223

Annex

List of Publications

Wunderlin, T., Junier T., Roussel-Delif L., Jeanneret N. and Junier P. (2013). Stage 0 sporulation gene A (*spo0A*) as molecular marker to study diversity of endospore-forming Firmicutes. *Environmental Microbiology Reports.* 5(6):911-24.

Bueche, M., **Wunderlin, T.**, Roussel-Delif L., Junier T., Sauvain, L., Jeanneret N. and Junier P. (2013). Quantification of endospore-forming Firmicutes by qPCR with the functional gene *spo0A*. *Applied and Environmental Microbiology.* 79(17):5302-12.

Wunderlin T., Corella J.P., Junier, T., Bueche, M., Girardclos S. and Junier P. (in press). Endospore-forming bacteria as new proxies to assess impact of eutrophication in Lake Geneva, (Switzerland-France). *Aquatic Sciences.*

Sauvain L., Bueche M., Junier T., Masson M., **Wunderlin T.**, Kohler-Milleret R., Gascon-Diez E., Loizeau J.L., Tercier-Waeber M.L. and Junier P. (in press). Bacterial communities in trace metal contaminated lake sediments are dominated by endospore-forming bacteria. *Aquatic Sciences.* doi 10.1007/s00027-013-0313-8.

Presentations at national or international conferences

1) Poster presentation at Annual PhD student meeting from Doctoral School of Organismal Biology, Neuchâtel, Switzerland. April (19.) 2010.

Survival in extreme environments - Natural diversity of spore-forming bacteria

Tina Wunderlin[1], Thomas Junier[2] & Pilar Junier[1]

Introduction

- Under environmental stress, some bacteria produce endospores, tough capsules housing the bacterias' full genetic information.
- The spores can survive for long periods of time and later germinate again into viable cells.
- The formation of endospores is a survival strategy found mainly in the group of *Firmicutes.*
- Spore-forming bacteria (i.e. *Bacillus, Clostridium*) are widely studied in the food and medical research.
- The natural diversity of spore-forming bacteria is not known.

Questions

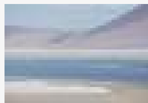
- What is the best method to isolate spores from environmental matrices to obtain DNA of only spore-forming bacteria.
- Is there a common core of genes involved in sporulation among different species of spore-forming bacteria?
- Can we identify new spore-forming microbial species?

Objective

Study the natural phylogenetic diversity of spore-forming bacteria by metagenomics.

Sampling sites

1. High salinity

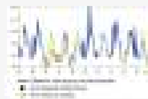


Salt desert, *Salar de Huasco*, Chile

2. High temperature and pressure

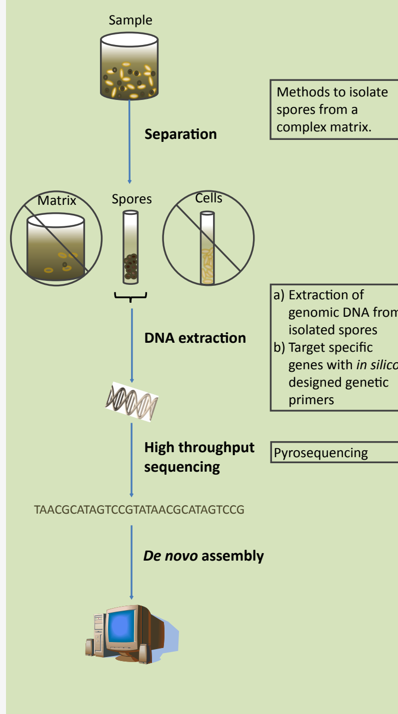
Geothermal research lab, Gross-Schönebeck, Germany

3. Dry-wet cycles



Fluctuating groundwater table, Bargen, Switzerland

Methodological Approach



Methods to isolate spores from a complex matrix.

a) Extraction of genomic DNA from isolated spores
b) Target specific genes with *in silico* designed genetic primers

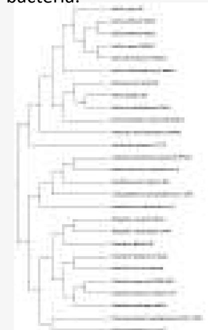
Pyrosequencing

First Results

- Preliminary tests indicate treatment with a strong oxidizing agent (i.e. Sodiumdodecyl sulftate) is effective to break cells and free spores.

