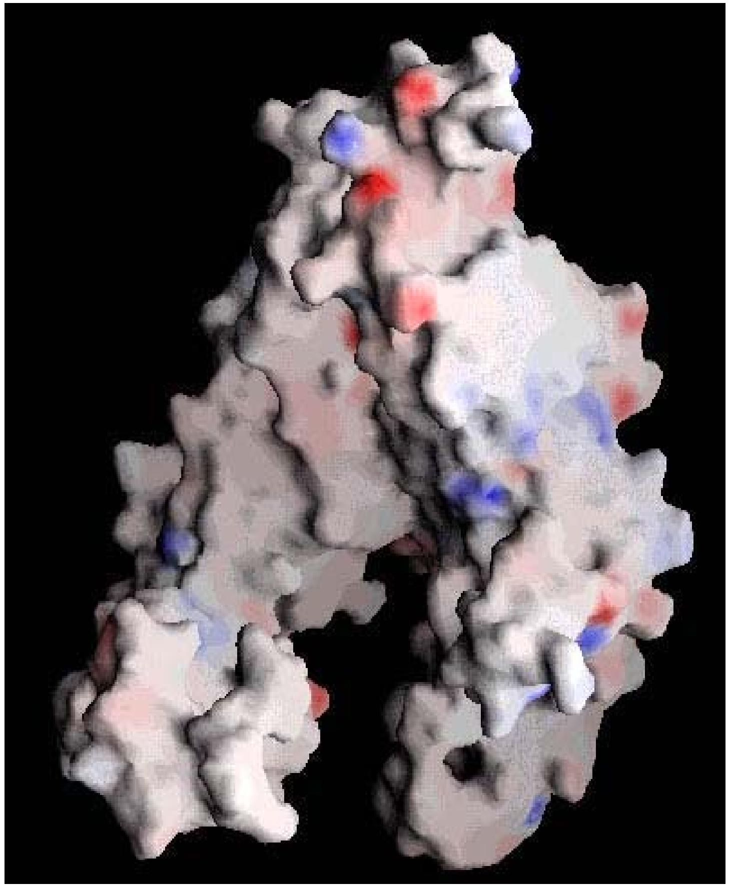
Phylum Korarchaeota-new phylum of yet uncultured archaea near the root of the Archaea branch, all are hyperthermophiles

-Most extremophilic members of the Domain Eukarya are red and green algae

Chronology of Life



Taq Polymerase

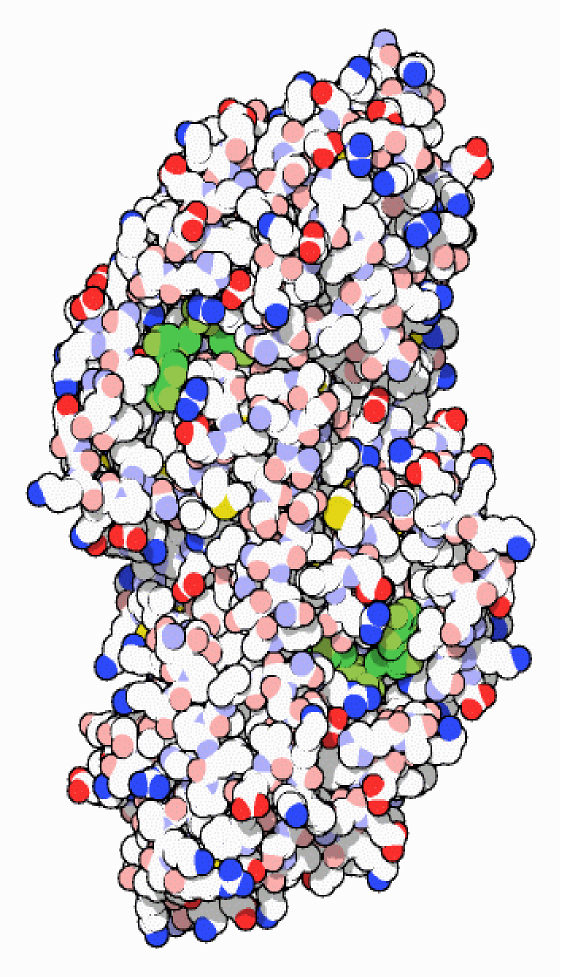


Isolated from the hyperthermophile *Thermus aquaticus*

Much more heat stable

Used as the DNA polymerase in the very useful Polymerase Chain Reaction (PCR) technique which amplifies DNA samples

Alcohol Dehydrogenase



-Alcohol dehydrogenase (ADH), is derived from a member of the archaea called *Sulfolobus solfataricus*

-It works under some of nature's harshest volcanic conditions: It can survive to 88°C (190ºF) - nearly boiling - and corrosive acid conditions (pH=3.5) approaching the sulfuric acid found in a car battery (pH=2)

-ADH catalyzes the conversion of alcohols and has considerable potential for biotechnology applications due to its stability under these extreme conditions

Bacteriorhodopsin

-Bacteriorhodopsin is a trans-membrane protein found in the cellular membrane of *Halobacterium salinarium*, which functions as a light-driven proton pump

