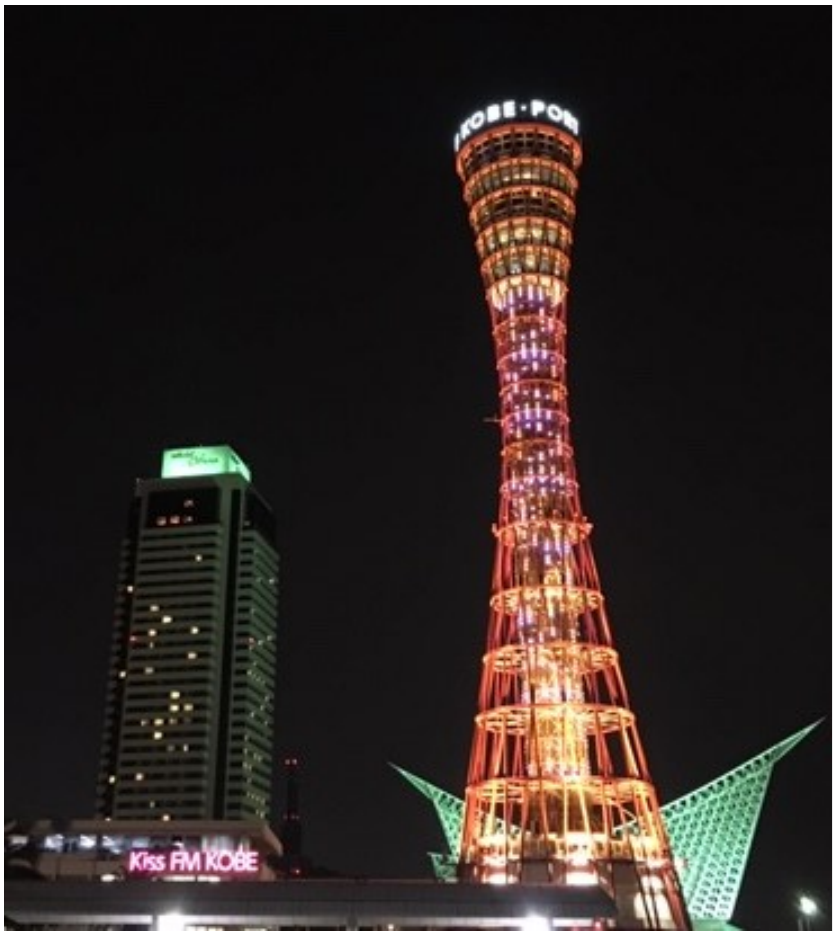
BACELL2023

2023 年度グラム陽性菌ゲノム機能会議



ABSTRACT BOOK

The Kobe Chamber of Commerce and Industry and Ariston Hotel

Kobe, Japan

20-23 November 2023

--- BACELL2020 comes back as BACELL2023!!

Since the *Bacillus subtilis* genome sequencing project meeting in Paris in 1990, the series of European Bacillus meetings, presently defined as “BACELL”, has been held annually as an international conference to discuss academic, industrial, and technological development of studies on *B. subtilis* and its related Gram-positive microorganisms.

As you remember, BACELL2020 was supposed to happen in April 2020 in Kobe, Japan. In March 2020, unfortunately, due to the epidemic of the new coronavirus, it had to be cancelled reluctantly. However, after all we have been through these four years, we are pleased to hold BACELL2023 here.

The Japanese Gram-positive Bacteria Genome Function Conference 2023 will be held jointly with BACELL2023, a monumental event of European-Japanese collaboration since 1990. It is an opportunity to exchange the latest research information on the genome function of not only *B. subtilis* but also Gram-positive bacteria in general.

We hope that BACELL2023 will be an ideal opportunity for the participants to disseminate their latest research results and serve as a beginning or a milestone to promote further EU-Japan cooperation.

Chair: Ken-ichi Yoshida, Kobe Univ

Co-chair (General affairs): Kei Asai, Tokyo Univ Agri
Accountants: Kenji Tsuge / Shu Ishikawa (auditor), Kobe Univ
External affairs: Hiromu Takamatsu, Setsunan Univ / Saori Kosono, University of Tokyo
Public relations: Nobuhiko Nomura, Tsukuba Univ

November 2023

BACELL2023 is organized by the local organizing committee and **National Bio-Resource Project**, and co-organized by the **Institute of Oceanic Research and Development, Tokai University**, and **Graduate School of Science, Technology and Innovation, Kobe University**.

BACELL2023 is financially supported by companies as follows:

Platinum supporter:
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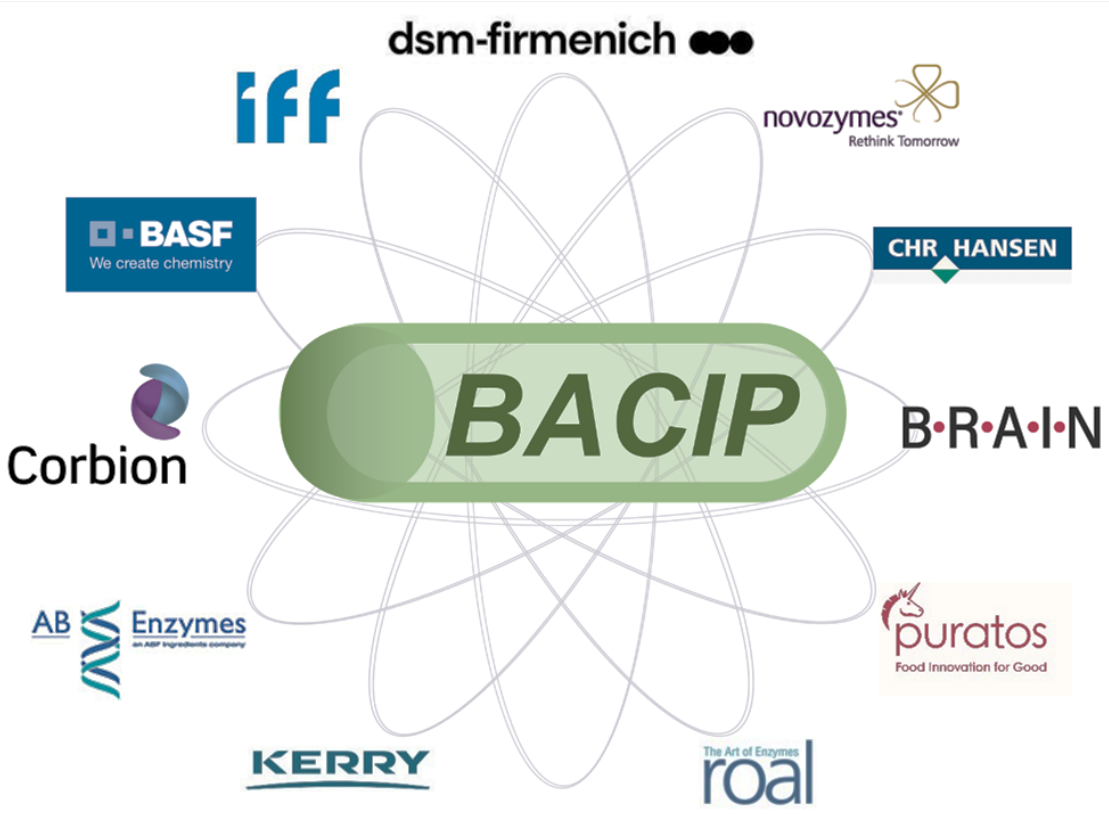
JSBBA Kansai

BACIP European student travel award

Thanking to the generous support of Bacillus Industrial Platform (BACIP), the 10 European students listed below have been selected to enjoy the travel award. The selection was done strictly based on the quality of the submitted abstracts:

Valentina Andrea Floccari (University of Ljubljana, Slovenia)
Cyprien Guérin (Université Paris-Saclay, France)
May Khider (Norwegian University of Science and Technology, Norway)
Veronika Kočárková (Institute of Microbiology of the CAS, Czech Republic)
Michael Kohlstedt (Saarland University, Germany)
Thomas Konjetzko (Institute for Bio- and Geosciences, Germany)
Sandra Maaß (University of Greifswald, Germany)
Shafagh Moradian (Queen Mary University of London, UK)
Frederic Schramm (Newcastle University, UK)
Frederik Völker (RWTH Aachen University, Germany)

BACIP also provided significant support for conference running costs.



BACELL2023-KOBE Programme

20 November 2023:

13:00- Registration
(KOBE Chamber of Commerce and Industry 2F Event Hall)

18:00-20:00 Welcome reception
(Ariston Hotel 2F)

21 November 2023:

09:00-10:15 Oral session 1:
Antimicrobials and toxins (Chair: Shu Ishikawa)
(KOBE Chamber of Commerce and Industry 3F Lecture Hall)

09:00-09:15 O-1 Yoshikazu Kawai (Sydney U, AU)
On the mechanism of lysis triggered by perturbations of cell wall synthesis in *Bacillus subtilis*

09:15-09:30 O-2 Jiri Pospisil (Czech Academy of Sciences, CZ)
ToxiCITY of *Bacillus subtilis*

09:30-09:45 O-3 Sanako Yoshida (Kobe U, JP)
Development and application of a novel bacteriocin, pallidocyclin, of a thermophilic bacterium *Aeribacillus pallidus* PI8

09:45-10:00 O-4 Katarzyna Mickiewicz (Newcastle U, UK)
B. subtilis as a model for studying host-pathogen interactions

10:00-10:15 O-5 Monika Ehling-Schulz (U Veterinary Medicine, AT)
ABC of CesCD: A bifunctional ABC transporter directing cereulide toxin synthesis in emetic *Bacillus cereus*

10:15-10:45 Coffee break
(KOBE Chamber of Commerce and Industry 2F Event Hall)

10:45-12:00 Oral session 2:
Evolution and mobile genetic elements/ Microbial interaction (Chair: Nozomu Obana)

10:45-11:00 O-6 Anna Dragoš (U Ljubljana, SI)
Multilevel control of *Bacillus subtilis* by SPbetaviruses – physiology, ecology and evolution perspective
11:00-11:15 O-7 Romain Briandet (INRAE Jouy, FR)
Bacillus velezensis kin consortia able to form a positive biofilm with antagonistic activity against pathogenic bacteria
11:15-11:30 O-8 Philippe Jacques (U Liege, BE)
Competition to commensalism, Trichoderma harzianum and Bacillus velezensis interaction is strongly cultural conditions dependent
11:30-11:45 O-9 Briana Burton (U Wisconsin-Madison, USA)
Molecular determinants of mosaic genomes resulting from interspecies natural transformation
11:45-12:00 O-10 Ines Mandić Mulec (U Ljubljana, SI)
Harnessing Bacterial Sociality: Understanding *Bacillus subtilis* - Pathogen Interactions for Effective Probiotic Applications

12:00-13:00 Lunch (Ariston Hotel 2F)

13:00-14:15 Rapid fire (62 Posters, 70 sec for each) (Chair: Saori Kosono)

14:15-15:30 Oral session 3:
Biotechnology 1 (Chair: Ritsuko Kuwana)
14:15-14:30 O-11 Biwen Wang (U Amsterdam, NL)
Inactivation of the conserved protease LonA increases xylanase production in *Bacillus subtilis* (The recorded presentation will be screened.)
14:30-14:45 O-12 Sandra Maaß (U Greifswald, DE)
From the Outer Space to the Inner Cell: Global Absolute Quantification of Extracellular, Membrane and Cytosolic Proteins in *Bacillus subtilis*
14:45-15:00 O-13 Frederik Völker (RWTH Aachen U, DE)
Harnessing the metabolic versatility of engineered *B. subtilis* 168 for enhanced production of the biopolymer poly-γ-glutamic acid
15:00-15:15 O-14 Kenji Tsuge (Kobe U, JP)
Long DNA synthesis using *Bacillus subtilis*
15:15-15:30 O-15 May Khider (NTNU, NO)
Production of 3-hydroxypropionic acid from methanol in recombinant *Bacillus methanolicus* MGA3

15:30-16:00 Coffee break

16:00-17:15 Oral session 4:
Biotechnology 2 (Chair: Ken-ichi Yoshida)

16:00-16:15 O-16 Varada Jagadeesh (Kobe U, JP)
SEAM-OGAB: Enabling rapid assembly of chimeric Non-Ribosomal Peptide Synthetase Gene Clusters in *Bacillus subtilis*

16:15-16:30 O-17 Sigrid Görgen (U Liege, BE)
Adapted cell factories for production of biosurfactant on cheap substrates

16:30-16:45 O-18 Cyprien Guérin (INRAE Jouy, FR)
Continuous culture in custom computer-controlled mini-bioreactors

16:45-17:00 O-19 Fujio Kawamura (Chiba U, JP)
Development of an ultra-transformation system in *Bacillus subtilis* 168

17:00-17:15 O-20 Colin Harwood (Newcastle U, UK)
A review of the regulatory frameworks for exploiting *Bacillus* species

17:15-18:30 Poster session 1 (Odd numbers)
(KOBE Chamber of Commerce and Industry 2F Event Hall)

22 November 2023:

09:00-10:15 Oral session 5:
Biotechnology 3 (Chair: Daisuke Imamura)

09:00-09:15 O-21 Michael Kohlstedt (U Saarland, DE)
Understanding redox metabolism in *Bacillus licheniformis* during heterologous protease production under industrially relevant conditions

09:15-09:30 O-22 Maliheh Vahidinasab (U Hohenheim, DE)
Genetic modification of *Bacillus subtilis* for improvement of antimicrobial lipopeptide biosynthesis

09:30-09:45 O-23 Naoki Miyamoto (Synplogen, JP)
Application of Combi-OGAB: fine-tuning growth-phase dependent promoters in biosynthetic gene cluster to create heterologous lethal antibiotic producer

09:45-10:00 O-24 Aysegul Oktem (U Groningen, NL)
Post-translational secretion stress regulation in genome-reduced *Bacillus subtilis*

10:00-10:15 O-25 Jan Maarten van Dijl (U Groningen, NL)
Engineering of *Bacillus* cell factories for recombinant protein production

10:15-10:45 Coffee break

10:45-12:00 Oral session 6:
Regulation (Chair: Kenji Tsuge)
10:45-11:00 O-26 Harald Putzer (Institut de Biologie Physico-Chimique, FR)
RNase Y autoregulates its synthesis in *Bacillus subtilis*
11:00-11:15 O-27 Jolanda Neef (U Groningen, NL)
Functional analysis of the small regulatory RNA S313 of *Bacillus subtilis*
11:15-11:30 O-28 Shafagh Moradian (Queen Mary U, UK)
Transcription attenuation as a source of genetic noise in *Bacillus subtilis*
11:30-11:45 O-29 Shinobu Chiba (Kyotosangyo U, JP)
Regulated translation arrest: a regulation mechanism of genes for bacterial protein localization machinery
11:45-12:00 O-30 Roland Hartmann (Philipps-Universität Marburg, DE)
6S RNAs in *Bacillus subtilis* – more than simple transcription inhibitors