Bacillus alvei with endospores

- A core of 6 common sporulation genes was identified in 27 genomes from spore-forming bacteria.



Phylogram with 6 concatenated common sporulation genes

[1] **Laboratory of Microbiology**. Institute of Biology, University of Neuchâtel, Switzerland
[2] Computational Evolutionary Genomics Group. University of Geneva, Switzerland

Université de Neuchâtel unine

2) Poster presentation at 4th European Spore Conference, Cortona, Italy. May (27.-29.), 2010.

New approaches to study the diversity of spore-forming microorganisms in natural communities

Tina Wunderlin[1], Thomas Junier[2] & Pilar Junier[1]

Introduction

The ecological diversity of spore-forming bacteria is far from being comprehensively assessed, mainly because it is difficult to specifically target this group within a complex microbial community. DNA extraction methods are often biased towards bacterial cells that are easy to lyse. Bacterial spores, with their cortex resistant to damage, are therefore often missed in microbial diversity studies from environmental samples.

Aims...

...to develop methods to specifically target the community of spore-forming bacteria in natural samples...for...

...studying the natural diversity of spore-forming bacteria in different environments.

Conclusion

To develop an effective and efficient method for targeting spore-forming bacteria in environmental samples, genomic approaches need to be combined with physical separation of spores from natural samples.

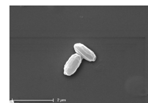


Fig. 1 – Characterization of *Bacillus subtilis* spores by Scanning Electron Microscopy (SEM)

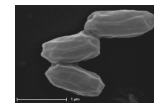


Fig. 2 – Spores of *B. megaterium* observed by SEM

Two different approaches

1) Separation of spores

Different chemical treatments have been tested for breaking up cells of three test strains (Gram + spore-forming, Gram + non spore-forming and Gram – non spore-forming).

A treatment with Sodiumdodecyl sulfate (SDS) or NaoH for 5 h inhibits growth (MPN counts) of cells from all tested strains without significant damage to the spores.

Bacteria used in separation tests:

A: *Bacillus alvei* sporulated culture
B: *Bacillus alvei* cell culture
C: *Escherichia coli* (Gram-.)
D: *Lactobacillus lactis* (*Firmicute*, non-sporulating, Gram+)

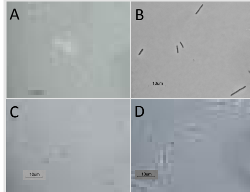


Fig. 4 – Microscopic images of cell cultures before treatment

Fig. 5 – Images of artificial community (mix of A, B, C and D) before and after treatment with NaOH (1 N) for 16 h.

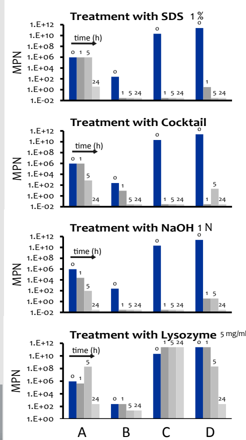


Fig. 3 - Growth (Most Probable Number) after chemical treatment of cell cultures for 1, 5 and 24 h, respectively.

Cocktail: mix of SDS (0.3%), NaOH (0.3 N), EDTA (0.3 mM) and Lysozyme (5 mg/ml).

2) Comparative genomics

A common core of 6 genes involved in sporulation was found among 27 genomes of spore-forming bacteria.

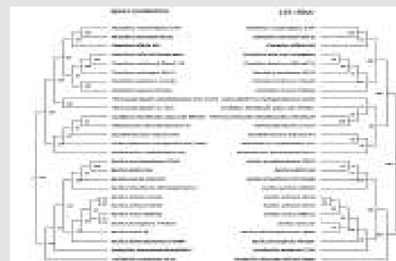


Fig. 6 – Phylogeny of 6 spore genes versus phylogeny of 16S rRNA of 27 genomes

Table 1 – Description of 6 common genes

Name	Gene symbol	Function
stage 0 sporulation protein A	*spoOA*	Global transcription regulator for sporulation
stage V sporulation protein T	*spoVT*	Global regulator activated by sigma G
stage V sporulation protein AC	*spoVAC*	Potential transmembrane protein with unknown function
stage V sporulation protein AD	*spoVAD*	Potential transmembrane protein with unknown function
stage IV sporulation protein B	*spoIVB*	Protease that activates processing of the pro-sigma K factor
spore protease	*gpr*	degradation of the small acid-soluble spore proteins (SASPs) during germination

Analysis of the 6 common sporulation genes and the corresponding phylogeny and conservation plots suggest *spo0A* and *gpr* genes are suitable for development of molecular markers.