-Can be used for electrical generation

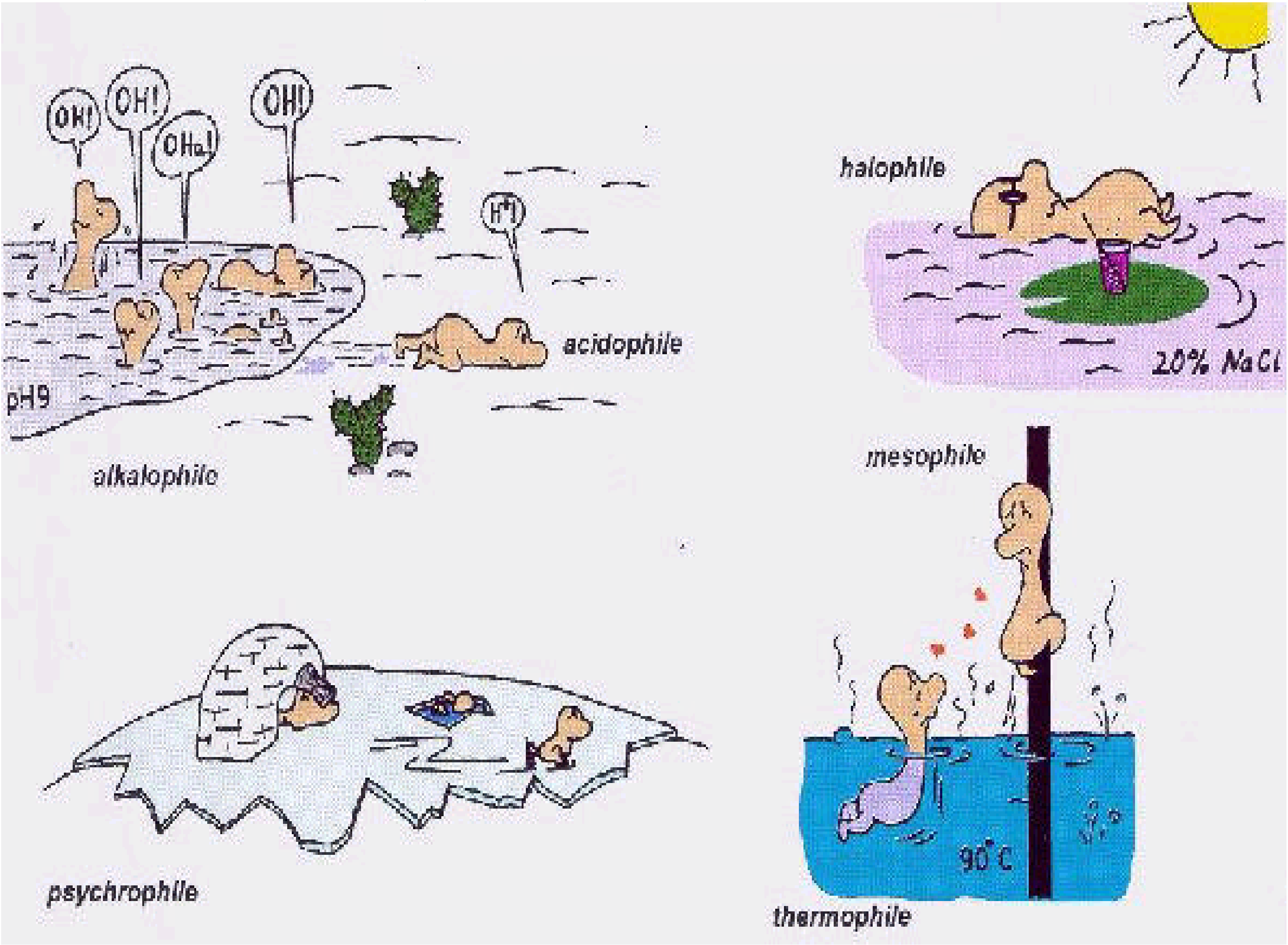
CONCLUSIONS

-Extremophiles are a very important and integral part of the earth’s biodiversity

They:

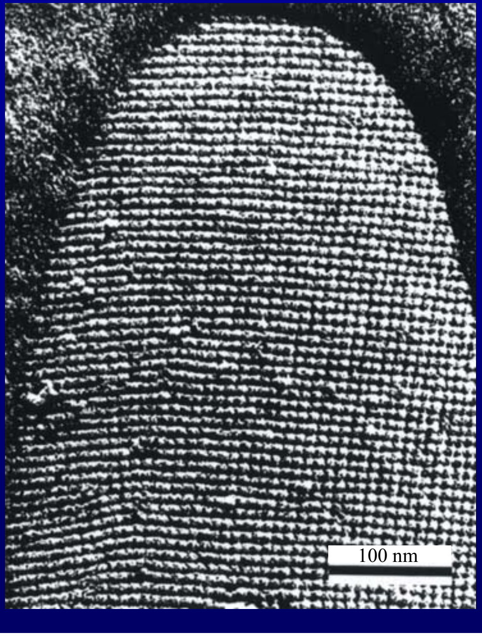
- reveal much about the earth’s history and origins of life
- possess amazing capabilities to survive in the extremes
- are proving to be beneficial to both humans and the environment
- may exist beyond earth

Questions?


OH!
OH!
OHa!
OH!
H^+!
acidophile
pH9
alkalophile
halophile
20% NaCl
mesophile
psychrophile
90°C
thermophile

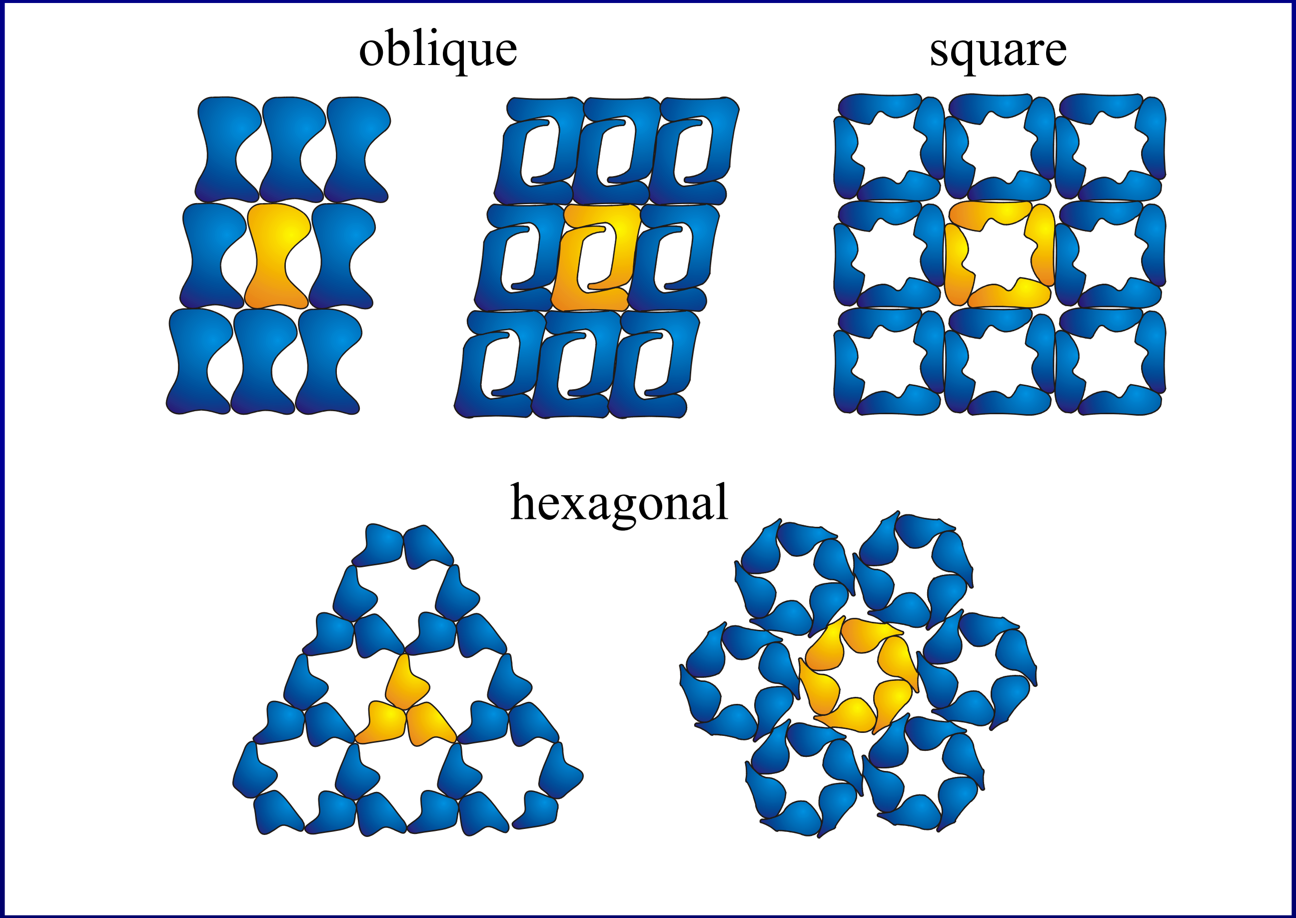
What are S-Layers ?
(Surface Layers)