12:00-13:00 Lunch (Ariston Hotel 2F)

13:00-14:30 Poster session 2 with coffee (Even numbers)

14:30-16:00 Oral session 7:
Sporulation and development/Stress response (Chair: Satoshi Matsuoka)
14:30-14:45 O-31 Libor Krásný (Czech Academy of Science, CZ)
Small subunits of RNA polymerase affect sporulation in *Bacillus subtilis*
14:45-15:00 O-32 Martin Robert (Kyoto U, JP)
Biofilming beyond *B. subtilis*
15:00-15:15 O-33 Pamila Osipova (Russian Academy of Sciences, RU)
Molecular mechanisms of resistance of *Bacillus pumilus* 25 isolated from ISS to antibiotics and oxidative stress
15:15-15:30 O-34 Danny K. Fung (U Wisconsin-Madison, USA)
Guanosine toxicity is associated with DNA damage and prophage activation in *Bacillus subtilis*

15:30-15:45 O-35 Sara Drais (U Strathclyde, UK)
Gingko Biloba leaf extract inhibits *Bacillus subtilis* biofilms and alters biofilm morphology in static and flow biofilm systems
15:45-16:00 O-36 Muktesh Kumar Sahu (IIT, IN)
NaCl induced lifestyle switching from sessile to motile state in *Bacillus subtilis*

16:00-16:15 Break (without coffee): Poster removal completed

16:15-17:00 Oral session 8:
Cell division/Others (Hiromu Takamatsu)
16:15-16:30 O-37 Frederic Schramm (Newcastle U, UK)
DnaA-boxes distant from the unwinding site promote helicase loader recruitment in a bipartite chromosome origin
16:30-16:45 O-38 Stuart Middlemiss (Newcastle U, UK)
Molecular motor tug-of-war regulates elongasome cell wall synthesis dynamics in *Bacillus subtilis*
Cancelled O-39 Liraz Chai (Hebrew U Jerusalem, IS)
A biophysical view of bacterial biofilms. From isolated components to multicellular organisms
16:45-17:00 O-40 Hironori Niki (NIG, JP)
Profiling a single-stranded DNA region within predicted G-quadruplexes in the *B. subtilis* genome

18:00- Banquet (Ariston Hotel 16F Party Hall)

22 November 2023:

09:45-17:00 Optional excursion: Himeji Castle
(On buses from KOBE Chamber of Commerce and Industry)

＜ Schedule ＞
09:45 (SHARP!): Ground floor of The Kobe Chamber of Commerce and Industry
10:00 departure:
11:30 arrival: Himeji Castle/Koko-en (Lunch and Entry)
15:00 departure:
16:30 arrival: Sannomiya
16:50 arrival: The Kobe Chamber of Commerce and Industry

Himeji Castle Himeji Castle is nicknamed "White Heron" Castle because of its white walls which are covered with white plaster. The reason for using white plaster is that it is fireproof. Like other surviving Japanese castles, Himeji Castle is built of wood, not stones, and so fire prevention is very important. As well as the fact that white plaster has fireproofing capabilities, it is also considered to be a strong reinforcement. Therefore, it covers both walls and almost every outside structure from eaves to pillars. The castle is built on the top of a hill called Himeyama, which is 45.6 meters above sea level. The main tower, which is the center and the symbol of the castle, is 46.4 meters high; namely, it stands 92 meters above sea level. Himeji Castle is famous for not only this huge main tower but also the highly effective and complicated defensive design, which is just like a maze. Even today, with the route clearly marked, many visitors are easily lost. You, too, may be lost in the labyrinth.

Abstracts for Oral presentations

O-1

On the mechanism of lysis triggered by perturbations of cell wall synthesis in *Bacillus subtilis*

Yoshikazu Kawai[1,2], and Jeff Errington[1,2]

[1] *Newcastle University, Newcastle Upon Tyne, UK;*
[2] *University of Sydney, Sydney, Australia*

Inhibition of bacterial cell wall synthesis by antibiotics such as β-lactams is thought to cause explosive lysis through loss of cell wall integrity. However, recent studies on a wide range of bacteria have suggested that these antibiotics also perturb central carbon metabolism, contributing to death via oxidative damage. Here, we genetically dissect this connection in *Bacillus subtilis* perturbed for cell wall synthesis, and identify key enzymatic steps in upstream and downstream pathways that stimulate the generation of reactive oxygen species through cellular respiration. Our results also reveal the critical role of iron homeostasis for the oxidative damage-mediated lethal effects. We show that a recently discovered siderophore-like compound (mirubactin C) sequesters iron and prevents its uptake, thereby counteracting oxidative damage via lipid peroxidation, likely by avoiding prooxidant effects of redox-active iron. Remarkably, we found that when the cell wall mutants are rescued by mirubactin C, morphological abnormalities such as bulging and twisting that are generally assumed to be a precursor to cell death by lysis, are largely unaffected. The main effect is on loss of the “pale” cellular appearance, often associated with the term lysis, which is presumably due to leakage of cell contents and reduced cytoplasmic density. Phase paling appears to be closely associated with lipid peroxidation. These results provide insights into the mechanisms of bacterial cell death and antibiotic action.

O-2

ToxiCITY of *Bacillus subtilis*

Jiří Pospíšil[1], Martin Hubálek[2], Hana Šanderová[1], and Libor Krásný[1]

[1] *Institute of Microbiology of the Czech Academy of Sciences v. v. i., Prague, Czech Republic*

[2] *Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences v. v. i., Prague, Czech Republic*

Bacteria use extracellular toxins to fight against each other. This project consists of two main parts. The first part focuses on identification of so far unknown extracellular toxic proteins/lipids produced by gram positive *Bacillus subtilis* 168 where genes for known toxins such as Sublancin 168 or WapA were deleted and culture filtrates of the Δ*sunA* Δ*wapA* strain were fractioned by Gel filtration. Toxicity of individual fractions was monitored by their effect on growth of *Bacillus megaterium*. We detected fractions that were toxic and contained a large protein complex that inhibited growth of *B. megaterium*. This complex is currently being characterized. The second part of the project is aimed on isolation of Sublancin 168, a known toxin (with unknown target) that kills gram-positive bacteria. A tagged version of Sublancin 168 was used to detect its target in several gram-positive bacterial species (*B. megaterium*, *B subtilis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*). Finally, we evaluated the synergy between Sublancin 168 and synthetically prepared antimicrobial compounds LPPOs (membrane targeting) in their ability to kill gram-positive or -negative bacterial species. In summary, novel toxins and/or their targets will be presented and discussed.

O-3

Development and application of a novel bacteriocin, pallidocyclin, of a thermophilic bacterium *Aeribacillus pallidus* PI8

Sanako Yoshida, Kyosuke Kita, Shu Ishikawa, and Ken-ichi Yoshida

Department of Science, Technology, and Innovation, Kobe University, 1-1 Rokkodai, Nada Kobe657-8501 Japan

Aeribacillus pallidus PI8 is a Gram-positive thermophilic bacterium that produces some antimicrobial substances against several bacterial species, including *Geobacillus kaustophilus* HTA426, another Gram-positive thermophilic bacterium. Bacteriocins are polypeptides synthesized ribosomally by various species of bacteria that have antimicrobial activities against the other bacterial species closely related to the producers. We considered that *A. pallidus* PI8 might produce a bacteriocin and sought to identify the genes involved in its production. *A. pallidus* PI8 turned out to possess the *pcynBACDEF* operon to produce pallidocyclin. *pcynA* encodes the precursor of pallidocyclin, *pcynF* functions as an immunity gene, and the others may be for maturation and secretion processes.

In this study, we establish an efficient and heterologous production of pallidocyclin in *Bacillus subtilis*. Pallidocyclin produced in *B. subtilis* was purified and subjected to mass spectrometric analyses to reveal its circular-peptide structure.

Pallidocyclin exerts toxicity to various bacterial species, including the one known to cause contamination in dairy products. This finding suggested that pallidocyclin might be applied to control the contaminant. On the other hand, thermophilic bacteria can be potential platforms for bioproduction, as higher temperatures have advantages such as reduced contamination and cooling costs and increased reaction efficiency. However, genetic modification research on thermophilic bacteria has not progressed due to a lack of thermostable antibiotics and resistance genes. Therefore, if pallidocyclin is used similarly to an antibiotic, *pcynF* could be a suitable novel selection marker for the genetic engineering of thermophiles, including *G. kaustophilus*.

O-4

B. subtilis as a model for studying host-pathogen interactions.

Yoshikazu Kawai, Stuart Middlemiss, Andrew Watson, Jeff Errington and **Katarzyna Mickiewicz**

Affiliations (Centre for Bacterial Cell Biology, Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne NE2 4AX, UK)

L-forms are cell wall-deficient bacteria, which are resistant to all classes of cell wall-targeting antibiotics and might contribute to recurrent infections, such as urinary tract infections and sepsis. Many Gram-positive and Gram-negative bacteria, including critical pathogens, such as *S. aureus* or *E. coli* can undergo L-form switching. Nevertheless, due to relative ease of handling and genetic trackability, it was *B. subtilis,* which was previously used to understand molecular basis of L-form division and replication. *B. subtilis* is a soil-dwelling bacterium, but also a commensal of human gut. Even though considered a non-pathogen, we have successfully used *B. subtilis* to study host-L-form interactions, in the context of cell lines and *Galleria mellonella* model. We showed induction of L-forms by the immune system and their long-term survival in the host, in the presence of cell wall-targeting antibiotics.

O-5

ABC of CesCD: A bifunctional ABC transporter directing cereulide toxin synthesis in emetic *Bacillus cereus*

Ehling-Schulz M.[1], Jenull S.[1], Chromikova Z.[1,2], Stancheva S.[1], M. Sulyok[3], Barak I.[2], and Gacek-Matthews A.[1]

[1] *Institute of Microbiology, Vetmeduni Vienna, Vienna, Austria,*
[2] *Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia,*
[3] *Institute for Bioanalytics and Agro-Metabolomics, University of Natural Resources and Life Sciences Vienna, Vienna, Austria*

Bacillus cereus is a human pathogen, which provokes a panoply of local and systemic diseases. The emetic strains, harboring a virulence megaplasmid similar to *B. anthracis* pXO1, produce the thermo- and pH- resistant toxin, cereulide. This non-ribosomal peptide is responsible for the emetic form of *B. cereus* foodborne disease and occasionally leads to rhabdomyolysis, acute liver failure, and death. The cereulide biosynthetic genes are organized within the *ces* operon, including the structural non-ribosomal peptide synthetase genes *cesA* and *cesB* as well as the putative ABC drug efflux transporter *cesC* and *cesD*.

Despite our extensive knowledge of the transcriptional regulation of cereulide biosynthesis, there is a lack of evidence concerning cereulide cellular localization, export, and mechanisms of host bacterium autoimmunity. Disruption of this putative ABC drug efflux pump leads to a cereulide negative phenotype, despite wild-type protein level of CesAB. These results suggest a scaffolding function of CesCD as a component of the toxin biosynthetic machinery. Subsequent *in vitro* and *in vivo* studies identified the ABC transporter CesC/D as an essential component of the non-ribosomal peptide biosynthetic machinery for cereulide toxin. This is the first report of an ABC transporter directly involved in the non-ribosomal peptide biosynthetic machinery. Using bioassays, it could be shown that CesCD contributes to bacterial host tolerance towards the cereulide toxin and the structurally analogous antibiotic valinomycin. These results suggest a binary function of CesCD as an essential component of the cereulide biosynthetic machinery on one hand as well as a defense mechanism on the other.

O-6

Multilevel control of *Bacillus subtilis* by SPβ viruses – physiology, ecology and evolution perspective

Virginie Grosboillot, Valentina Floccari, Maja Popović, Polonca Štefanič, Anna Munk, Helge Federssen, Robert Hertel, Ákos T. Kovács, Mikael Lenz Strube, Marc Bramkamp, and **Anna Dragoš**

Department of Microbiology, Biotechnical Faculty, University of Ljubljana
Department of Biotechnology and Biomedicine, Technical University of Denmark
Institute for Biotechnology, BTU Cottbus-Senftenberg
Institute of General Microbiology, Kiel University

Phages are viruses that infect and kill bacteria, but some of them also integrate into bacterial chromosome and establish long-term reproductive alliance with their host, when they can profoundly impact bacterial physiology, ecology, and evolution. *Bacillus subtilis* and other closely related species, can host SPβ viruses – temperate phages which characterize with regulatory switch lifestyle, rather large genomes, and tendency to form chimeras during superinfection. Therefore, they can control the host at several levels: interruption of functional attachment genes, introducing of new genetic arsenal and recombination within the host.

We screen publicly available, complete genomes of *B. subtilis* for presence of SPβ viruses using prophage-prediction tools. We isolate diverse representatives of SPβ viruses from their native hosts and use them to lysogenize model host strain to further access phage impact on *B. subtilis*. We also use available transcriptomics data to extract growth conditions where host control by phage may manifest, or phage genes which are the most relevant for such control. Finally, we use experimental evolution, phenotypic assays, interbacterial competition assays and genetic engineering, to address mechanisms of host control by phage.

Genomic analysis reveals that SPβ viruses are abundant, as they are present in roughly 40% of *B. subtilis* isolates. Transcriptomics data suggest that certain phage genes are silent during lytic cycle and activate only under specific growth conditions, pointing out towards physiological states of the host that could be controlled by the prophage. Further research with isogenic bacterial host lysogenized with different SPβ

viruses, demonstrates strong impact of these phages on physiology and ecology of their host species, modifying features such as growth rate, cell shape, colony morphology, sporulation dynamics or bacteriocin production and competitive fitness.

We believe, based on our observations so far, that SPβ viruses play an important role in physiology, ecology, and evolution of its host species, affecting traits that can be relevant for applications of *B. subtilis*. By reshuffling their genetic modules, SPβ viruses may escape domestication and accelerate evolution of the host.