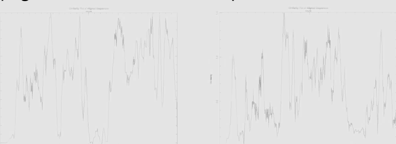


Fig. 7 – Conservation plot of *spoOA* gene

Fig. 8 – Conservation plot of *gpr* gene

3) Poster presentation at Annual Assembly of the Swiss Society of Microbiology (SSM), Zurich, Switzerland. June (24.-25.), 2010.

New approaches to study **diversity of spore-forming microorganisms** in natural communities

Tina Wunderlin[1], Thomas Junier[2] & Pilar Junier[1]

Spores…

…are highly resistant cell states formed by some bacteria, mainly from the phylum Firmicutes.
…are ubiquitously found in most environments (soil, water, air).
…have lower cell lysis efficiency and are often missed with conventional DNA extraction methods.
…are therefore often neglected in microbial diversity studies.

Objective

In this project, we are developing methods to specifically target the spore-forming fraction of natural microbial communities.

We want to study the natural diversity of spore-forming bacteria in different environments.

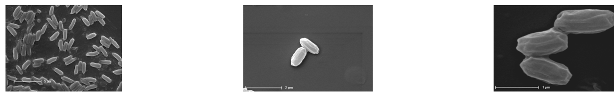


Fig. 1, 2 and 3 – Characterization of *Bacillus* spores by Scanning Electron Microscopy (SEM)

Methodological approaches:

1) Comparative genomics

We found 6 genes involved in sporulation that are conserved among 27 genomes of spore-forming bacteria.

Fig. 4 – Phylogeny of 6 spore genes versus 16S rRNA

Analysis of the 6 common sporulation genes and the corresponding phylogeny and conservation plots suggest that *spooA* and *gpr* genes are suitable for development of molecular markers.

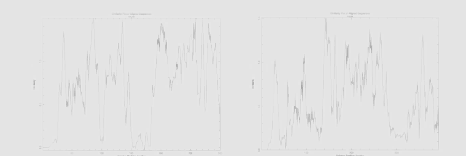


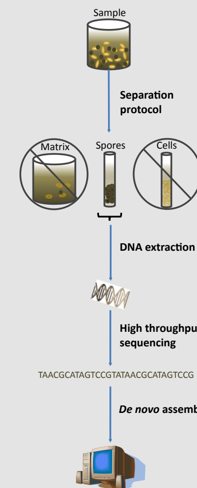
Fig. 5 – Conservation plot of *spooA* gene Fig. 6 – Conservation plot of *gpr* gene

Table 1 – Description of 2 best conserved sporulation genes

Gene	Symbol	Function
Stage 0 sporulation protein A	*spooA*	Global transcription regulator for sporulation
Spore protease	*gpr*	Degradation of the small acid-soluble spore proteins (SASPs) during germination

2) Separation and extraction protocol

The second approach is to develop a protocol to separate and extract spores from samples, i.e. from residual biomass and environmental matrix.



The spore fraction is separated from samples before being subjected to DNA extraction. The diversity will then be analyzed by sequencing and genome annotation.

Initial tests on different strains grown in the lab show that a combined treatment with lysozyme and a mix of NaOH and SDS, is effective to break most vegetative cells without damaging the spores.

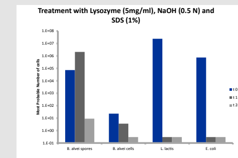


Fig. 7 – Cell counts (Most Probable Number) of re-grown cultures (*Bacillus alvei* spores and cells, *E. coli* as Gram- and *Lactobacillus lactis* as Gram+ control) after treatment with lysozyme, NaOH and sodium dodecyl sulfate (SDS).