- The most common surface structure in prokaryotic organisms (bacteria, archaea).
- Highly ordered protein arrays composed of single protein or glycoprotein subunits.
- The simplest type of membranes developed during biological evolution.
- They completely cover the cell surface during all stages of bacterial growth and division.



Freeze-etched preparation of a cell exhibiting a square S-layer lattice.

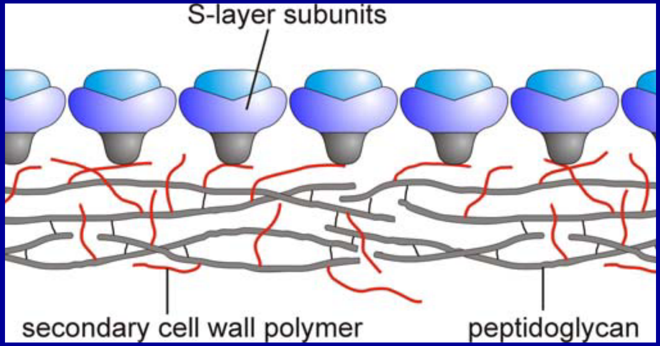
Lattice Types of S-layer Proteins



Binding Mechanism of S-Layer Subunits

In bacteria, the S-layer subunits are linked to each other and to the underlying cell envelope layer by non-covalent interactions.

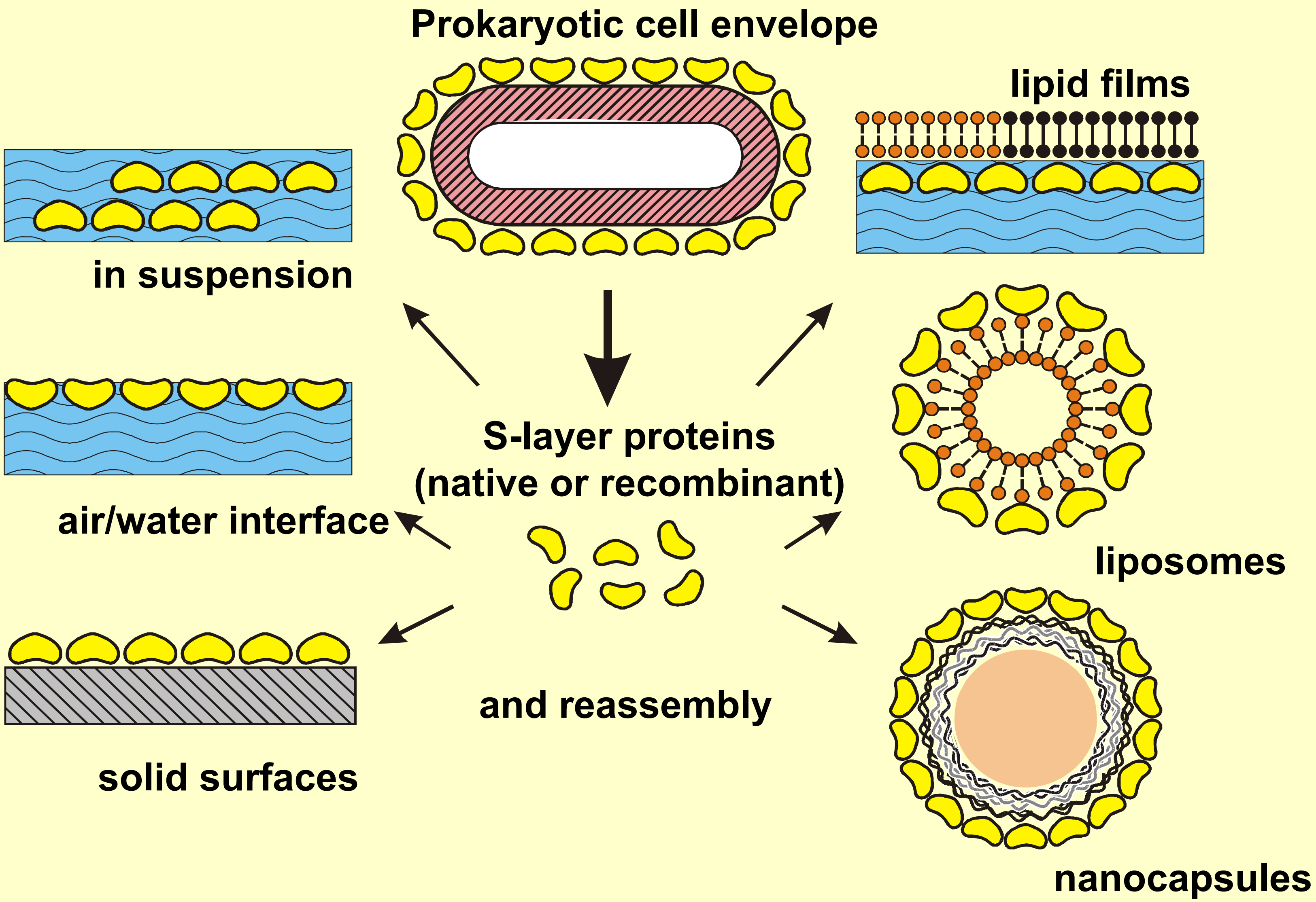
The N-terminal region of S-layer proteins from Gram-positive organisms recognizes a distinct type of secondary cell wall polymer as the proper binding site.