O-7

Bacillus velezensis kin consortia able to form a positive biofilm with antagonistic activity against pathogenic bacteria

Virgile Guéneau[1,2], Laurent Guillier[3], Guillermo Jimenez[2], Julia Plateau-Gonthier[2], Marie-Françoise Noirot-Gros[1], Pascale Serror[1], Mathieu Castex[2] and **Romain Briandet**[1]

[1] *Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350, Jouy-en-Josas, France*

[2] *Lallemand SAS, 31702, Blagnac, France*

[3] *French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Laboratory for Food Safety, Salmonella and Listeria Unit, 94701, Maisons-Alfort, France.*

The use of antimicrobials contributes to resistance, environmental pollution, and undesirable microbial establishment. To address this, harnessing beneficial microorganisms as an alternative to chemical inputs has gained importance within the One Health concept. We propose a methodology utilizing phenotype analysis, High Content Screening Confocal Laser Scanning Microscopy (HCS-CLSM), genetically modified fluorescent strains, and image analysis for rational strain selection. This approach evaluated *Bacillus* spp. in multi-species biofilm models, revealing three *Bacillus velezensis* strains with strong antagonistic effects against pathogens. These strains formed a stable consortium, enhancing biofilm colonization without compromising their antagonistic capabilities. Kinetic HCS-CLSM studies demonstrated the significant antagonistic effect of *B. velezensis* against *Enterococcus cecorum*, following a lotka-volterra prey-predator model. Additionally, pre-established *B. velezensis* biofilms had a greater impact on *Salmonella enteritidis* growth compared to simultaneous introduction with the pathogen. This highlights the specific effect of *B. velezensis* biofilms on *S. enteritidis*. Overall, the study emphasizes the potential of specific *B. velezensis* strains in combating pathogens and forming resilient biofilm communities. The proposed methodology provides a systematic approach for strain selection, enabling the development of innovative alternative to chemical antimicrobial strategies.

O-8

Competition to commensalism, *Trichoderma harzianum* and *Bacillus velezensis* interaction is strongly cultural conditions dependent

B. Fiffani[1,2], F. Delvigne[1], V. Phalip[2] and **P. Jacques[1]**

[1] *Microbial Processes and Interactions, TERRA Teaching and Research Centre, Joint Research Unit BioEcoAgro UMRt 1158, University of Liège- Gembloux Agro-Bio Tech, Gembloux, Belgium*

[2] *Joint Research Unit BioEcoAgro UMRt 1158, University of Lille, Villeneuve d'Ascq, France*

Cocultures of two strains with potential biocontrol activity of plant diseases, *Trichoderma harzianum* IHEM5437 and *Bacillus velezensis* GA1, a strong antifungal lipopeptide producing strain, were performed in different nutritional conditions. In a rich medium, *Bacillus* completely inhibited *Trichoderma* growth. Using *Bacillus* mutant strains deficient in different secondary metabolites, we showed that iturin and fengycin are responsible for this antagonistic activity. A drastically different trend was observed in a medium where a nitrogen nutritional dependency is imposed. Indeed, in this minimum medium which contains nitrate as sole nitrogen source, a cooperation between the bacteria and the fungus is established. It is reflected by the growth of both species as well as the reduction of the expression of *Bacillus* genes encoding lipopeptide synthetases. Interestingly, the growth of the bacteria in the minimum medium was enabled by the amendment of the culture by the fungal supernatant, which, in this case, ensures a high production yield of lipopeptides. These results highlight, for the first time, that *Trichoderma harzianum* and *Bacillus velezensis* are able, in specific environmental conditions, to adapt their metabolism to grow together.

O-9

Molecular determinants of mosaic genomes resulting from interspecies natural transformation

Jonathan Lombardino[1], Tanya G. Falbel[1], Ananya Hota[2], and **Briana M. Burton**[1]

[1] *University of Wisconsin – Madison, Wisconsin, USA*

[2] *Howard University, Washington D.C., USA*

Although observations of horizontal gene transfer (HGT) events are common in the bacterial domain, one mechanism of HGT, natural transformation, has been refractory to unbiased genome-scale studies. Natural transformation involves exchange of nucleic acid without the requirement of mobile element mediators from the donor. As a result, genomes of natural transformants lack signature sequences for identification and tracking of donor nucleic acid segments. We present an unbiased genome-scale study of transfer events obtained from a diverse set of *Bacillus* donor species genomes transformed into *Bacillus subtilis* as a recipient. Analysis of the transfer events revealed previously unreported patterns of short, mosaic transfer events frequently linked in proximity. These events are enriched for orthologous regions of high identity, and likely contribute to transformation “hotspots” between divergent *Bacilli*. Comparing wild type and recombination mutant recipients, we define key molecular inputs to the structure of these mosaic transformant genomes, including contributors to the lengths of transfer events and the nature of the mosaic patterns. Together the results challenge traditional preconceptions of gene exchange during transformation and suggest transformable bacteria have broader access to allelic exchange and assortment of their genetic variation than previously appreciated.

O-10

Harnessing Bacterial Sociality: Understanding *Bacillus subtilis* - Pathogen Interactions for Effective Probiotic Applications

Eli Podnar[1], Katarina Šimunović[1], Tjaša Danevčič[1], Polonca Štefanič[1], Kraigher Barbara[1], Anja Klančnik[2], Sonja Smole-Možina[2], Hans Steenackers[3], Bram Lories[3], and **Mandić Mulec Ines[1]**

[1] University of Ljubljana, Biotechnical Faculty, Department of Microbiology, Ljubljana, Slovenia

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The global rise of antibiotic-resistant pathogenic bacteria in livestock and food processing environments has become a pressing concern, necessitating the search for alternative control strategies. Probiotics offer a promising solution. Our recent findings highlight *Bacillus subtilis* PS-216 as a potential probiotic candidate. Through *in vitro* studies, we have demonstrated that PS-216 efficiently inhibits a variety of pathogenic bacteria and disrupts preformed biofilms of the prevalent foodborne pathogen, *Campylobacter jejuni*, while exhibiting remarkable survival in harsh gastric conditions. Furthermore, when administered to broilers for 21 days, PS-216 spores in drinking water result in significant weight gain and a decrease in *C. jejuni* levels in the cecum.

Despite the growing popularity of *B. subtilis* strains as probiotics, the molecular mechanisms governing pathogen - *B. subtilis* interactions remain poorly understood. Recent results on interactions between *B. subtilis* PS-216 and foodborne pathogens (*C. jejuni* and *Salmonella* Typhimurium), in biofilms, will be presented. Results reveal the potent inhibitory effect of PS-216 on the growth of these two pathogenic bacteria. Notably, the combat outcome hinges on two antibiotic loci (*pks, bac*) responsible for the synthesis of bacillaene and bacylisin, respectively. Moreover, nutrient availability and interactions with the pathogen play a crucial role in modulating the P_{pksC} promoter activity, influencing interspecies competition dynamics. Interestingly, in nutrient-poor conditions, *S.* Typhimurium suppresses PS-216 spore development through a stress response-dependent pathway, revealing intriguing molecular determinants driving this interspecies contact-dependent conflict.

This study provides valuable insights into *Bacillus subtilis*-pathogen interactions, harnessing bacterial sociality for effective pathogen control in food systems.

O-11

Inactivation of the conserved protease LonA increases xylanase production in *Bacillus subtilis*

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Bacillus subtilis is widely used for the industrial-scale production of enzymes due to its capacity to efficiently secrete proteins. However, this efficiency is protein-dependent and varies dramatically. The molecular mechanisms underlying this variation are largely unknown. For example, while most commercially relevant enzymes have their production peak in the stationary phase, the secretion of the industrial important xylanase XynA begins already at the end of logarithmic growth and reduces in the stationary phase. The reason for this is not known. By using transcriptome (RNA-seq) and ribosome (Ribo-seq) profiling techniques, we were able to show that the reduction of XynA production in the stationary phase is neither a consequence of reduced transcription nor reduced translation, suggesting that intracellular proteolytic degradation might play a role. Indeed, when we deleted the conserved and general protein quality protease encoding gene *lonA*, we found that the production of XynA continues in the stationary phase leading to an improved production of this protein by more than 50 %.

O-12

From the Outer Space to the Inner Cell: Global Absolute Quantification of Extracellular, Membrane and Cytosolic Proteins in *Bacillus subtilis*

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Absolute quantification of proteins is a prerequisite to model biological processes in the context of systems biology and for the global determination of protein stoichiometries. However, data obtained with current approaches for proteins from compartments other than the cytosol fall short of requirements in this field. Here we present new approaches for large-scale absolute quantification of two challenging subset of proteins, namely membrane and extracellular proteins. The nature of these proteins requires enrichment and concentration steps during sample preparation that must be quantified to obtain correct protein amounts reflecting native protein abundance in the cell culture. The straightforward protocols for absolute quantification of membrane-associated and extracellular proteins use appropriate standard proteins to calculate correction factors that reveal the extend of protein enrichment and concentration during sample preparation. Shotgun mass spectrometric data is subsequently calibrated using commercially available universal protein standards.

The resulting data sets provide new insights into protein abundances in different cellular compartments and the extracellular medium. Thus, alterations in the arrangement of membrane proteins and putative membrane complexes could be sensitively detected. In addition, accurate determination of changes in protein abundances at different locations inside and outside the cell contributes to a deeper understanding of protein secretion. This simple and inexpensive method for quantitative proteomic studies can be applied to the entire spectrum of physiologically relevant conditions, ranging from environmental stresses to biotechnological production of small molecules and proteins.

O-13

Harnessing the metabolic versatility of engineered *B. subtilis* 168 for enhanced production of the biopolymer poly-γ-glutamic acid

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For several decades, the synthesis of the poly(amino acid) poly-γ-glutamic (γ-PGA) acid has been studied extensively as a natural product across various *Bacillus* species. Depending on the cultivation conditions and the genetic circuit selected for γ-PGA production, the biopolymer exhibits varying molecular weights and distributions of D- and L-glutamic acid. This yields diverse product characteristics, making γ-PGA an excellent candidate for utilization in multiple fields of application, ranging from medical- to agricultural use. However, much is still to be discovered regarding the complex interplay of carbon- and nitrogen metabolism during glutamate polymerization. Therefore, we reached out to study different metabolic engineering targets, aiming to unravel their potential involvement in biopolymer synthesis while also increasing product titer and yield. First, we enabled γ-PGA synthesis in the domesticated, non-γ-PGA producing laboratory strain *B. subtilis* 168 by a promoter exchange and subsequently streamlined the γ-PGA synthesis pathway by multiple gene deletions associated with the organism's overflow metabolism. With this strategy, the γ-PGA titer was increased by 41%, however a severe accumulation of pyruvate was observed in combination with increased acetate production. These obstacles were individually tackled by multiple gene deletions and insertions to redirect glycolytic flux towards the TCA cycle, further improving the strain's performance. Moreover, we constructed a strain lacking the regulator of carbon catabolite repression CcpA, therefore enabling the simultaneous consumption of substrate mixtures. Since CcpA has a pivotal role in glutamate homeostasis and the deletion results in glutamate auxotrophy, we investigated strategies to reenable glutamate *de novo* synthesis.

O-14

Long DNA synthesis using *Bacillus subtilis*

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Recent expansion of bio-economy society has led to requirement of synthesis of longer DNA and construction of more complex DNA library than conventionally constructed using *Escherichia coli*. Over the years, I have developed a variety of *B. subtilis*-based methods for manipulating long DNA that meet these needs. One of them, OGAB method, uses a plasmid transformation system of *B. subtilis* to synthesize up to 100 kb of long DNA by assembling as much as 50 of DNA fragments in one step. In 2016, I started a long DNA synthesis biofoundry at Kobe University for the purpose of verifying the possibility of social implementation of this method. This facility is capable of carrying out all processes starting from the chemical synthesis of the DNA fragments used in OGAB method and finally confirming the sequence of the synthesized long DNA. It had constructed a lot of long DNAs for several national projects of Japan on request. Furthermore, as part of the application of long DNA constructed by OGAB method, I devised an efficient long DNA combinatorial library construction method called Combi-OGAB method using the principle of OGAB method. In 2017, based on these technologies, I started a venture business Synplogen. This company is not only engaged in long DNA synthesis service, but also conducts research and development of virus vectors for gene therapy by applying OGAB method. I will introduce an overview of long DNA synthesis technology using *B. subtilis* and its application.

O-15

Production of 3-hydroxypropionic acid from methanol in recombinant *Bacillus methanolicus* MGA3

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The production of platform chemicals relies on chemical synthesis processes, which have negative impacts on the environment. Although microbial cell factories offer an environmentally friendly alternative to produce value-added compounds, conventionally used production hosts grow on plant-based feedstocks such as molasses and sugarcane, which compete with the food industry for resources. Next-generation feedstocks such as methanol are therefore attractive alternatives.

Bacillus methanolicus MGA3 is a thermophilic methylotroph with increasing industrial relevance and an extensive toolbox for genome editing and metabolic engineering. It was engineered for production of various compounds, such as acetoin or amino acid derivatives, cadaverine and γ-aminobutyric acid.

3-hydroxypropionic acid (3-HP) is a pyruvate derivative used for production of various chemicals. The conversion of malonyl-CoA to 3-HP is catalysed by the enzyme malonyl-coenzyme A reductase (MCR). *Chloroflexus aurantiacus* possesses a gene encoding a bifunctional MCR which catalyses a two-step conversion of malonyl-CoA to 3-HP, while other species such as *Saccharolobus solfataricus* and *Metallosphaera sedula* possess two genes encoding separate enzymes: MCR, and malonic semialdehyde reductase (MSR). Here, we tested *B. methanolicus* MGA3 strains heterologously overproducing MCR or MCR together with MSR from different donors, and a split version of MCR from *C. aurantiacus*. The activity of the enzymes in the recombinant *B. methanolicus* strains corresponded to 3-HP accumulation. The highest activity (0.6 U mg-1) and production (0.09 g/L) was displayed in the strain producing *M. sedula*-derived MCR and MSR. The next step in improvement of the 3-HP-producing strains will be co-factor regeneration by the activity of various transhydrogenases.

O-16

SEAM-OGAB: Enabling rapid assembly of chimeric Non-Ribosomal Peptide Synthetase Gene Clusters in *Bacillus subtilis*

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Microorganisms are prolific producers of essential therapeutic agents, many of which undergo biosynthesis through modular enzymatic assembly line processes, involving polyketide synthases, non-ribosomal peptide synthetases (NRPSs), or a combination of both. Modifying these biosynthetic gene clusters offers a promising route to discover bioactive natural products too complex for chemical synthesis. However, current engineering techniques face significant challenges due to the substantial size (10-150 kb), prevalence of sequence repeats, and difficulty in defining an optimal fusion point for manipulation.

To address these issues, our study introduces the "Seamed Express Assembly Method (SEAM)" coupled with the Ordered Gene Assembly in *Bacillus subtilis* (OGAB) to reconstruct fully functional NRPS gene clusters. The SEAM-OGAB method strategically introduces restriction enzyme sites as seams at module borders within the C-A linker, facilitating efficient gene assembly.

Efficiency of SEAM-OGAB was demonstrated by constructing the *ppsABCDE* NRPS gene cluster (38.4 kb), producing plipastatin, a cyclic decapeptide in *B. subtilis*. Despite challenges posed by extensive direct repeat sequences in the plipastatin gene cluster, introducing amino acid level seams into the C-A linker did not hinder NRPS function, resulting in successful plipastatin production.

SEAM-OGAB offers a distinct advantage over previous assembly methods by enabling combinatorial shuffling of NRPS gene modules, allowing for rapid construction of chimeric NRPSs. To demonstrate, we successfully swapped plipastatin NRPS and surfactin NRPS modules, resulting in the generation of novel lipopeptides in *B. subtilis* through chimeric gene assembly. The SEAM-OGAB method thus presents a promising tool for accelerating the development of complex bioactive compounds with potential therapeutic applications.