This protocol is then tested with artificial communities and communities mixed with environmental matrix (sand, soil) before being applied to environmental samples.

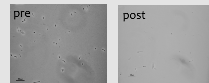


Fig. 8 and 9 – Image of artificial community (mix of 4 lab cultures) before and after treatment with NaOH (1 N).

[1] Laboratory of Microbiology. Institute of Biology, University of Neuchâtel, Switzerland
[2] Computational Evolutionary Genomics Group. University of Geneva, Switzerland

4) Poster presentation at ISME Conference, Seattle, Washington, USA. August (22.-27.), 2010.

New approaches to study the **diversity of endospore-forming microorganisms** in natural communities

Tina Wunderlin & Pilar Junier

Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, Switzerland

Endospores…

…are resistant cell states formed by certain bacteria to survive harsh environmental conditions.

…are ubiquitous in most environments.

…are often resistant to cell lysis and therefore missed with conventional DNA extraction methods.

…are often neglected in microbial diversity studies.

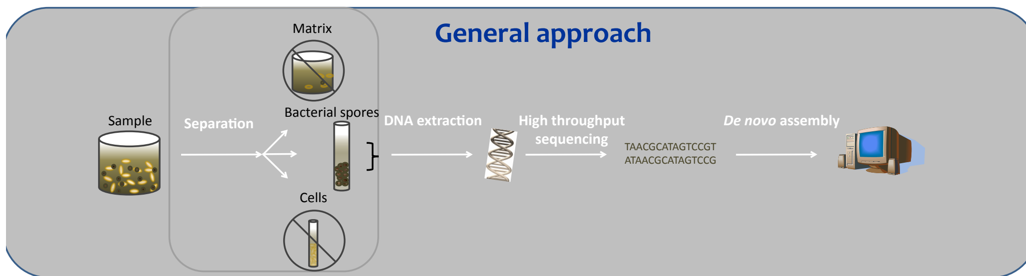
Fig 1: Endospores of *Paenibacillus alvei* in phase contrast microscopy.

Objective

The aim of this study is to look at the natural diversity of spore-forming bacteria in different environments.

For that we will first develop a method to specifically target the spore-forming fraction of natural microbial communities.

General approach



1) Screening for best method

The spores fraction is isolated using treatments that damage the membranes of vegetative cells without damaging the spores.

Initially the different treatments were tested on four strains grown in the lab. The method was evaluated by looking at the recovery (re-growth) of cells after treatment.

Fig 2 - Strains used for separation experiments

Table 1- Comparison of different treatments used to destroy vegetative cells: Effeciency was measured as the percentage of re-grown bacteria compared to the untreated control. For the values: 0=75-100%; 1=50-75%; 2= 25-50 %; 3= 0-25%

	EDTA	NaOH	Lysozyme	Sodium-dodecyl-sulfate (SDS)	Lysozyme - NaOH + SDS	Dry heat	Sonication	UV radiation	Wet heat	Wet heat	Wet heat - Lysozyme - NaOH + SDS	Wet heat - Lysozyme - NaOH + SDS
	1 mM	0.5 N	5 mg/ml	1 %	10 mg/ml - 0.5 N + 1 %	85 °C	40 W	30 W/cm², 20 cm	85 °C	65 °C	65 °C- 10 mg/ml - 0.5 N + 1 %	85 °C – 10 mg/ml - 0.5 N + 1 %
Duration (min)	300	60	60	60	60 – 60	20	0.5	2	20	10	20 - 60 - 60	10 - 60 - 60
***P. alvei* - endospores**	3	2	0	0	0	0	0	3	3	0	0	3
***P.alvei* – veg. cells**	0	3	0	3	3	0	2	3	3	3	3	3
***E. coli* - Gram-**	0	3	3	3	3	3	3	3	3	3	3	3
***L. lactis* - Gram+**	0	3	3	3	3	3	3	3	3	3	3	3

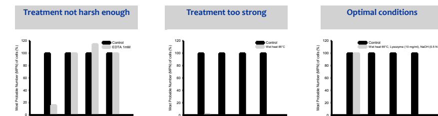


Fig. 3– Cell counts (Most Probable Number) of re-grown cultures after treatment. Count presented as % cell numbers in the control (untreated) sample. 1= *P. alvei* spores; 2= *P. alvei* cells; 3= *E. coli*; and 4= *L. lactis*.