SCWPs are covalently linked to the peptidoglycan backbone.

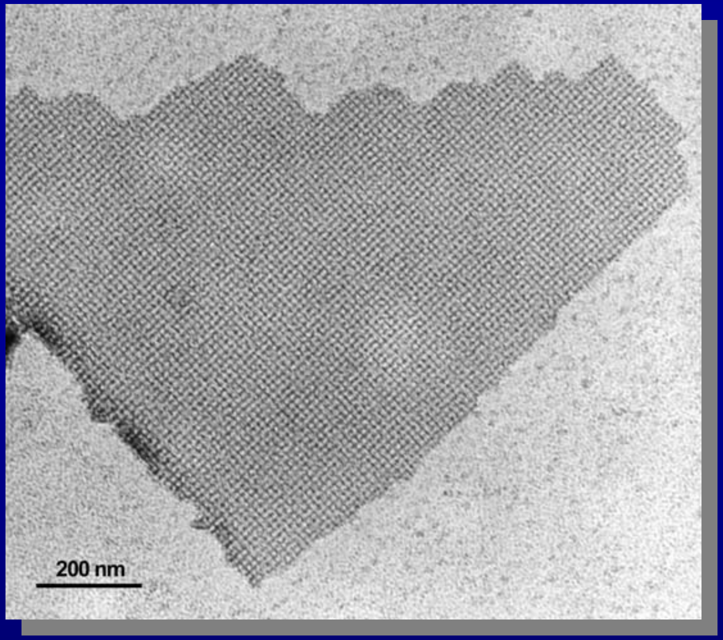
S-layer subunits can be extracted from cell wall fragments with chaotropic agents (e. g. GHCl) and form self-assembly products after removal of the disintegrating agent.

S-layer Reassembly

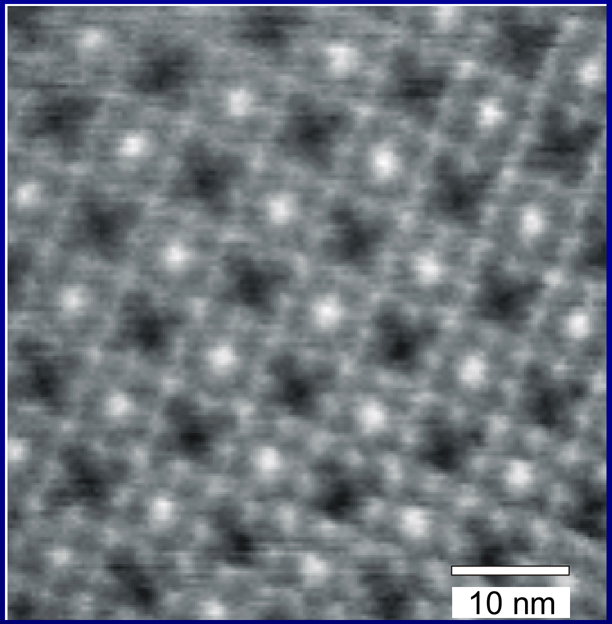


SbpA, the S-layer Protein of *B. sphaericus* CCM 2177

SbpA, 1,268 amino acids long, assembles into a square lattice.



Self assembly product
in suspension



AFM image of a square
(p4) S-layer lattice

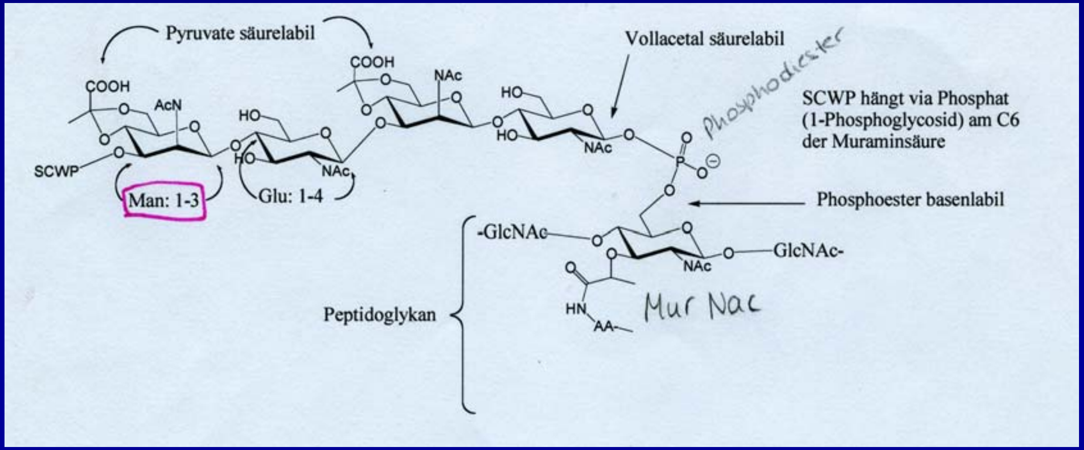
The N-terminal part carries an SLH-domain (3 SLH-motifs).