O-17

Adapted cell factories for production of biosurfactant on cheap substrates.

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We are interested in lipopeptides produced by *Bacillus* strains, such as surfactins, fengycins and iturins. These lipopeptides are composed of a cyclic peptide chain of L and D-amino acid residues linked to a linear b-hydroxy or b-amino fatty acid chain. These molecules have shown various antimicrobial, antitumoral, antibiofilm activities and environmental applications. Unfortunately, due to the cost of the culture medium, the cost price of these lipopeptides is yet too high.

The aim of our project is to adapt *Bacillus* cells to different cheap culture media to produce lipopeptides in high yield. Two different cheap substrates were chosen. One is a rich media that is a by-product obtained after bioethanol distillation and, the second one is a poor substrate, composed of the maize and miscanthus straws.

Catabolic pathways involved in the use of the different carbon (glycerol, xylose, …) and nitrogen substrates present in the tested cheap culture media were analyzed to detect limiting steps in their use by the *Bacillus* cells.

Several metabolic engineering strategies are coupled, one is the re-direction of carbon flow (deletion of competitive pathways) toward the metabolic pathway of the production of lipopeptides and the second is a specific genetic adaptation to the cheap substrates to produce specific enzymes to degrade substrates and increase carbon and nitrogen sources assimilations.

Our results show, (a) that is possible to grow *Bacillus* strains and produce lipopeptides from all these crude cheap media and (b) the genetic modification to redirect the carbon flow, coupled with the production of specific enzymes to assimilate carbon and nitrogen sources increase the production of lipopeptides.

O-18

Continuous culture in custom computer-controlled mini-bioreactors

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Molecular microbiologists seldom use bioreactors despite the interest of these continuous culture systems. In order to facilitate their implementation, we developed a new modular system of computer-controlled mini-bioreactors which takes advantage of the opportunities offered by small-scale digital fabrication (Fablabs) and the spread of powerful programmable and connected microcontrollers that led to the Internet of Things (IoT).

The combination of modularity and small culture volumes makes it possible to implement complex experimental designs involving real-time monitoring and coordinated control of several bioreactors. Envisioned applications range from experimental and directed evolution to study of physiological adaptation. As a proof of concept, we applied our modular system to maintain *Bacillus subtilis* cultures in various conditions: from simple parallel chemostats to a cascade of two chemostats that maintains a continuous culture of phages. Genomic data on the evolution of resistance to increasing ethanol stress will be presented.

O-19

Development of an ultra-transformation system in *Bacillus subtilis* 168

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When grown in LB rich medium containing a metal ion, the transformation frequency of *Bacillus subtilis* 168 using PCR-generated donor DNA, but not homologous chromosomal DNA, was considerably enhanced at early stationary phase. The transformation frequency was improved by removing both the template phage, SPβ, and the defective phage, PBSX, from the chromosome of strain 168. The P_{mtlA}-*comKS* cassette (1) was integrated into the *leuB* locus of the chromosome of the double-deletion mutant of ΔSPβ and ΔPBSX, resulting in an ultra-competent SAK8 ΔSPβ ΔPBSX Δ*leuB*::P_{mtlA}-*comKS trpC2* strain. Induction of both *comK* and *comS* increased the transformation frequency of SAK8 for PCR DNA by at least 20-fold. In this ultra-transformation system, when transforming with PCR DNA carrying the *spo0A5oc* (Lys5 AAA->ocher UAA) mutation or the 31-based pair deletion mutant of *spo0A* (*spo0AΔ31*), a transformation frequency of approximately 30% was obtained. These results indicate that mutants with either a single base change or short deletions can be constructed without any selection. Furthermore, quintuple transformants were obtained simultaneously, using five different PCR DNAs carrying *cat*, *erm*, *spc*, *spo0A5oc* and *trpC2*$^{+}$ markers.

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O-20

A review of the regulatory frameworks for exploiting *Bacillus* species

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The genome sequencing landscape used by the *Bacillus subtilis* community to sequence the genome of this bacterium is unrecognisable today. It involved more than 30 laboratories worldwide using a variety of cloning and sequencing strategies. Twenty-five years later, just as the power of genomics has dramatically improved our understanding of individual organisms, so too has it revolutionised our understanding of taxonomic relationships among organisms. It has long been recognised that the genus *Bacillus* is derived from more than one common evolutionary ancestor. Until recently, the *Bacillus* consisted of ~280 validly published species, including two important species clades; the 'Subtilis clade' that includes the type strain of the species and the 'Cereus clade' that includes important human and animal pathogens. Because the type strain for the genus is a member of the Subtilis clade, it would be expected that the members of the Cereus clade would be transferred to a new genus. However, the International Code of Nomenclature of Prokaryotes (ICNP) does not recommend this transfer if the renaming confusion could endanger human health.

A genomic study of >300 ***Bacillaceae*** species resulted in a plethora of new genera. This causes considerable problems when defining 'Generally Recognised a Safe' (GRAS) and 'Qualified Presumption of Safety' (QPS) status. I will discuss the implications of strain identification in relation to antibiotic resistance and production. This is important given the enormous potential of members of the ***Bacillaceae*** for environmentally compatible and fossil fuel replacing technologies associated with crop protection, pre- and probiotics, biofuel production, biocatalysis etc.

O-21

Understanding redox metabolism in *Bacillus licheniformis* during heterologous protease production under industrially relevant conditions

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Bacillus licheniformis is well-known for its remarkable protein secretion capacity, explaining its extensive use as an industrial host for the biotechnological production of enzymes such as alkaline proteases, amylases, lipases, and cellulases[1].
A comprehensive understanding of its microbial central carbon metabolism is crucial to elucidate the complex relationship between cellular physiology and enzyme secretion capacity[2].
Pioneering studies have revealed initial interesting findings on the metabolic state of *Bacillus* during enzyme production [3]. However, these studies were conducted under simplified and artificial steady-state conditions, which only partially overlapping with real industrial settings limiting their value for analyzing industrial enzyme production, typically conducted in large-scale fed-batch fermentations, characterized by high cell density and dynamic shifts in cell growth, nutrient supply, and product secretion.
In this study, we combined endo- and exometabolomics, enzyme activity measurements, and a novel ^{13}C metabolic flux approach. The latter used the ^{13}C-labelling pattern of free intracellular amino acids, capable to precisely resolve dynamic flux changes.
This newly developed approach allowed to describe the adaptations in carbon, energy, and redox metabolism of protease-producing *B. licheniformis* during different stages of the fermentation process.
For the first time, we could monitor short-term shifts in metabolic pathway activities during biomass growth and early and late protease production stages.
The additional assessment of the activity and co-factor specificity of NADPH-forming dehydrogenases, as well as the balancing of reducing equivalents, revealed a shortage in the supply of redox power during important process phases.

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O-22

Genetic modification of *Bacillus subtilis* for improvement of antimicrobial lipopeptide biosynthesis

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Bacillus species are widely recognized for their ability to function as efficient cell factories, producing valuable secondary metabolites such as surfactin, fengycin, and iturin families of cyclic lipopeptides. These lipopeptides possess diverse applications across multiple industries. Surfactin, a robust biosurfactant produced by *Bacillus* species, has been extensively studied, while iturins and fengycin are known for their potent antifungal properties. However, the natural production levels of lipopeptides by wild-type *Bacillus* strains do not meet industrial requirements, necessitating genetic modifications to develop high-yield lipopeptide producer strains and the implementation of bioprocess engineering techniques to enhance antimicrobial lipopeptide production. To engineer *Bacillus* strains with high lipopeptide yields, it is crucial to understand the molecular mechanisms that regulate surfactin synthetase, particularly the *srfA* operon, and its interaction with global regulators involved in cell differentiation, including Spo0A, AbrB, and DegU. In our research group, we have achieved surfactin production of up to 26.5 g/L through high cell density fed-batch fermentations, which is the highest reported yield in scientific literature. Furthermore, we have constructed a constitutive plipastatin mono-producer strain to investigate fengycin production in *Bacillus subtilis*. Our findings indicate that full plipastatin production relies on the expression of surfactin synthetase or some of its components, *degQ* expression, and activation of the DegU-P response regulator. In conclusion, there are promising prospects for advancing lipopeptide production through a combination of genetic modification and bioprocess engineering strategies, enabling their widespread utilization in various industrial applications.

O-23

Application of Combi-OGAB: fine-tuning growth-phase dependent promoters in biosynthetic gene cluster to create heterologous lethal antibiotic producer.

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Combi-OGAB (**Combi**natorial-**O**rdered **G**ene **A**ssembly in **B**acillus subtilis) is a powerful technology to construct diverse DNA library in one pot and to accelerate screening the desirable clone from the library. In this study, we demonstrate Combi-OGAB can realize to create heterologous lethal antibiotic producer. When biosynthetic gene cluster (BGC) is identified by metagenome and the produced antibiotic shows toxic to host bacteria, the heterologous producers cannot be created. To overcome this, we utilized Combi-OGAB to mono-cistronically fine-tune growth-phase dependent promoters in antibiotic BGCs to optimize production phase to suitable phase to increase productivity and phenotypic resistance. We, here, selected *Bacillus subtilis* and Gramicidin S (GS) as a host bacterium and an antibiotic agent, respectively. Combi-OGAB screening cycles brought the GS producer with about 30 mg/L, which is over 30-fold higher than lethal concentration of GS against this producer. Selected three promoters have different transcription timing in growth, and their transcription strength is not necessarily strong. These results suggest that our strategy can optimize production phase of lethal antibiotic GS against *B. subtilis* to suitable phase for maximized phenotypic resistance with host bacteria's nature to create heterologous lethal antibiotics producers.

O-24

Post-translational secretion stress regulation in genome-reduced *Bacillus subtilis*

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The Gram-positive bacterium *Bacillus subtilis* is an attractive host for industrial enzyme production. Researchers have been improving this cell factory for decades using different strategies. In recent years, genome-reduced strains of *B. subtilis* have become a popular area of research. For instance, the engineered strain PG10, lacking 36% of the parental genome, was shown to be superior in the production of staphylococcal antigens compared to the parental strain 168 (Aguilar Suárez et al., 2019). Another study comparing the 168 strain and the midi*Bacillus* strain IIG-Bs-27-47-24, which lacks one third of the parental genome, revealed that the so-called secretion stress responses are altered in genome-reduced cells producing the staphylococcal protein IsaA (Aguilar Suárez et al., 2021). In the present study, we show that the serine proteases AprX and AprE degrade the quality control proteases HtrA and HtrB, which explains the elevated levels of HtrA and HtrB in genome-reduced *B. subtilis* strains. The roles of AprX and AprE as determinants for the extracellular HtrA levels were identified by investigating the phylogeny of genome-minimized strains, and verified by individual gene deletions and complementation studies. Furthermore, the *htrA* promoter activity in the parental strain 168 and several genome-reduced strains was measured in real-time to further elucidate the regulation of *htrA* transcription. Altogether, we conclude that the expression of HtrA and HtrB is not only subject to regulation at the transcriptional level, but also at the post-translational level. The post-translational regulation is exerted by the serine proteases AprX and AprE.

O-25

Engineering of Bacillus cell factories for recombinant protein production

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Bacteria are widely used as cell factories for the production of enzymes and pharmaceutical products that, respectively, replace wasteful chemical processes and support human health and wellbeing. However, not all societal demands can be adequately met with the current bacterial production strains. Genome engineering can solve this problem through the creation of novel strains with desired properties. This view is underscored by our studies on genome-minimized strains of the bacterial cell factory *Bacillus subtilis*, which is used to produce enzymes and vitamins at industrial scale. New genome-reduced strains have a higher capacity for the synthesis and secretion of difficult-to-produce proteins, while suffering less from detrimental 'protein production stresses'. In addition, such strains show an enhanced capacity for the production of disulphide-bonded proteins, including engineered antibodies. Lastly, genome-engineered strains may deliver more protein products, while producing less biomass. Altogether, our findings highlight the great potential of genome engineering to develop new-generation bacterial cell factories for industrial applications that satisfy the ever-increasing demands for high quality enzymes and pharmaceutical proteins.

O-26

RNase Y autoregulates its synthesis in *Bacillus subtilis*

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Control of gene expression requires the instability of messenger RNA. In *Bacillus subtilis*, RNase Y is the major decay-initiating endoribonuclease affecting global mRNA stability. There is thus a need to keep the activity of RNase Y in a tightly controlled steady supply. Here, we show how this key enzyme regulates its own synthesis by modulating the longevity of its mRNA. Autoregulation is achieved through cleavages in two regions of the *rny* (RNase Y) transcript : i) within the open reading frame, immediately inactivating the mRNA for further rounds of translation; ii) in the *rny* 5′ UTR, creating entry sites for the 5′ exonuclease J1. This links the functional inactivation of the transcript by RNase J1 to translation efficiency, depending on the ribosome occupancy at the translation initiation site. By these mechanisms, RNase Y can initiate degradation of its own mRNA when the enzyme is not occupied with the degradation of other RNAs and thus prevent its overexpression beyond the needs of RNA metabolism.

O-27

Functional analysis of the small regulatory RNA S313 of *Bacillus subtilis*

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Small regulatory RNA (sRNA) molecules play increasingly recognized roles in bacterial gene regulation, especially in adaptive responses to changing environmental conditions. However, the specific biological functions of many sRNA molecules remained enigmatic. In the present study, we addressed the role of the sRNA S313 of *Bacillus subtilis*. Our investigation was motivated by a genome-wide sRNA target prediction, which suggested a role for S313 in modulating the expression of the elemental iron transport system EfeUOB. Here, S313 was predicted to target the *efeU* transcript. Accordingly, the absence of S313 led to a specific growth phenotype that was also observed for *efeU* mutant strains when introduced into medium lacking NaCl. This phenotype is characterized by stalled growth, lysis and subsequent recovery of the S313 or *efeU* mutant bacteria. Importantly, in absence of NaCl, the S313-deficiency was shown to have far-reaching consequences, as evidenced by major rearrangements in bacterial metabolism and decreased resistance to antibiotics. However, efforts to reintroduce S313 for complementation of the observed phenotypes proved unsuccessful, suggesting that deletion of S313 led to second site suppressor mutations. To identify such mutations, whole-genome sequencing was performed on independently obtained S313 deletion mutants. This revealed a frameshift mutation in the *efeU* gene that was consistently observed for all sequenced S313 mutants. Moreover, sequencing revealed a consistent 38-bp deletion in the PhoP-P binding site upstream of the promoter of the *pstS* gene for a phosphate transporter. Collectively, these findings indicate a critical role for S313 in the iron and phosphate metabolism of *B. subtilis*.