2) Best method verified on samples

The best method to damage cells without damaging spores was tested on three different samples:

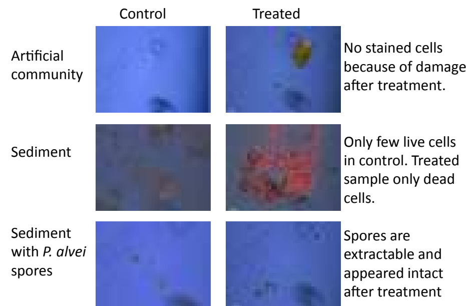
A) artificial community (mix of four strains used in screening)

B) sediment sample from Lake Loclat, Switzerland

C) sediment sample amended with *P. alvei* spores.

Samples were analyzed using Live/Dead BacLightTM bacterial staining kit (invitrogen) and observed by fluorescence microscopy.

	Control	Treated	
Artificial community			No stained cells because of damage after treatment.
Sediment			Only few live cells in control. Treated sample only dead cells.
Sediment with *P. alvei* spores			Spores are extractable and appeared intact after treatment



Conclusion

The most efficient treatment to break up vegetative cells is wet heat at 65°C with a successive digestion using lysozyme, SDS and NaOH.

Spores can be separated from sediment samples by extraction and subsequent treatment to destroy all bacterial cells without apparent damage.

Further tests have to be conducted to verify the absence of residual DNA from cells present in the treated samples.

The extraction method for sediment needs to be optimized to separate all spores from particle aggregates in the sample

We acknowledge funding from the Swiss National Science Foundation through grant No. PZ00P3_126330

5) Oral presentation at Anniversary of F.A. Forel Institute, Geneva, Switzerland. September (17.), 2010.

Laboratoire de Microbiologie
de l'Université de Neuchâtel
www2.unine.ch/lamun

Spore-forming bacteria
in lake sediments

Tina Wunderlin & Pilar Junier

Symposium Institute F.-A. Forel
Geneva, September 17th 2010

6) Poster presentation at Swiss meeting of Microbial Ecology, Engelberg, Switzerland. February (2.-4.), 2011.

Metagenomics on a microbial sub-population – finding molecular markers for endospore-forming bacteria

Tina Wunderlin, Thomas Junier, Manon Brenier, Ludovic Roussel-Delif, Nicole Jeanneret and Pilar Junier

Laboratory of Microbiology, University of Neuchâtel, Switzerland

Bacterial endospores are…

Paenibacillus alvei with endospores (phase-contrast microscopy)

Characterization of *Bacillus subtilis* endospores (Scanning Electron Microscopy SEM)

Bacillus megaterium endospores (SEM)

…resistant cell states formed by selected bacteria to endure unfavorable environmental conditions

…ubiquitous in most environments

…difficult to lyse with conventional DNA extraction methods

…underrepresented in microbial diversity studies

Our objective is to investigate the diversity of the spore-forming fraction of microbial communities in different environments.

We therefore designed molecular markers that target genes exclusive to this group of bacteria.

1) Sequences of proteins involved in sporulation from 27 spore-forming species were retrieved from the Comprehensive Microbial Resources database.