MAKQNKGRKFFAASATAALVASAIVPVASAAQ**VNDYNKISGYAKEAVQSLVDQGVIQGDTNGNFNPLNTVTRAQAAEIFTKA**LELEADGDVN**FSDVKKGAWYYNSIAAVVANGIFEGVSANEFAPN**
KSLTRSEAAKVLVDAFGLEGSESLSQ**FADASQVKGWAKSALETAVANGIFTGSEENGKLNLKPNAAITRQDFAVVF**ARTLDLAVET**VDASVKAINNTTVEVTFDEEVDNVEALKFKIEGLEVKNAAV**
KQTNKKVVVLTTEAQTADKEYVVTLDGEEIGKFKGIEAVVPKSIVLKTTNTQGKVGNQVTLTADVGVKAAGIPVTFNVDAPTGSLNKDAVVEVYTNAEGIASYSYTQYAPDADDVTVYPTGAPQLRA
FGTVYWGVDNILTIEEVTTGNTLANGVKKTYKVTFKDPKTGAALTNKKLNVSFVENTNVAFNAISKATVTNPSSGLTVTPYQTTTGLQEEIQVTTDSNGQATFVVSGTNTAVTPYVFVDGSSSVLGV
STVTGTNNVTQATTANKKWEATELTATAAQVKFEGAQLNHQITVERDGEEEAAAQYGSKLNGREYKVKVLDKDGKPYANGLVNIGLDEVLDRNLNTNSKAQFANVTDGTALTLNPGTSQQGKIKL
NSKGEATVVLYGAKGEVGTPVVWIDQNTSQNNQSGVLEDGEPFFKAPVSNFQYERVMGAEFTVEGKTSNQNVGTDGIANYAFALTNQSGKTLTNLIGKMTYEIRNTSGQPIEVKTPISYRDQNNV
SSTLFKIEEYGSITISADVISTSNAAFTIETAPNGKAATVVVTPSFVTANNFDASADNNLNGVVTDARDFNKHVTAPALTATFGNNKEVGTNYTGTVQYFNKDKKTITFNGKNAVKYAGESGKTYKYF
GLGSTPISTADDFITQLQNNAANGSVTVTYKVVDNEVQFYIVSFNNDGAANAVKPEPTAVAPTDGTVAFTNTSFNASANQTITVTDADLNADATVAETTTVKVTSGTVVYTLTATETGVNTGVFTATL
TAAQLAVLSDGVITATYEDVKNTTGSSVTRTATATLNTVVGQVNYNAAATTAYSEGNPVAATLTGTGLTQVIGGGALVMKINGTQFTTNVTAINPGTSVADSAAAVAGEINAAAVAAGIITAPATVAT
VNGTNNIVITSPTAAGSTVEVVSNAATGTGLVVASASATTSAPVKAAWKFTLTTTPAVGEKITVKVGNVEKTYTVALGDTLTTIETALNTAFGTDFDVTLAAGSNVFTVTQDVAAPTTAELTVTITK

For binding to the negatively charged SCWP, the 3 SLH-motifs and a 58 amino acids long SLH-like motif are required.

```
1 SbpA  33-  82 VNDYNK I SG      Y AKEA  VQS LVDQGVIQ GD     TNGN FNPLNT        VT   RAQA AE I FTKA
2 SbpA  93-142  FSDVKK    G      AWYYNS I AAVVANGI FE GV     SANE FAPNKS        L T  RS EA AKVLVDAF
3 SbpA153-202   FADASQVKG        WAKSA   LE TAVANGI FT GSEEN GKLNL KPNAA        I T  R QDFAVVF
4 SbpA213-270   VDASVKAI NNTTVEVTF DEE   VDNVEAL KFKI EGLEVKNA     A  VKQTNKKVVVLTTE   AQT  A
```

Secondary Cell Wall Polymer of *B. sphaericus* CCM 2177

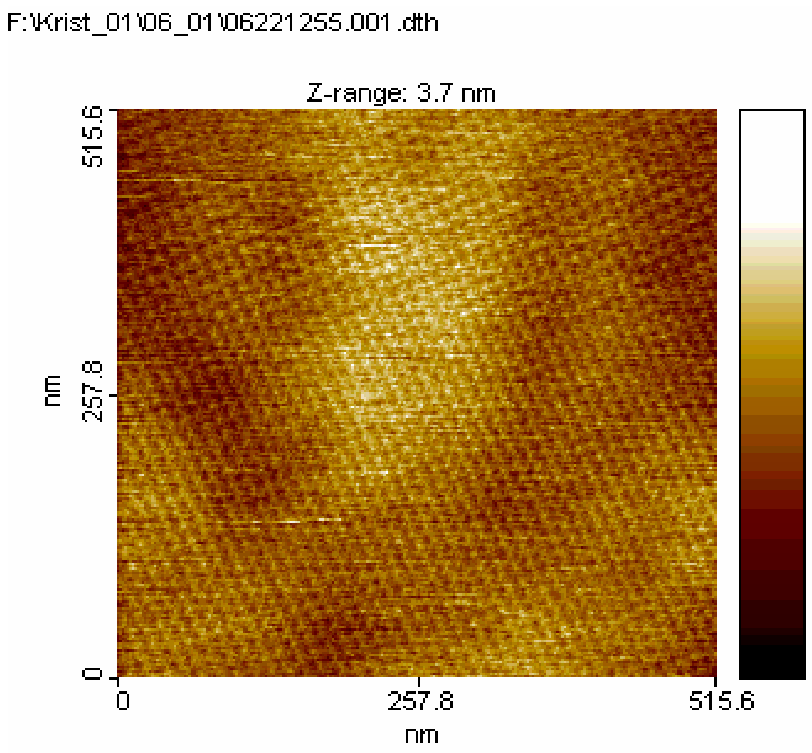


→3)-[4,6-*O*-(1-carboxyethylidene)]$_{\sim 0.5}$-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→

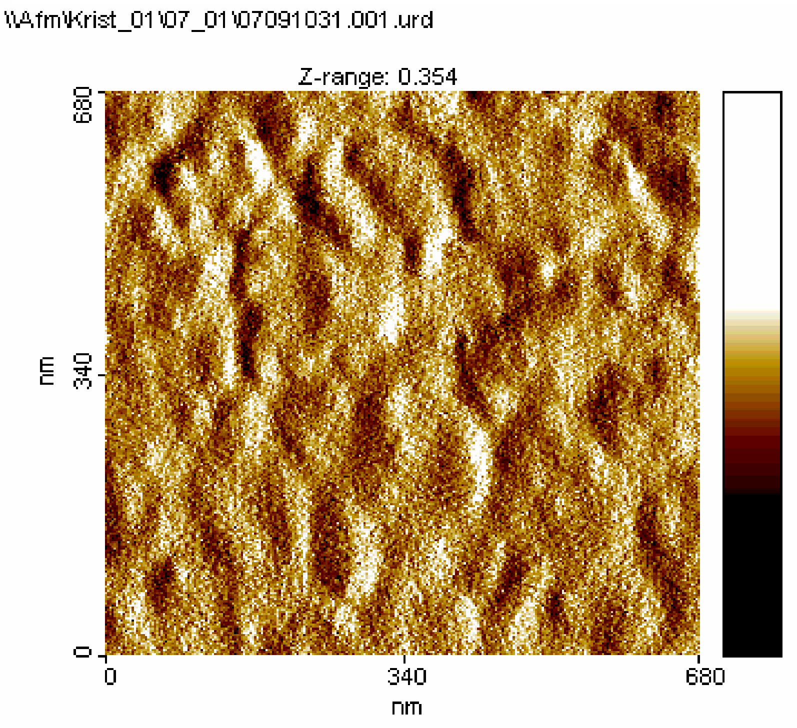
- SCWP specific for SLH-domain of SbpA contains pyruvic acid residues (pyruvate ketals) as negatively charged component.
- For SbpA, carrying an SLH-domain, the pyruvic acid residues are crucial for the recognition and binding process.