O-28

Transcription attenuation as a source of genetic noise in *Bacillus subtilis*

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The conversion of the information encoded within genes into physiological output (gene expression) is inherently stochastic, resulting in cell-to-cell heterogeneity (noise) within a population of genetically identical cells. In bacteria, noisy gene expression could serve as a bet-hedging strategy to ensure the survival of pre-adapted individual cells during fluctuating unfavorable conditions. Hitherto, research into the sources of gene expression noise has mainly focused on the mechanisms of transcription initiation; however, the impact of regulatory events after transcription has been initiated is largely unknown. Transcription attenuation, i.e., the premature termination of transcription after initiation, is an RNA-based mechanism of gene regulation in bacteria that controls the expression of genes required for amino and nucleic acid biosynthesis. In this study, we analysed the attenuation-controlled transcription of the tryptophan (*trp)* operon in *Bacillus subtilis*. We quantified individual molecules of *trp* mRNA in single cells using single-molecule RNA fluorescence in situ hybridisation (RNA FISH) and determined the level of noise of *trp* expression when cells were exposed to varying tryptophan concentrations. In parallel, we compared single-cell to bulk-level *trp* expression measurements from traditional reporter assays. Our data provides new insights into the heterogeneity of *trp* expression within *B. subtilis* populations and details of expression states of individual genetically identical cells subjected to the same environment, which remain obscured in traditional bulk-level measurements.

O-29

Regulated translation arrest: a regulation mechanism of genes for bacterial protein localization machinery

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Regulatory arrest peptides exert various cellular functions by mechanisms involving regulated translational arrest. A class of bacterial arrest peptides, also known as monitoring substrates, are encoded upstream of the *sec* or *yidC* genes, which encode a component of protein localization machinery, and feedback-regulate their gene expression. In this study, we addressed how universal the arrest peptide-mediated gene regulation for protein localization machinery is in bacteria. To identify putative monitoring substrates, we conducted global bioinformatic screening through 30,000 bacterial genomes and subsequent in vivo and in vitro analyses to identify novel arrest peptides encoded upstream of *sec*/*yidC* genes from a wide range of bacteria. These analyses led to the identification of more than ten arrest peptides. Some arrest peptides appeared to share a similar amino acid sequence, such as R-A-P-P, suggesting that a specific sequence could serve as a common evolutionary seed that could overcome the species-specific structure of ribosomes to evolve arrest peptides. We also identified some arrest peptides having distinct arrest-inducing sequences. Our study highlights a universally repeated pattern of evolution, in which many different bacteria have evolved Sec/YidC-related arrest peptides likely for regulatory purposes, resulting in a patchy but widespread phylogenetic distribution. We propose that regulated translation arrest is a universal means by which bacteria regulate the expression of genes for components of protein localization machinery.

O-30

6S RNAs in *Bacillus subtilis* – more than simple transcription inhibitors

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Bacterial 6S RNAs regulate transcription via binding to the active site of RNA polymerase (RNAP) holoenzymes (1). *Bacillus subtilis* expresses two 6S RNA paralogs, 6S-1 and 6S-2 RNA, whose gene knockouts lead to different phenotypes in a wild-type strain (NCIB 3610): The ∆6S-1 RNA strain grows to lower optical density during extended stationary phase, while the ∆6S-2 RNA strain shows derepressed biofilm formation, retarded swarming activity and accelerated spore formation. The ∆6S-1&2 double deletion strain displays prolonged lag phases of growth under oxidative, high salt and alkaline stress conditions, in addition to decelerated spore formation (2). We performed transcriptome (RNA-seq, qRT-PCR) and proteome (MS) analyses to understand these phenotypes. The interaction of 6S-1 and 6S-2 RNAs with different RNAP holoenzymes (σ^A, σ^B, σ^D or σ^F) was studied *in vitro* by 6S RNA inhibition of transcription at strong and weak DNA promoters. 6S-1 RNA was found to bind to σ^B- and σ^F-RNAP, though weaker than to σ^A-RNAP, but not to σ^D-RNAP. 6S-2 RNA was found to be an effective transcription inhibitor only at weak σ^A-depend. promoters. We further show that σ^A with domain 4 and C-tail deletions still maintains the ability to initiate DNA transcription and to bind 6S-1 RNA, suggesting that this part of the housekeeping sigma factor is less essential in *B. subtilis* than in *E. coli*. Finally, we studied processing/decay of 6S-1 and 6S-2 RNAs in *B. subtilis*, revealing a key – but mechanistically different – role of RNase J1 in the metabolism of both 6S RNA paralogs.

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O-31

Small subunits of RNA polymerase affect sporulation in *Bacillus subtilis*

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Sporulation, the ultimate response to stress in *Bacillus subtilis*, depends on temporally and spatially regulated gene expression mediated by RNA polymerase (RNAP), the central enzyme of transcription, and a cascade of alternative σ factors. Here we describe the effects of small, non-essential subunits of RNAP, δ and ω, on sporulation in the model Gram-positive bacterium *B. subtilis*. A comprehensive set of experiments was performed including deletion analysis, transcriptome and proteome characterization, microscopy, and *in vitro* mechanistic studies. The experiments revealed synergistic interplay between δ, ω and the RNAP core and σ factors, and showed essentiality of δ and ω in early stages of sporulation for stimulation of gene expression by the master regulator of this process, Spo0A. A model of the involvement of δ and ω in the sporulation cascade will be presented and discussed.

O-32

Biofilming beyond *B. subtilis*

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Biofilms represent a collective mode of microbial growth in which cells are embedded in a complex extracellular matrix of proteins, polysaccharides, and other components. Biofilms are thought to provide a unique and protective environment for cells. *B. subtilis* is arguably the most established model to study bacterial biofilms growing on solid media. The intricacies of its patterns are both beautiful and intriguing and there is growing evidence for complex differentiation and embryo-like features within these structures. Inspired by these developments, we are using *Escherichia coli* (*E. coli*) as an alternative model to study cellular differentiation in biofilms while harnessing the versatile genetic resources available for this organism. We are doing time-lapse imaging of *E. coli* biofilms (biofilming) to capture the growth and developmental patterns as well as to visualize spatio-temporal changes in cellular metabolic activity using fluorescent reporters. I would like to present some of our early-stage findings regarding such patterns of growth and gene activity and contrast our results with those that have been obtained by others using *B. subtilis*. I'd also like to discuss the parallels and some possible differences between these two bacterial models and how together they may reveal further unsuspected complexity of cellular activity in biofilms.

O-33

Molecular mechanisms of resistance of *Bacillus pumilus* 25 isolated from ISS to antibiotics and oxidative stress

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Spore-forming bacteria are residents of the International Space Station (ISS) and can be potential bio-destructors of structural materials leading to malfunctions of various equipment (1). These bacteria show increased resistance to various stress factors. The molecular mechanisms of resistance of ISS bacterial strains to stress are poorly understood. *Bacillus pumilus* 25 strain was isolated as part of the sanitary and microbiological monitoring of the environment on the ISS. The aim of the work was to determine the molecular mechanisms of resistance of the *B. pumilus* 25 strain to oxidants, antibiotics, as well as to analyze the expression levels of genes associated with the cellular response to DNA damage and the level of hydrogen sulfide production. The strain was identified using 16S RNA sequencing and MALDI-TOF analysis. Stress and antibiotic resistance were determined using standard microbiological methods. Gene mRNA levels were measured by real-time PCR. The results show that *B. pumilus* 25 exhibits increased resistance to oxidative stress, such as hydrogen peroxide 35%, cumene hydroperoxide (100%) and tert-butyl hydroperoxide (100%) and to most antibiotics tested: ampicillin, fosfomycin, chloramphenicol, zeocin, nalidixic acid. The toxic effects of nalidixic acid and zeocin are associated with the formation of double-stranded breaks in DNA and chromosome fragmentation (2). We suggested that resistance to these antibiotics may be associated with the increased activity of the DNA double-strand break repair systems. Indeed, in the *B. pumilus* 25 strain we found increased expression of the ku and ligD genes encoding key components of the non-homologous end joining system by an average of 9-11 times as compared to the “terrestial” strain (3). Resistance to antibiotics and oxidizing agents may also be associated with increased production of hydrogen sulfide and antioxidant defense enzymes, catalases and superoxide dismutases, as we previously showed in other strains isolated on the ISS (4,5).

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O-34

Guanosine toxicity is associated with DNA damage and prophage activation in *Bacillus subtilis*

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Guanosine triphosphate (GTP) is an essential nucleotide that supports a wide range of cellular biosynthetic and bioenergetic processes. In bacteria, GTP can be synthesized *de novo* or through nucleobase salvage pathways, both of which are inhibited by the nucleotide alarmone (p)ppGpp to control GTP homeostasis. In the absence of (p)ppGpp, bacteria can accumulate excess GTP leading to cell death through unknown mechanisms. Here we report that this “death by GTP” is associated with DNA damage and activation of prophages SPβ and PBSX via the SOS response in *B. subtilis*. We found that the expression of LexA-repressed SPβ and PBSX operons as well as the viral quorum-sensing regulator *aimR* were induced in cells under guanosine toxicity. In addition, production of SPβ was detected in guanosine-killed populations, and lethality due to GTP excess was reduced by ~10-fold in mutants lacking the prophages. Furthermore, the prophage-associated lethality requires the SOS response, as a prophage-harbouring strain with non-cleavable LexA had disrupted prophage induction by guanosine and increased survival to the same extent as the prophage-removed mutant. Finally, formation of RecA-GFP foci was greatly increased in cells with excess GTP, suggesting that deleterious accumulation of GTP leads to DNA damage as a trigger to prophage activation. Taken together, our work suggests that (p)ppGpp contributes to reduce DNA damage and prophage activation by preventing GTP overaccumulation.

O-35

Gingko Biloba leaf extract inhibits *Bacillus subtilis* biofilms and alters biofilm morphology in static and flow biofilm systems.

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Biofilm development is fundamental for bacterial resilience. Biofilms represent a problem across clinical and environmental settings, due to their resistance to antimicrobials. In this study, the antibiofilm effect of *Gingko biloba* leaf extract is studied in both static and flow cell *Bacillus subtilis* biofilms.

Using standard microbiological techniques, fluorescent imaging and molecular techniques, it was possible to study the behaviour of *B. subtilis* biofilms at different concentrations of *G. biloba*. Results show that the extract has an antibiofilm activity rather than bactericidal, inhibiting biofilm formation but having a smaller impact on bacterial growth. The biofilm inhibitory effect applies to both agar and flow cell biofilms. Impacts on biofilm morphology are also observed. Confocal laser scanning microscopy reveals a concentration-dependent effect on pellicle biofilm development and morphology, resulting in highly organized filaments. Initial experiments to assess the effect of flow alone on biofilms show the development of multiple multicellular filaments, with rope-like structures and cellular aggregates. Fluorescent microscopy also shows localized fluorescence in the centre of the bacterial cells, indicating a change in DNA expression.

To further investigate the effect of *G. biloba* on genetic expression, RT-qPCR was performed. Data analysis indicated an initial increase in genes responsible for autolysis, sporulation, competence and biofilm development at 50 µg/mL. This was followed by a sharp decrease in genetic expression of the above genes at 75 µg/mL and more, suggesting that *G. biloba* modulates biofilm development at a genetic level.

While biofilms still remain a challenge, these findings suggest that *G. biloba* could be used as a safe antibiofilm agent, including biofilms that develop under a fluid flow.

O-36

NaCl induced lifestyle switching from sessile to motile state in *Bacillus subtilis*

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Bacteria often reside in a multicellular community embedded within a self-produced matrix known as biofilm. Numerous environmental and chemical cues such as pH, temperature, nutrient availability, salt concentration, and metabolites produced by microorganisms can influence biofilm's biophysical and biochemical properties. However, the underlying molecular mechanisms governing the resultant behaviour remain elusive. Here, we outline the effect of NaCl (at physiologically relevant concentration) on biofilm dynamics in an undomesticated strain of *Bacillus subtilis*. Increase in cytosolic concentration of NaCl leads to decrease in robustness of pellicle and increase in lateral expansion of macrocolony. Gene expression analysis shows upregulation of motility-related genes and downregulation of biofilm matrix genes which is mediated through a transcription factor *sigD*. Moreover, NaCl-induced effects, such as changes in pellicle architecture, lateral expansion rate, and exopolysaccharide production, were nullified using Δ*sigD* and Δ*srfAC* mutants. Increase in NaCl concentration proved to result in switching *B. subtilis* from its sessile to motile state. At cellular level, bacteria often display super-diffusive motion spanning nanoseconds, even in absence of NaCl. However, upon assessing the temporal changes brought about by NaCl, we noticed a fascinating transition from sub-diffusive behaviour of individual bacterial cells to rapid diffusive behaviour. In addition, uptake of NaCl was found to reduce heterogeneity and increase flagellar production in a subpopulation of biofilm, which corroborates with its effect in

causing increase in motility. These findings imply bacteria may have competitive advantages in the new environment due to changes in cellular dynamics brought on by NaCl.

O-37

DnaA-boxes distant from the unwinding site promote helicase loader recruitment in a bipartite chromosome origin

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The duplication of genetic material is essential to life. In bacteria DNA replication begins with the master initiator DnaA binding to DnaA-box sequences in the chromosomal origin, followed by DNA strand separation and replicative helicase loading. Despite the universal presence of multiple DnaA-boxes in bacterial origins, the functional and spatial requirements underlying their number and activity is not understood. This is especially true for bipartite origins such as found in *Bacillus subtilis*, where an additional cluster of 17 DnaA-boxes (*incAB)* is essential for replication despite being spatially separated (~1300bp) from the unwinding site (*incC,* 7 boxes). We genetically investigated the spatial and functional relationship between *incC* and *incAB in vivo*. Surprisingly, we find that *incAB* still promotes replication initiation when transposed 2.1 megabase pairs away from *incC*. Following DnaA, in *B. subtilis*, the essential auxiliary initiators DnaD and DnaB are recruited to the origin for helicase loading. Using ChIP we show that the deletion of *incAB* results in a decreased enrichment of auxiliary initiators at *incC*, suggesting their delivery to the unwinding site by *incAB*. Fittingly, we identify a mutation in *dnaB,* increasing the initiator's affinity for DNA and DnaD, to suppress the deletion of *incAB*. Finally, we show that the *dnaB* suppressor allows the extreme minimization of the origin to just three DnaA-boxes as opposed to the native 24. Altogether, our results uncover surprising flexibility in the spatial layout of a bacterial origin and provide new insight into *oriC* function and minimal sequence requirements for bacterial DNA replication initiation.