Species name	Group	Environment	Temperature optima
Alkaliphilus metalliredigens QYMF	Clostridia	Borax leachate ponds	Mesophile
Alkaliphilus oremlandii OhILAs	Clostridia	Freshwater USA	Mesophile
Bacillus amyloliquefaciens FZB42	Bacilli	Soil	Mesophile
Bacillus anthracis A0248	Bacilli	Human isolate from USAMRIID, Ohio	Mesophile
Bacillus anthracis Sterne	Bacilli	Soil	Mesophile
Bacillus cereus 03BB102	Bacilli	Human blood	Mesophile
Bacillus cereus Zk	Bacilli	Zebra carcass in Namibia	Mesophile
Bacillus licheniformis ATCC 14580	Bacilli	Soil	Mesophile
Bacillus pumilus SAFR-032	Bacilli	Soil	Mesophile
Bacillus subtilis 168	Bacilli	X-ray irradiated strain in Marburg in 1947	Mesophile
Bacillus thuringiensis Al Hakam	Bacilli	Severe human tissue necrosis	Mesophile
Bacillus weihenstephanensis KBAB4	Bacilli	Soil	Psychrotolerant
Candidatus Desulforudis audaxviator MP104C	Clostridia	Fracture water from a borehole at a depth of 2.8 km in a South African gold mine	Mesophile
Carboxydothermus hydrogenoformans Z-2901	Clostridia	Hot swamp from Kunashir Island, Russia	Hyperthermophil
Clostridium beijerinckii NCIMB 8052	Clostridia	Freshwater, Soil	Mesophile
Clostridium botulinum B Eklund 17B	Clostridia	Marine sediments taken off the coast of Washington, USA	Mesophile
Clostridium difficile 630	Clostridia	Clinical isolate Switzerland	Mesophile
Clostridium botulinum A2 Kyoto-F	Clostridia	Infant botulism in Kyoto, Japan in 1978	Mesophile
Clostridium kluyveri DSM 555	Clostridia	Mud of a canal in Delft, The Netherlands	Mesophile
Clostridium perfringens SM101	Clostridia	Soil	Mesophile
Geobacillus thermodenitrificans NG80-2	Bacilli	Oil reservoir formation water taken at a depth of 2000 m	Thermophile
Heliobacterium modesticaldum Ice1	Clostridia	Hot spring microbial mats and volcanic soil in Iceland	Thermophile
Lysinibacillus sphaericus C3-41	Bacilli	Mosquito breeding site in China in 1987	Mesophile
Pelotomaculum thermopropionicum SI	Clostridia	Thermophilic upflow anaerobic sludge blanket reactor	Thermophile
Thermoanaerobacter pseudethanolicus ATCC 33223	Clostridia	Thermal springs in Yellowstone National Park	Thermophile
Thermoanaerobacter sp. X514	Clostridia	Anaerobic enrichment culture from a deep subsurface sample (2000 m below the surface)	Thermophile
Desulfotomaculum reducens MI-1	Clostridia	Heavy metal contaminated sediment	Mesophile

2) With bi-directional BLAST of the sporulation proteome we found 6 genes with conserved sequences (cut-off at e-value of 10^{-60}).

Schematic representation of endospore formation and germination and the contribution of the six conserved genes in the pathway.

3) Two of these genes (*spo0A* and *gpr*) are suitable for molecular markers because…

a) they contain conserved and variable regions (conservation plot for *spo0A*).

b) their phylogeny is similar to the 16S rRNA gene (trees).

Conservation plot (made with plotcon software from emboss) showing the nucleotide similarity among the 27 species (y-axis) over the entire sequence of the *spo0A* gene (x-axis).

Phylogenetic trees of 16S rRNA and *spo0A* genes are similar, suggesting that primers for *spo0A* could be used for phylogeny. Maximum-Likelyhood trees were constructed with PHYML (Guindon, 2005) and drawn with Newick Utilities (Junier, 2010).

4) The sequences were aligned and searched for conserved regions (20 bp) that could function as primers for PCR amplification.
A selection of possible primers was tested on spore-forming and non spore-forming (control) strains.
(two examples of *spo0A* primers shown)

Primer set 1, expected fragment length 509 bp — **Primer set 2**, expected fragment length 275 bp

Images of electrophoresis in 1.7% agarose gel with bands of the expected fragment size for DNA extracts from spore-forming strains without amplification in the control strains (100 bp ladder from Promega).
1: *Bacillus subtilis*, 2: *Clostridium pasteurianum T*, 3: *Geobacillus thermoleovorans*, 4: *Escherichia coli*, 5: *Lactobacillus lactis* (non-spore forming Firmicute), 6: Blank

- Our primers are promising markers for spore-forming bacteria, since amplification occurs only in DNA extracts from this group.
- These markers can be used for amplification, sequencing and phylogeny as well as quantitative PCR.

We acknowledge funding from the Swiss NSF through Grant No. 31003A-132358/1 and No. PZ00P3_126330

Université de Neuchâtel unine