Ilk et al., 1999, J. Bacteriol, Vol. 181, p. 7643-7646.

SbpA Recrystallised on Gold Chips Precoated with SCWP



precoated with SCWP



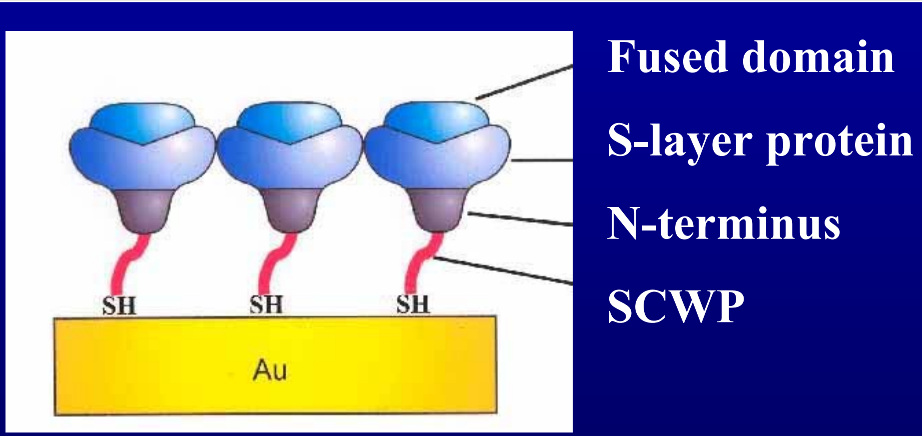
without SCWP

Functional S-layer Fusion Proteins

Fusion of functional peptide sequences or protein domains to the C-terminus of the S-layer protein.

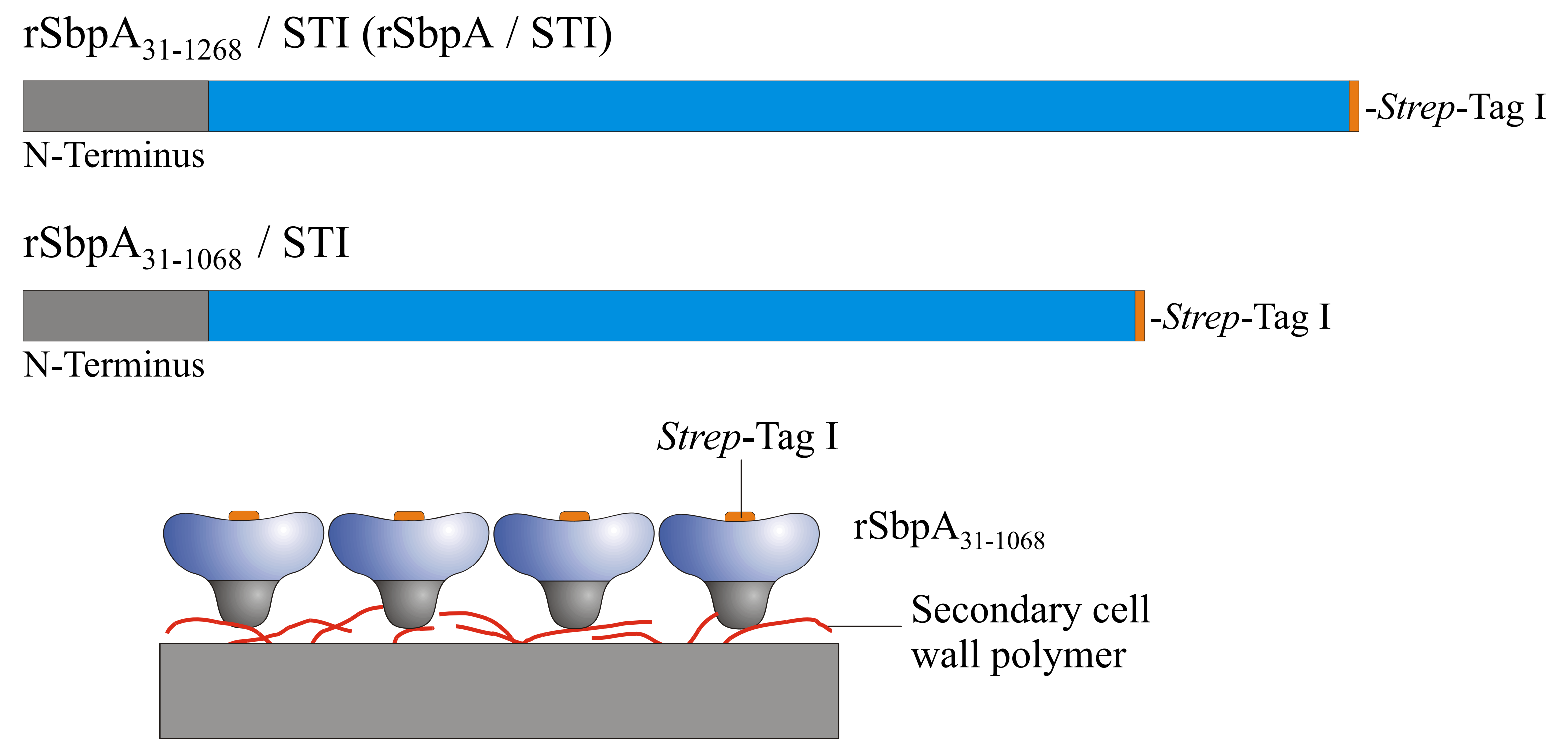
Fusion of the functional domain does not interfere with the self assembly properties.