O-38

Molecular motor tug-of-war regulates elongasome cell wall synthesis dynamics in *Bacillus subtilis*

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Most rod-shaped bacteria elongate by inserting new cell wall material into the inner surface of the cell sidewall. This is primarily performed by a highly conserved protein complex, the elongasome, which moves processively around the cell circumference and inserts long glycan strands that act as barrel-hoop-like reinforcing structures, thereby giving rise to a rod-shaped cell. However, it remains unclear how elongasome synthesis dynamics and termination events are regulated to determine the length of these critical cell-reinforcing structures. To address this, we developed a method to track individual elongasome complexes around the entire circumference of *Bacillus subtilis* cells for minutes-long periods using single molecule fluorescence microscopy. We found that the *B. subtilis* elongasome is highly processive and that processive synthesis events are frequent terminated by rapid reversal or extended pauses. We found that cellular levels of RodA regulate elongasome processivity, reversal and pausing. Our single molecule data, together with stochastic simulations, show that elongasome dynamics and processivity are regulated by molecular motor tug-of-war competition between several, likely two, oppositely oriented peptidoglycan synthesis complexes bound to the MreB filament. Our data, thus, demonstrate that molecular motor tug-of-war is a key regulator of elongasome dynamics in *B. subtilis*, which likely also regulates the cell shape via modulation of elongasome processivity.

O-39 (Cancelled)

A biophysical view of bacterial biofilms. From isolated components to multicellular organisms

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Biofilms are multicellular microbial communities that encase themselves in a secreted network of biopolymers and attach to surfaces and interfaces. From a soft matter perspective, biofilms are regarded as colloidal hydrogels, with the cells playing the role of colloids and the extracellular matrix (ECM) compared with a cross-linked hydrogel. However, from a biological perspective, biofilms are heterogeneous communities that organize in space and time into functionally distinct subgroups, in a process resembling differentiation in higher organisms. Biofilm heterogeneity has been demonstrated at the cellular level, but the molecular level has been neglected. In this talk I discuss the soft matter and biological perspectives of biofilms, focusing on the properties of water, ECM, and metal ions. In addition, using simultaneous synchrotron X-ray diffraction/fluorescence (XRD/XRF), we portrayed the dominant structural features in Bacillus subtilis biofilms and mapped them in space and time. Particularly, we revealed molecular-level structural hierarchy in biofilms, that followed their macroscopic morphology. Mapping the XRD and XRF signatures of intact biofilms in space and time allowed us to suggest an inclusive view of biofilm development, linking the ECM and the spores via the transport of water and metal ions. Our findings demonstrate that biofilm heterogeneity is not only affected by local genetic expression and cellular differentiation but also by passive effects resulting from the physicochemical properties of the molecules secreted by the cells. This study unravels the importance of molecular-level heterogeneity in shaping biofilm physiology and development.

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O-40

Profiling a single-stranded DNA region within predicted G-quadruplexes in the *B. subtilis* genome

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Guanine-rich sequences that contain a core of stacked G-tetrads can occasionally form non-canonical nucleic acid topologies known as G-quadruplexes (G4). G4 DNA is believed to play a significant role in regulating genes, including transcriptional and translational processes, DNA replication, and genome stability. In the *B. subtilis* genome, G-quadruplexes can be predicted using phylogenetic clustering, which relies on differences in the loop sequences. To confirm the presence of the G4 structure within a cell, we attempted to detect the single-stranded DNA (ssDNA) region within the predicted G4 structure. Non-denaturing sodium bisulfite treatment catalyzed the conversion of cytosines to thymines via uracils (CT-conversion) at the locally melted DNA of a bacterial genome. By utilizing next-generation sequencing, we obtained the actual CT-conversion rate. In principle, the CT-conversion rate is an accurate guide for detecting the formation of the ssDNA segment. Therefore, the CT-conversion rate accurately indicates the formation of the G4 structure.

Abstracts for Poster presentations

List of Poster presentations:

P-1 Biwen Wang (U Amsterdam, NL)
Transposon insertion sequencing of a minimal *Bacillus subtilis* genome

P-2 Biwen Wang (U Amsterdam, NL)
Induction of CtsR-regulated chaperones improves xylanase production in *Bacillus subtilis*

P-3 Biwen Wang (U Amsterdam, NL)
Gintool: Transcriptome analysis using regulation directionality information

P-4 Biwen Wang (U Amsterdam, NL)
Lethal ROS production upon membrane depolarization of dormant *Bacillus subtilis* cells

P-5 Ryosuke Kadoya (Sugiyama Jogakuen, JP)
Development of probiotic agents containing *Bacillus natto* against *Campylobacter* food poisoning

P-6 Valentina Andrea Floccari (U Ljubljana, SI)
Aberrant *Bacillus subtilis* cell's morphology emerges as consequence of active lysogeny

P-7 Romain Briandet (NRAE Jouy, FR)
Tracking *Bacilli* swimmers in microalgae biofilms

P-8 Virginie Grosboillot (U Ljubljana , SI)
SPβ-like viruses trigger changes in *Bacillus subtilis* behaviour under defined environmental conditions

P-9 Yotaro Isamu (Tsukuba U, JP)
The potential of membrane vesicles as immunostimulant modulating *Clostridioides difficile* colonization

P-10 Miku Matsushita (Tsukuba U, JP)
Isolation and characterization of bacterial-derived extracellular membrane vesicles in aged mice gut

P-11 Polonca Stefanic (U Ljubljana, SI)
Kin discrimination and cooperative behaviours in *Bacillus subtilis*

P-12 Stephen Lander (Northwestern U, USA)
Secreted nuclease facilitates extracellular DNA (eDNA) repurposing during biofilm development

P-13 Anne-Gaëlle Planson (INRAE Jouy, FR)
Construction and directed evolution of *B. subtilis* synthetic consortia

P-14 Yuzheng Wu (Kobe U, JP)
Luciferase luminescence of colonies to assess NADPH levels in *Bacillus subtilis* cells

P-15 Nunthaphan Vikromvarasiri (RIKEN, JP)
Metabolic engineering design based on flux balance analysis to improve bio-production from glycerol in *Bacillus subtilis*

P-16 Koki Tanaka (Tokyo Agri U, JP)
Analysis of segmented filamentous bacteria genome function in *Bacillus subtilis*

P-17 Katsumi Amano (Tokyo Agri U, JP)
Introduction and heterologous expression of heliobacterial photosynthetic gene cluster in *Bacillus subtilis*

P-18 Satoru Watanabe (Tokyo Agri U,JP)
Gene expression profile of *CyanoBacillus*, carrying chimeric genome of *Bacillus subtilis* and cyanobacterium *Synechocystis* sp. PCC 6803

P-19 Takahiro Morita (Tokyo Agri U, JP)
Development of the genetic engineering methods of *Cyanobacillus*

P-20 Hirotaka Matsubara (Amano enzyme, JP)
Genome analysis of *Bacillus amyloliquefaciens* industrial strains and its application to heterologous protein production

P-21 Junko Yamamoto (Shinshu U, JP)
Establishment of gene introduction into alkaliphilic bacteria using conjugation system consisting with Type IV secretion system (T4SS) and conjugative factor RP4

P-22 Taiki Kanzaki (Shinshu U, JP)
Determination of the *oriT* minimum region in conjugation between *Escherichia coli* and *Bacillus subtilis*

P-23 Claudia Borgmeier (BRAIN Biotech, DE)
Modulation of a built environment microbiome by a *B. subtilis* strain

P-24 Wakana Suda (Tokyo Agri U, JP)
Effect of restriction/modification on plasmid transfer in *Bacillus subtilis* natto

P-25 Rina Nogami (Kobe U, JP)
Elucidation of the poly-γ-L-glutamic acid (γ-L-PGA) synthesis mechanism and its mutational impacts in *Bacillus subtilis*

P-26 Thomas Konjetzko (FZ Jülich, DE)

Unravelling the potential of thermophilic *Geobacillus* spp. as chassis organisms for bioplastic upcycling

P-27 Etienne Dervyn (INRAE Jouy, FR)
OSIRIS: Orthogonal Sigma for Internal Resources Implementation towards Synthesis

P-28 Ryosuke Fukuda (Tsukuba U, JP)
Temperature-dependent gene regulation for environmental adaptation in *Clostridium perfringens*

P-29 Mitsuo Ogura (Tokai U, JP)
Regulatory mechanism of the operon containing genes encoding 5-oxoprolinase and manganese importer

P-30 Ahmad Altoun (Marburg U, DE)
Regulation of bacterial transcription by 6S RNAs

P-31 Veronika Kočárková (Czech Academy of Science, CZ)
Novel transcription factors in *Bacillus subtilis*

P-32 Yuzuki Shimada (Saitama U, JP)
Analysis of *sigI* regulation via lipoteichoic acid synthase in *Bacillus subtilis*

P-33 Ling Juan Wu (Newcastle U, UK)
Global regulatory role of ParB through *parS*-mediated autoregulation and gene silencing

P-34 Richard Daniel (Newcastle U, UK)
Differentiation between old and new peptidoglycan is required for coordinated cell growth in Bacteria

P-35 Matthieu Jules (INRAE Jouy, FR)
(p)ppGpp sets the level of tRNA charging through continuous regulation of translation initiation

P-36 Anne-Gaëlle Planson (INRAE Jouy, FR)
Greedy reduction of *Bacillus subtilis* genome yieldsemergent phenotypes of high resistance to a DNA damaging agent and low evolvability

P-37 Naoko Tsuji (Kyoto Sangyo U, JP)
Isolation of novel translation arrest peptides with RAPP and RGPP sequence motifs

P-38 Koichiro Masuda (Tokyo Agri U, JP)
Analysis of the suppressor strain, which recovered the survivability of *Bacillus subtilis* sigma factor minimizing strain

P-39 Sari Ikawa (Tokyo Agri U, JP)

In vivo comparative analysis of SigA /RpoD family in *Bacillus subtilis*

P-40 Miho Omote (Tokyo Agri U, JP)
Analysis of regulatory mechanism of RNA polymerase expression under nutrient starvation conditions in *Bacillus subtilis*

P-41 Natsumi Kimura (Tokyo Agri U, JP)
Effect of the acetylation state of 2-oxoglutarate dehydrogenase complex on sporulation in *Bacillus subtilis*

P-42 Teppei Kawakami (Tokyo Agri U, JP)
Analysis of Arg phosphorylation site of SigA in *Bacillus subtilis* sporulation initiation.

P-43 Ritsuko Kuwana (Setsunan U, JP)
Comparative analysis of thioflavin T and other fluorescent dyes for fluorescent staining of *Bacillus subtilis* vegetative cell, sporulating cell, and mature spore

P-44 Nozomu Obana (Tsukuba U, JP)
A novel conserved protein complex controls sporulation in *Clostridium*

P-45 Nobuki Kuwabara (Hosei U, JP)
Non-secreted intercellular signal transduction during sporulation in *Bacillus subtilis*

P-46 Tsutomu Sato (Hosei U, JP)
Identification of CgeA as a glycoprotein that anchors polysaccharides to the spore surface in *Bacillus subtilis*

P-47 Maja Popović (U Ljubljana, SI)
Disruption of phage integration gene leads to unexpected changes in *Bacillus subtilis*

P-48 Alexandre D'Halluin (Institut de Biologie Physico-Chimique, FR)
The sporulation specific 3' exoribonuclease KapD is involved in the spore crust and outer coat formation in *B. subtilis*

P-49 Daisuke Seo (Kanazawa U, JP)
Investigation of a physiological role of the ferredoxin-$NADP^+$ oxidoreductase paralog found in *Bacillus subtilis*

P-50 Hiroko Fukuda (Shinshu U, JP)
Application of a high-throughput colony growth measurement system to *Bacillus subtilis* under different pH circumstances

P-51 Yuma Okubo (Chiba U, JP)
B. subtilis $tRNA_{Arg}$ is indispensable for the competence development

P-52 Koichiro Akiyama (NIG, JP)
Divergent cell shapes by a mutation in the IIA domain of MreB

P-53 Shota Suzuki (Rikkyo U, JP)
Adaptive evolution of *oriC* through in vitro propagation in RCR

P-54 Tomoaki Okado (Saitama U, JP)
Expression analysis of genes involved in propidium iodine permeability in *Bacillus subtilis*

P-55 Kimihiro Abe (NIID, JP)
Spo0A-dependent membrane vesicle production in *Bacillus subtilis*

P-56 Ryuji Yamazawa (Setsunan U, JP)
YabG is a novel arginine-specific cysteine protease

P-57 Shigeki Kada (Meg Milk, JP)
Exploration of D-amino acid producing lactic acid bacteria by bioassay using *Bacillus subtilis* mutants.

P-58 Takashi Inaoka (NARO, JP)
Isolation of the suppressor mutations that restore the growth of *zwf* mutant in *B. subtilis*

P-59 Koya Sakuma (Nagoya U, JP)
Dual-wield NTPases: An uncharacterized protein family mined from AlphaFold Protein Database conserved among *Bacilli* and *Clostridia*

P-60 Akiko Soma (Chiba U, JP)
Identification of a minimum set of tRNA repertoire in *B. subtilis*

P-61 Masakazu Furuta (Osaka Metro U, JP)
Comparative analysis of growth retardation effects on *Bacillus subtilis* spores via diverse microbial control methods: heat, gamma-rays, UV, and essential oils from spices for the development of optimal combined processes

P-62 Shinya Kaneko (Titech, JP)
Novel approach to the genome (giant DNA) synthesis

P-1

Transposon insertion sequencing of a minimal *Bacillus subtilis* genome

Zihao Teng, **Biwen Wang**, Frans van der Kloet, and Leendert Hamoen

Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands.

One of the most basic biological question is what is necessary to maintain a living cell. Genome reconstruction methods with simple *Mycoplasmas* have suggested that fewer than 500 genes are sufficient. Interestingly, more than 30 % of these genes have an unknown function. A more complicated bacterium that has a cell wall, such as *Bacillus subtilis*, will need more genes to function. However, it is not known which ones. A systematic gene deletion study identified only about 200 essential genes, presumably because many essential processes are executed by redundant pathways. To find the essential set of genes to run *B. subtilis*, a stepwise gene deletion strategy has been applied that resulted in the minimal strain PG10, with a 37 % reduced genome. Unfortunately, the stepwise reduction of the genome also affected growth, and further genome minimization is hampered by increasingly aberrant growth characteristics, which makes it difficult to determine what genes are essential for *B. subtilis* to grow. However, PG10 still contains approximately 2600 genes. To examine how many of these genes are essential for PG10 growth, we performed transposon insertion sequencing (Tn-seq) and found that an additional 133 genes became essential, of which a large proportion have an unknown function. We have further characterized two of these.

P-2

Induction of CtsR-regulated chaperones improves xylanase production in *Bacillus subtilis*

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Bacillus subtilis is extensively used for the commercial production of enzymes due to its efficient secretion system. However, the efficiency of secretion varies greatly between enzymes, and despite many years of research, optimization of enzyme production is still largely a matter of trial-and-error. To identify relevant secretion bottlenecks, genome-wide transcriptome analysis seems a useful tool, yet to this day, only a limited number of transcriptome studies have been published that focus on enzyme secretion in *B. subtilis*. Here examined the effect of high-level expression of the commercially important endo-1,4-β-xylanase XynA on the *B. subtilis* transcriptome using RNA-seq. We used an in house developed gene set enrichment analysis tool, GINtool, to interpret regulatory changes. Rather unexpectedly, we found a reduced-expression of several protein chaperones, including ClpC, ClpE and ClpX, when XynA was overproduced. Expression of these chaperones is controlled by the transcriptional repressor CtsR. CtsR levels are, in turn, directly controlled by regulated proteolysis, involving ClpC and its cognate protease ClpP. Preventing this downregulation by knocking out the interrelated transcriptional repressor CtsR resulted in increased XynA production by more than 25 %. The Clp chaperones are conserved in most organisms, suggesting that repressing this protease might be a generally method to improve protein production.