Specific interactions between SbpA and the SCWP are exploited for an oriented binding of S-layer fusion proteins on solid supports.



Building of Nanoarrays

Investigation of the Structure - Function Relationship of SbpA



rSbpA$_{31\text{-}1268}$ / STI (rSbpA / STI)

-*Strep*-Tag I

N-Terminus

rSbpA$_{31\text{-}1068}$ / STI

-*Strep*-Tag I

N-Terminus

Strep-Tag I

rSbpA$_{31\text{-}1068}$

Secondary cell wall polymer

- Truncation did not interfere with the self-assembly or recrystallisation properties.
- Deletion of 200 amino acids increased accessibility of the C-terminus.

rSbpA Fusion Proteins

with integrated functional peptide sequences or protein domains

N-Terminus

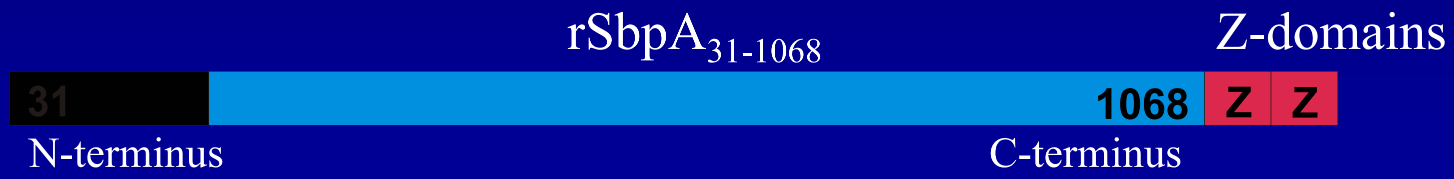
$rSbpA_{31\text{-}1068}$

Functional Domain

$rSbpA_{31\text{-}1068}$ / Bet v1 (161 aa)	(major birch pollen allergen)
$rSbpA_{31\text{-}1068}$ / cAb (117 aa)	(variable region of a camel antibody)
$rSbpA_{31\text{-}1068}$ / ZZ (116 aa)	(IgG-binding domain)
$rSbpA_{31\text{-}1068}$ / EGFP (238 aa)	(enhanced green fluorescent protein)

Sára et al, 2005, In Biological and Pharmaceuticals Nanomaterials. C. Kumar, ed. Wiley-VCH, Germany, p. 353.
Sára et al., 2005, Micro- and Nanoengineering proceeding, Elsevier.

Fusion Protein rSbpA$_{31\text{-}1068}$/ZZ



Z-domain: Analogue of the "B" binding domain of Protein A from *Staphylococcus aureus*

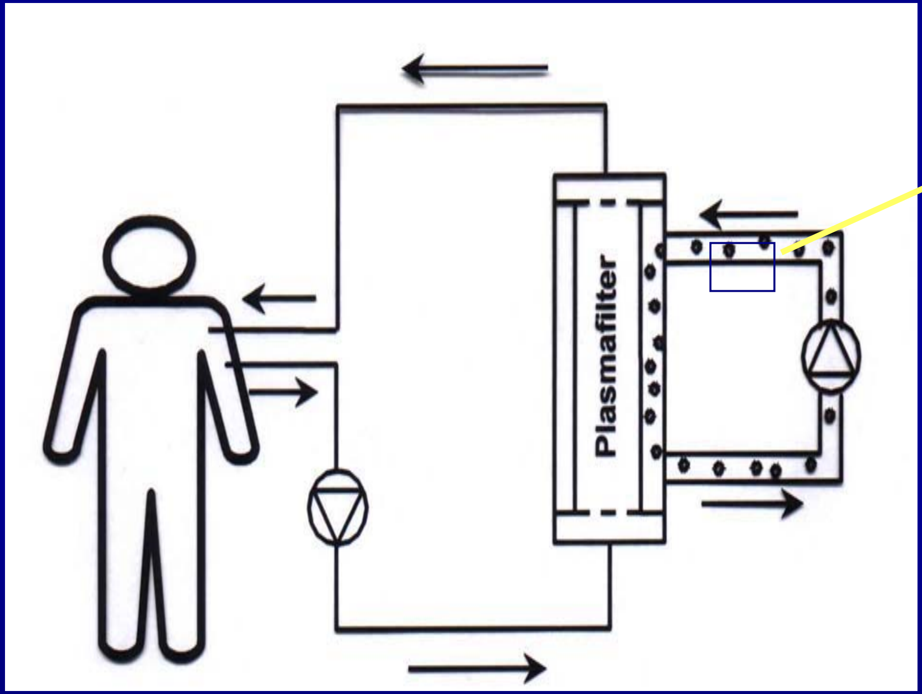
Consisting of 58 aa

Binding the Fc-component of IgG

Völlenkle et al., 2004, Appl. Environ. Microbiol., Vol. 70, p. 1514-1521.
Highlighted in: Nature Reviews Microbiology, 2004, Vol. 2, p. 353.

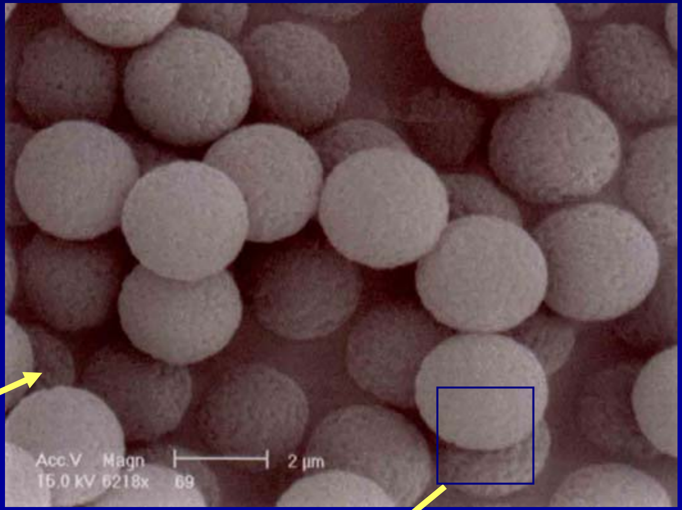
Microspheres Based Detoxification-System