P-3

Gintool: Transcriptome analysis using regulation directionality information

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To infer the biological meaning from transcriptome data, it is useful to focus on regulons, i.e. sets of genes that are regulated by the same regulator. Unfortunately, current gene set enrichment analysis tools do not consider whether a gene is activated or repressed by a regulator. This distinction is crucial when analysing regulons, since a regulator can work as an activator of certain genes and as a repressor of other genes, yet both sets of genes belong to the same regulon. Therefore, simply averaging expression differences of the genes of such a regulon will not properly reflect the activity of the regulator. What makes it more complicated is the fact that many genes are regulated by different transcription factors, and current transcriptome analysis tools are unable to indicate which regulator is most likely responsible for the observed expression difference of a gene. To address these challenges, we developed the gene set enrichment analysis program GINtool. Additional features of GINtool are novel graphical representations to facilitate the visualization of gene set analyses of transcriptome data, the possibility to include functional categories as gene sets for analysis, and the option to analyse expression differences within operons, which is useful when analysing prokaryotic transcriptome data.

P-4

Lethal ROS production upon membrane depolarization of dormant *Bacillus subtilis* cells

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The bactericidal activity of several commonly used antibiotics have been shown to partially rely on the production of reactive oxygen species (ROS). Bacterial persister cells, an important cause of recurring infections, are tolerant to these antibiotics because they are in a dormant state. However, even dormant cells must maintain a membrane potential. Here we used *Bacillus subtilis* as model system to study the effect of membrane depolarization on dormant cells. Surprisingly, we found that membrane depolarization also leads to ROS production. In contrast to previous studies, this does not require the Fenton reaction and results primarily in superoxide radicals. Genetic analysis revealed that Rieske factor QcrA, the iron-sulfur subunit of complex III, is a primary source of superoxide radicals. Interestingly, the membrane distribution of QcrA changed upon membrane depolarization, suggesting a dissociation of complex III. Our data reveal an alternative mechanism by which antibiotics can cause lethal ROS levels, and may partially explain why membrane-targeting antibiotics are effective in eliminating persisters. The current status of the project will be presented.

P-5

Development of probiotic agents containing *Bacillus natto* against *Campylobacter* food poisoning

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This research project focused on developing a probiotic agent using *Bacillus natto* to prevent food poisoning by *Campylobacter jejuni*. *B. natto* has been recognized for its ability to regulate the intestinal microflora and inhibit pathogenic microorganisms. Compared to antibiotics, *B. natto* exhibits a robust inhibitory effect on the growth of various pathogenic microorganisms, while being non-pathogenic to humans and animals. To develop the probiotic agent, this study addressed the following issues: 1) Searching for *B. natto* strains with growth inhibitory activity against *C. jejuni*, 2) Examining the inhibition of *C. jejuni* growth in the intestine through *B. natto* administration, and 3) Observing changes in the intestinal microflora upon *B. natto* administration.

The antibacterial effect of *B. natto* against *C. jejuni* was strain dependent. The efficacy of antimicrobial activity was assessed in terms of intensity and stability, which varied among the strains. Furthermore, when mice were fed a *B. natto* strain with high antibacterial activity, its effectiveness against *C. jejuni* in the intestine was observed. Notably, significant changes were observed in intestinal bacterial microflora after *B. natto* administration, with no adverse effects on mouse health. These results suggested that certain strains of *B. natto* may possess antimicrobial activity against *C. jejuni* in the intestine. This study provides valuable insights into the potential use of *B. natto* as a probiotic for the prevention of *Campylobacter* food poisoning. Further research is necessary to determine the safety and efficacy of *B. natto* as a probiotic specifically for this purpose.

P-6

Aberrant *Bacillus subtilis* cell's morphology emerges as consequence of active lysogeny

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Temperate bacteriophage – bacterial host interaction has always been one of the most controversial in the microbial world. We investigate the lysogenic conversion exerted by RSphages,able to follow the active lysogeny life cycle (or Regulatory switch mechanism), regulating gene expression of the host through integration/excision from host functional genes. Our research focuses on investigating the interaction between SPβ-viruses and *Bacillus subtilis*, a widespread beneficial bacterium. We observed a strong influence on host morphology exerted by a specific SPβ-virus, resulting in aberrant spherical-shaped cells as opposed to the conventional rod shape. This phenotype manifests in late exponential phase and during sporulation, being stable and heritable. Combining Transmission Electron Microscopy analysis and Fluorescence Microscopy's time-lapse experiments we investigated potential differences between the wild type and its spherical-shaped lysogenic version, questioning processes like cell-elongation, cell-division and sporulation. Moreover, we discovered that superinfection with a second homologous SPβ-virus restores the conventional rod-shaped morphology, suggesting a phage-phage interplay which results in protecting the host from the cell morphology change. Interestingly, we also observed that the double lysogeny has strong impact on host fitness, influencing growth dynamics also correlated with spontaneous phage release. This observation, together with controversial sequencing results, suggests a mixed population of lysogens as a consequence of recombination events between the two integrated prophages. The outcome of this research will bring us closer to understanding the role of prophages in controlling bacterial hosts, highlighting mechanisms still unknown, crucial knowledge to control bacteria for biotechnological and medical applications.

P-7

Tracking Bacilli swimmers in microalgae biofilms

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Are microorganisms within biofilms always immobilized in their organic matrix?
Not necessarily... We have demonstrated that certain bacilli biofilms spontaneously contain a subpopulation of motile bacteria capable of navigating through the matrix in three dimensions (Houry *et al.*, 2012, Ravel *et al.*, 2022). These continuous movements create a network of transient pores that enhance material transfers within the biofilm and allow irrigation of the deeper layers. The vascularization of biofilm matrices by motile bacteria can facilitate the penetration of specific substances with substantial toxic or beneficial effects.
In particular, we had proven that such swimming bacteria could help tackling undesirable biofilms by either to boost disinfectant effectiveness or through self-produced antimicrobial compounds.
In this ground-breaking study, we delve into the swimming capabilities of bacilli species within microalgae biofilms. Microalgae biofilms have gained increased interests due to their high productivity potential. Here, we explore the potential of using bacilli swimmers within microalgal biofilms to further increase biomass productivity. Swimmers criss-cross a biofilm of bacteria, but are they able to vascularize a microalgae biofilm knowing that these microorganisms are at least three times larger? Using confocal microscopy, we characterized the trajectories of the bacilli within photosynthetic biofilms. Although the microalgae biofilm becomes increasingly thick with increasingly production of matrix (Fanesi *et al.*, 2019), bacillus swimming is still observable. This poster highlights the heterogeneity of this phenomenon in a complex biofilm. Exploring these vascularization phenomena could enhance the entry of nutrients or the release of molecules such as O_2 trapped in biofilms, and thus promote their growth and productivity.

Houry A, Gohar M, Deschamps J, Tischenko E, Aymerich S, Gruss A, Briandet R. Bacterial swimmers that infiltrate and take over the biofilm matrix. Proc Natl Acad Sci U S A. 2012 Aug 7;109(32):13088-93. doi: 10.1073/pnas.1200791109.

Ravel G, Bergmann M, Trubuil A, Deschamps J, Briandet R, Labarthe S. Inferring characteristics of bacterial swimming in biofilm matrix from time-lapse confocal laser scanning microscopy. Elife. 2022 Jun 14;11:e76513. doi: 10.7554/eLife.76513.

Fanesi A, Paule A, Bernard O, Briandet R, Lopes F. The Architecture of Monospecific Microalgae Biofilms. Microorganisms. 2019 Sep 13;7(9):352. doi: 10.3390/microorganisms7090352.

P-8

SPβ-like viruses trigger changes in *Bacillus subtilis* behaviour under defined environmental conditions

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A third of the prophages predicted in *Bacillus subtilis* strains are SPβ-viruses. SPß-like prophages have large genomes, around 130,000 bp and integrate in host functional genes such as *spsM*, all located within 1% of their host genome. Because they feature a reversible active lysogenic lifestyle, meaning they actively switch between intra and extrachromosomal stages without lysing their host, they affect their host behaviour switching on and off those particular genes. However, little is known about the triggers and the manner they alter their host behaviour and a majority of SPß gene functions are still unknown. To gain insight in prophage-host interactions, we first developed a bioinformatic pipeline to generate the graphical synteny between a group of prophages, identifying 11 well-conserved genes among the group, 7 of them with unknown function. Another 17 SPß genes appear unique to the phage, all with unknown function. We then used publicly available transcriptomic data to assess what genes are potentially involved in the prophage excision under given conditions. We noticed that conserved genes globally present low expression variation over the different conditions, although most of them show an increased expression under certain stress conditions. In addition, this analysis allowed us to establish potential correlation between genes or between genes and conditions, based on their expression pattern, indicating their potential role during those processes. Taken together those data show which conditions influence the prophage genes expression profile and whether this behaviour is to be expected in other related prophages, highlighting the relation between prophage genes, their function and their effect on its host.

P-9

The potential of membrane vesicles as immunostimulant modulating *Clostridioides difficile* colonization

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Many bacteria produce membrane vesicles (MVs), which are bacterial lipid bilayer nanostructures, are loaded with various bacterial-derived components, and may stimulate the host immune response. Previous studies show that nasal immunization of MVs induces the production of intestinal secretory IgA antibodies specific to the MV producer, influencing the intestinal microbiota composition. These findings suggest the potential of MVs as an immunostimulant for modulating host immune response and precise control of gut microbiota. In this study, we focus on *Clostridioides difficile*, a gram-positive enteric pathogen that causes chronic and nosocomial colitis, such as antimicrobial-associated diarrhea and pseudomembranous enteritis. We isolated MVs produced by *C. difficile* and tested whether MV nasal immunization may inhibit the colonization of this pathogen and colitis in the murine intestinal tract.

In Gram-positive bacteria, cell lysis mediated by prophage-encoded genes, holin and endolysin, induces MV production. We constructed the holin-endolysin-inducible expressing strain in *C. difficile* and found that holin-endolysin expression enhanced MV production also in *C. difficile.* To assess the potential of vaccination with MV, we immunized mice with MVs, and then the mice were challenged with *C. difficile* infection. We observed the inhibition of *C. difficile* colonization and amelioration of infection-induced diarrhea and weight loss in the mouse intestine immunized with holin-endolysin-induced MVs.

This study suggests the potential of MVs as a platform for developing immunostimulants to control *C. difficile* infection and modulate the intestinal colonization of specific gut bacteria.

P-10

Isolation and characterization of bacterial-derived extracellular membrane vesicles in aged mice gut

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The intestinal microbiota has a variety of biological roles in host immune homeostasis and inflammatory responses. Membrane vesicles (MVs), spherical nanostructures with diameters ranging from 20 to 400 nm produced by bacteria, are considered one critical mediator for the microbiota-host interaction. MVs are released extracellularly and transport various bacterial components to surrounding cells. Previous studies using MVs produced by gut bacteria isolates suggest that MVs play pivotal roles in modulating host physiological states. However, which bacteria actively release MVs in the intestinal tracts is still unclear.

In this study, to identify bacterial taxa releasing MVs in the intestinal tract, we isolated MVs from murine feces. We compared the bacterial taxonomic compositions of feces- and MV-derived DNA by 16S rRNA amplicon sequencing. We expected bacterial taxa enriched in MV-derived DNA to produce MVs in the murine intestine predominantly. We isolated MVs from mice of different weeks old and identified bacteria taxa enriched in the MV fractions. We detected the genus *Turicibacter* frequently in the MV-derived DNA of aged mice. Since *Turicibacter* has been reported to have some relation with chronic inflammation, *Turicibacter*-derived MVs might be related to age-related chronic inflammation in the host intestine.

Currently, we are evaluating the inflammatory activity of MVs produced by gut resident *Turicibacter* and will discuss the relationship MVs produced in aged host intestine and chronic inflammation.

P 11

Kin discrimination and cooperative behaviours in *Bacillus subtilis*

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Bacillus subtilis is a soil-dwelling bacterium with diverse social life and interactions that can lead to horizontal gene transfer. While sexual isolation in *B. subtilis* is known to facilitate DNA uptake from closely related microorganisms, we recently discovered that antagonistic interactions between less closely related strains increased recombination, contrary to existing beliefs. This increased DNA uptake was attributed to the induction of competence between non-kin strains, enhancing potential adaptation rates. However, the mechanisms underlying kin discrimination (KD) remain poorly understood. In this study, we elucidate novel ways through which KD stabilizes cooperation in *B. subtilis* during swarming, pellicle formation, and coexistence at the microscale. First, we performed an experiment in which we mixed a Δ*srfA* mutant with it's wt parent strain and staged the mixed population against kin and a non kin swarm. We discovered that non-kin strain eliminated Δ*srfA* mutants from the mixed population. Moreover, our experimental evolution studies demonstrated that swarming-deficient cheaters evolved more frequently in contact with kin strains compared to non-kin strains, providing further evidence of non-kin-driven elimination of cheaters. Furthermore, we investigated the impact of KD on coexistence during pellicle formation at macro and micro scales using microfluidic chambers. As expected, we found that both macro and micro-scale interactions resulted in the dominant strain outcompeting the non-kin strain, while kin strains formed mixed populations. These results highlight the importance of KD for community stability and provide support for the theory that the evolution of cheaters in bacterial populations is impeded by kin discrimination.

P-12

Secreted nuclease facilitates extracellular DNA (eDNA) repurposing during biofilm development

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DNA is the genetic code found inside all living cells, yet DNA also has unique structural properties that can be utilized outside the cell. While extracellular DNA (eDNA) is found in bacterial biofilms, whether it persists as a structural feature or can be repurposed for further cellular activity remains unclear. Here, by studying model *Bacillus subtilis* biofilms, we report that eDNA acts as a temporary scaffold that is repurposed for cell growth later in development. Specifically, we used a fluorescent dye to show that eDNA is produced throughout early biofilm development before being removed in a single coordinated pulse. Chemical, genetic, and proteomic approaches reveal that a secreted nuclease is responsible for eDNA removal. Biofilms that are deficient for this nuclease produce the eDNA scaffold but fail to remove it during biofilm development, resulting in increased starvation and dysregulated biofilm development. Our results demonstrate a dynamic and active role of eDNA coordinated by an extracellular nuclease that facilitates robust biofilm development.