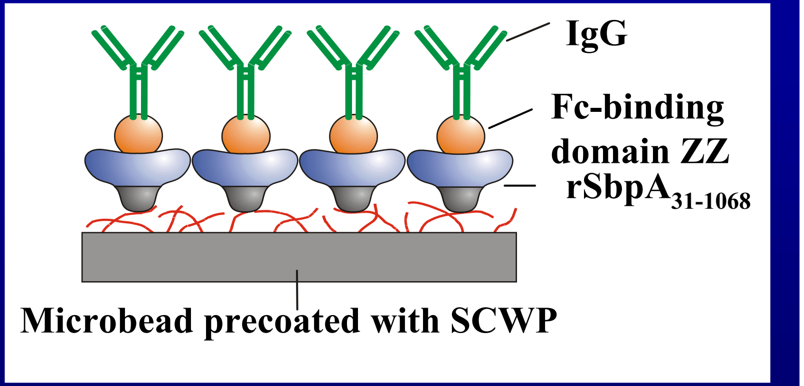
Secondary circuit
(plasma +microspheres)



Primary circuit
(blood)

Cellulose beads, 3 µm, coated with SCWP





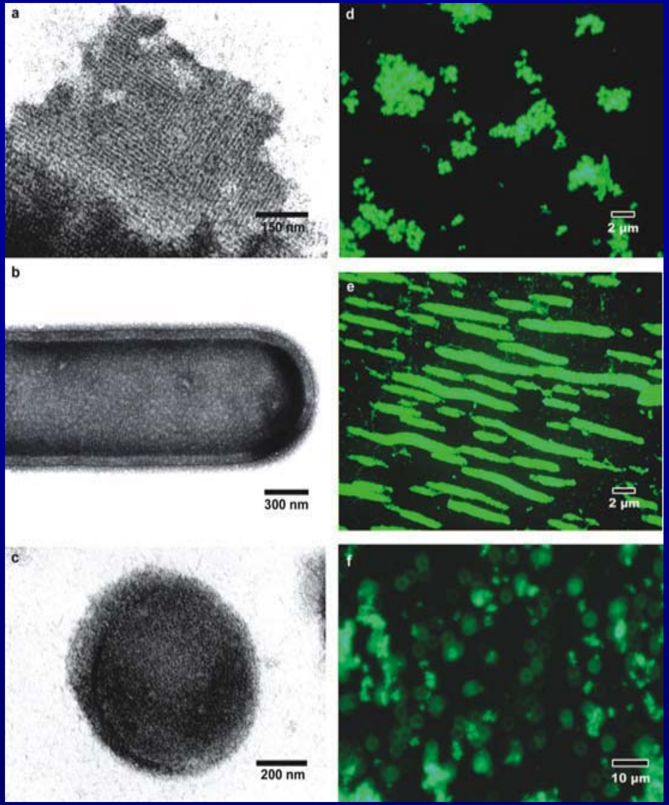
Endotoxin contamination: < 1 EU / ml → Application in biological systems

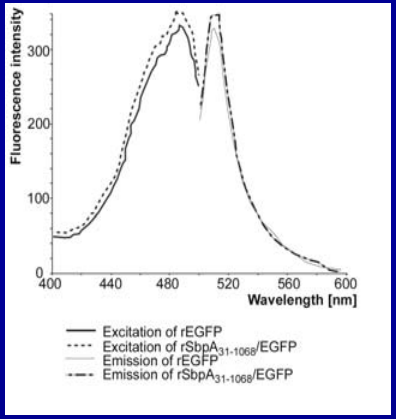
rSbpA-Enhanced Green Fluorescent Fusion Protein

rSbpA$_{31-1068}$/EGFP

-EGFP

N-Terminus





Fluorescence excitation and emission spectra of rEGFP and of the fusion protein. Both exhibiting identical excitation peaks at 488 nm and emission at 507 nm.

Electron micrographs of negatively stained preparations and fluorescene micrographs

(a,d) Self-assembly products formed by rSbpA/EGFP

(b,e) rSbpA/EGFP recrystallized on peptidoglycan-containing sacculi of *B. sphaericus* CCM 2177

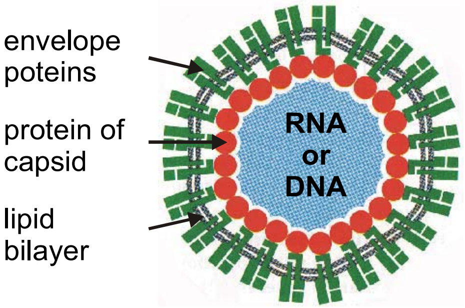
(c,f) Positively charged liposomes coated with the fusion protein rSbpA/EGFP

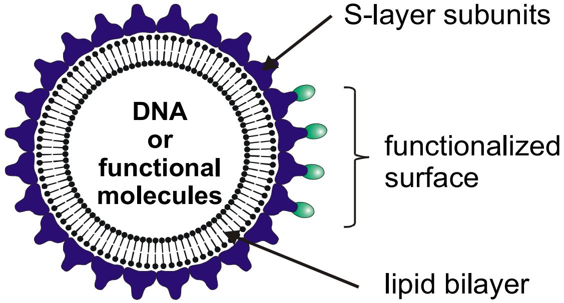
S-Layer Coated Liposomes (Biomimetic Viruses)

Higher mechanical and thermal stability
Functionalisation by coating with S-layer fusion proteins

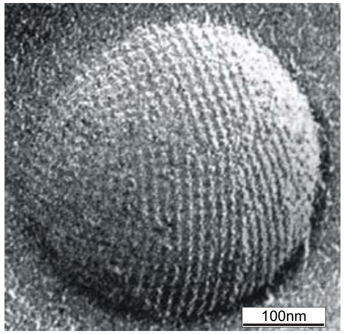
Application potential:

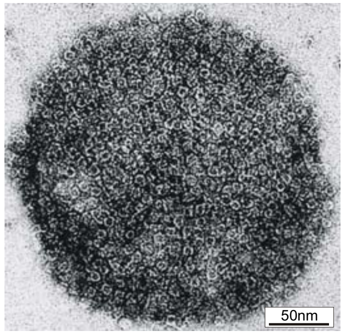
Drug-targeting and Drug-delivery systems
Immune therapy
Gene therapy (Inclusion of DNA)





Freeze-etched preparation of an S-layer coated liposome





Negatively stained preparation of S-layer coated liposome after immobilization of ferritin