P-13

Construction and directed evolution of *B. subtilis* synthetic consortia

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In natural ecosystems, microorganisms coexist in communities and engage in complex interactions that influence ecosystem structure, stability, and functionality. However, the precise mechanisms governing these interactions remain largely unexplored. One approach to investigate these mechanisms is the rational design of interactions within model organisms.

In this study, we have constructed synthetic consortia of *B. subtilis* that exhibit obligate mutualistic interactions through amino acid cross-feeding. These interactions have the potential to partition metabolic roles among individuals, thereby establishing a division of labor.

To identify compatible cross-feeding strains, we cultivated 36 auxotrophic *B. subtilis* strains in pairwise co-cultures. Of the 630 possible combinations, 70 consortia exhibited biomass accumulation greater than 50% of that of the wild-type strain. From this set, three consortia were selected for further characterization. The {Δ*argG*; Δ*metE*}, {Δ*leuA*; Δ*pheA*}, and {Δ*lysA*; Δ*thrC*} consortia showed the ability to grow up to 40 generations in minimal media, with growth rates corresponding to 25%, 25%, and 50% of the wild-type growth rate, respectively. The proportions of each partner within the consortia reached consortia-specific equilibria.

A directed evolution experiment was performed in chemostat culture to optimize the fitness of the {Δ*lysA*; Δ*thrC*} consortium. This consortium was continuously grown for 380 generations. Preliminary screening has identified variants with a 1.4-fold increase in growth rate compared to the non-evolved consortium. These variants are being characterized to unveil the underlying molecular mechanisms responsible for improved division of labor in order to reengineer and refine cross-feeding dynamics.

P-14

Luciferase luminescence of colonies to assess NADPH levels in *Bacillus subtilis* cells

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Genetic modifications in *Bacillus subtilis* have allowed the conversion of *myo*-inositol into *scyllo*-inositol, a promising therapeutic agent for Alzheimer's disease. This conversion comprises two reactions catalyzed by two distinct inositol dehydrogenases, IolG and IolW. The IolW-mediated reaction requires the intracellular regeneration of NADPH, and there appears to be a limit to the endogenous supply of NADPH.

The primary mechanism of NADPH regeneration in this bacterium remains unclear. The *gdh* gene of *B. subtilis* encodes a sporulation-specific glucose dehydrogenase that uses $NADP^+$ as its essential cofactor. When *gdh* functioned constitutively, the intracellular NADPH levels were elevated to enhance the inositol conversion. In addition, a bacterial luciferase derived from *Photorhabdus luminescens*, depending on the reducing power of NADPH for its luminescence, became more luminescent in the cells in liquid culture and colonies on culture plates. The results indicated that the luciferase luminescence represented intracellular NADPH levels. Thus, luciferase can be applied to screen for mutants with elevated NADPH levels. We select colonies with higher luciferase luminescence upon EMS mutagenesis to obtain mutants with elevated intracellular NADPH levels, which may further enhance the inositol conversion.

Elevating NADH levels in *B. subtilis* should have uses beyond inositol conversion. For example, it is directed to reduce acetoin into 2,3-butanediol, which could be material to produce perfumes, aviation fuel, etc. We are currently investigating this possibility.

P-15

Metabolic Engineering Design Based on Flux Balance Analysis to Improve Bio-production from Glycerol in *Bacillus subtilis*

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Flux Balance Analysis (FBA) is a mathematical optimization technique for the simulation of metabolic fluxes in genome-scale metabolic models using single-level linear programming. *Bacillus subtilis*, a “generally recognized as safe” organism that can grow at high levels of fermentation products and does not generate endotoxins, is an ideal platform microorganism for bioengineering to improve the production of various useful compounds. Glycerol is a promising alternative substrate for use as a renewable, low-cost, and non-food competitive feedstock for bio-industrial platforms. This study aimed to enhance 2,3-butanediol (2,3-BD) production from glycerol in *B. subtilis* using a “step-by-step” gene deletions technique to identify gene knockout targets to improve the metabolic pathway. As a result, *B. subtilis* was bioengineered to enhance 2,3-BD production from glycerol following FBA results via a published genome-scale model, iYO844. Four genes, *ackA*, *pta*, *lctE*, and *mmgA*, were knocked out step by step, and the effects thereof on 2,3-BD production were evaluated. *lctE* knockout led to a substantial increase in 2,3-BD production. Interestingly, *mmgA* deletion can improve 2,3-BD production, which was first discovered in this study. Moreover, we compare the use of FBA and OptKnock techniques. We found that FBA provided a higher accuracy and effective approach for metabolic design and manipulation to enhance bio-production from glycerol in *B. subtilis*. Besides, we found that *zwf* deletion can improve lactate production, which has never been reported. The strategy developed in this study, using FBA combined with experimental validation, is expected to provide a novel platform for bioengineering research to enhance bioconversion.

P-16

Analysis of segmented filamentous bacteria genome function in *Bacillus subtilis*

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Segmented filamentous bacteria (SFB) are non-pathogenic, Gram-positive bacteria that are colonized in the intestinal tracts of a wide range of mammals. SFB are known to be involved in induction of the host's immune system. However, SFB is non-culturable and it is difficult to analyse the gene function. *Bacillus subtilis* possesses high transformation and homologous recombination ability and is closely related to SFB. In this study, A large part of SFB genome was introduced into *B*. *subtilis* genome to analyse its function. First, we focused on mechanisms of SFB sporulation initiation. SFB-Spo0A, a key transcriptional regulator in sporulation, complemented *B*. *subtilis*-Spo0A. We also found that a gene (SFBM_0504), which codes for kinase, was suggested to phosphorylates *B*. *subtilis*-Spo0A directly but act like phosphatase against SFB-Spo0A. We also focused on the flagella formation of SFB because they are presumed to be deeply involved in induction of the host's immune mechanism. The SFB flagella-formation genes consisting of five gene clusters (approximately 57kb in total length) have been introduced all together into *B*. *subtilis* genome using a large-scale DNA introduction method.

P-17

Introduction and heterologous expression of heliobacterial photosynthetic gene cluster in *Bacillus subtilis*

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Bacillus subtilis has excellent natural transformation ability and can be used as a host to introduce and maintain long heterologous genomes. The longest record of this was the construction of *B. subtilis* with a genome of the cyanobacterium *Synechocysits* sp. PCC 6803 (Itaya, et al., 2005 PNAS, 102, 15971-15976). However, functional expression of introduced foreign genes, including photosynthesis-related genes, has not yet been achieved in this organism. We focused on the photosynthetic gene cluster (PGC) of *Heliobacteria*, a photosynthetic bacterium that performs anoxygenic photosynthesis, as a target for the large-scale expression of exogenous genes in *B. subtilis*. *Heliobacteria* belongs to the phylum Firmicutes as does *B. subtilis*, and examination of the PGC sequence revealed that the promoter and SD sequences were similar to the consensus sequence of *B. subtilis*.

To express PGC functionally in *B. subtilis*, we introduced the PGC of *Heliobacterium modesticaldum* Ice1 (38 genes, about 56 kbp) into the *B. subtilis proB* site. The expression of PGC was examined at the transcriptional level by RNAseq, and it was found that 74% (28 genes) of the total genes were transcribed in these cells. On the other hand, the transcription levels of the remaining 26% (10 genes) encoding the chlorophyll *g* metabolic enzymes were extremely low, indicating that these genes were not expressed according to *B. subtilis* rules. In this presentation, we would like to discuss our research and prospects for PGC expression in *B. subtilis*.

P-18

Gene expression profile of CyanoBacillus, carrying chimeric genome of *Bacillus subtilis* and cyanobacterium *Synechocystis* sp. PCC 6803

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Bacillus subtilis BEST 7613 (CyanoBacillus), carrying the chimeric genome of *B. subtilis* BEST 7003 and photosynthetic cyanobacterium *Synechocystis* sp. PCC 6803, has been established by the mega-cloning technology (Itaya, et al., 2005 PNAS, 102, 15971-15976). Whole-genome sequence analysis of the isolate and parental B. subtilis strains provided clues for identifying single nucleotide polymorphisms (SNPs) in the 2 complete bacterial genomes in one cell (Watanabe et al., 2012 J. Bacteriol., 194, 7007). The behavior of CyanoBacillus is just *B. subtilis*: this strain sustains heterotrophic growth and rod-shaped morphology. In order to reveal the transcriptome in CyanoBacillus, we carried out RNA-seq analysis. Interestingly, *Synechocystis* genes were hardly expressed in CyanoBacillus.

The chimeric genome strain CyanoBacillus has raised a number of poorly argued issues. CyanoBacillus does not contain the ribosomal RNA (*rrn*) operon of *Synechocystis*, because the *rrn* operon inhibits the further cloning of other genomic regions of *Synechocystis* to *B. subtilis* genome. RNA-seq and northern analysis of the genealogy of CyanoBacillus revealed that the expression of *Synechocystis rrn* operon disturbed the cloning of Synechocystis genome. In this poster presentation, the barriers relying in between *B. subtilis* and *Synechocystis* will be discussed.

P-19

Development of the genetic engineering methods of *Cyanobacillus*

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Using the *Bacillus subtilis* genome vector system depending on the high natural transformation ability of *B. subtilis*, a synthetic microorganism called *Bacillus subtilis* BEST 7613, also known as "Cyanobacillus" was established by combining the entire genome of *Synechosystis sp.* PCC 6803 (Cyanobacteria) with the genome of *B. subtilis* BEST 7003. However, Cyanobacillus do not possess several important Cyanobacteria genes involved in transcription and translation, and genes derived from Cyanobacteria were rarely expressed and functional. The purpose of this study was to express the genes located in Cyanobacteria regions and analyse the biological features of Cyanobacillus by using genetic engineering techniques such as genome editing.

Cyanobacillus possesses many antibiotic resistance markers in the process of construction, and then useful selection markers for functional analysis is not available. It also shows low natural competence ability, which makes genome handling difficult. Therefore, we focused on conjugational transfer, in which bacteria are linked to each other and give and take DNA. In our laboratory, a system was constructed in which pLS20 and the mobilizable plasmid pCJ were introduced into 168 strains as donor bacteria, and pCJ was transferred to recipient bacteria. Using this system, we are planning to perform genome editing using CRISPR-Cas9. We transferred pCJ to Cyanobacillus and confirmed that the frequency of appearance of Cyanobacillus transconjugant was the same as when *Bacillus subtilis* was used as the recipient bacterium. Currently, we are working on removing antibiotic resistance markers from Cyanobacillus by means of this method.

P-20

Genome analysis of *Bacillus amyloliquefaciens* industrial strains and its application to heterologous protein production

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Bacillus amyloliquefaciens has been used as an industrial alpha-amylase producing strain for nearly 80 years. This industrial strain has been created through repeated mutation breeding, but there has been no analysis of which mutations improve enzyme productivity. In this poster, we will discuss the identification and application of genes involved in enzyme productivity in this strain. We first analysed mutations related to enzyme productivity and transformability by NGS analysis. To analyse mutation points for enzyme productivity, we focused on nonsense mutations and frameshift mutations. The analysis showed that loss of function of the gene named No. 30 had the highest enzyme productivity enhancing effect. When the mutation effect was reproduced by No.30 gene disruption(Δ30 strain), not only show the improved secretory productivity of the endogenous enzyme gene by 2.3-fold compared to the non-disrupted strain, but the enzyme productivity was also increased by 1.3- to 4.9-fold when the exogenous enzyme gene was introduced. It was suggested that this approach could be applied in the analysis of other series of mutant breeding production lines. Analysis of transforming factors showed that, contrary to expectations, there were few mutations in competency-related genes previously reported in *B. subtilis*. It is suggested that elements related to competency may differ from model organisms in industrial strains that have been subjected to mutation breeding for many years. These candidate genes found in this study that are involved in the acquisition of transgenic potential and in the improvement of enzyme productivity will be investigated to elucidate their functions.

P-21

Establishment of gene introduction into alkaliphilic bacteria using conjugation system consisting with Type IV secretion system (T4SS) and conjugative factor RP4

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Alkaliphilic bacteria are a type of extreme environment microorganism and their optimum pH range for growth is above 9. These bacteria's ability on producing valuable metabolites and alkalitolerant enzymes making them promising organisms in industrial applications. Researchers are not only interested in the usefulness of alkaliphilic bacterial metabolites but also in the alkaline adaptation mechanisms that enable them to grow under high pH environments. Currently, research on alkaliphilic bacteria is limited due to the lack of the general gene introduction methods for them. Therefore, the objective of this study is to establish a versatile gene transfer method for broad species in alkaliphilic bacteria.

In our previous studies, we searched for gene introduction method from *Escherichia coli* to *Bacillus subtilis* and confirmed that a conjugation system consisted with a Type IV secretion system (T4SS) and a conjugative factor RP4 derived from *Pseudomonas* was available. In this study, we attempted this conjugation system to transfer genes from *E. coli* into alkaliphilic bacteria. We selected *Bacillus halodurans* C-125 as a model strain for alkaliphilic bacteria because it is a close relative of *B. subtilis*. Therefore, we aim to use *B. halodurans* C-125 as a starting strain to introduce genes into other alkaliphilic bacteria.

P-22

Determination of the *oriT* minimum region in conjugation between *Escherichia coli* and *Bacillus subtilis*.

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Conjugation is the phenomenon by which genes from a bacterium are transferred to another cell via contact point and is a useful method for gene introduction. Since it enables us long-chain DNA transfer and inter species gene transfer, conjugation becomes more and more important tool.

Conjugation has several transfer mechanisms. In particular, the RP4 system is widely used as a gene introduction method because of its ability to transfer DNA to various species. A *cis* acting region, origin of transfer (*oriT*), and a few *trans* acting protein subunits play important roles in initial steps. The subunits have their individual binding site within *oriT* and form a complex called relaxosome. The relaxosome initiates the system, accordingly. Since the structure of *oriT* affects gene transfer efficiency, it is important to determine the minimal region of *oriT*. We already minimized *oriT* region for conjugation among *Escherichia coli*.

The aim of this study is to determine the *oriT* minimum region for *Bacillus subtilis* as a recipient. We prepared several plasmids having partially deleted *oriT*s and subsequently measured conjugation efficiency. In conclusion, we found that the *oriT* minimum region is specific to recipient bacterial species.

P-23

Modulation of a built environment microbiome by a *B. subtilis* strain

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The built environment (BE) has become the default ecosystem for the vast majority of humans living in industrialized countries. The bacteria, fungi, and viruses that colonize these environments together with human inhabitants and anthropogenic chemical and physical inputs ultimately shape the BE microbiome. The composition of which can have a significant impact on human health. An exemplary BE inhabitant microbial genus being associated with negative effects such as malodour and potential pathogenicity is *Moraxella*. Members of this genus are able to tolerate detergents, desiccation or UV irradiation and therefore frequently colonize bathroom, kitchen or laundry surfaces by forming biofilms that withstand removal attempts typically applied for sanitation purposes. In the present study, we explored the feasibility of modulating a built environment microbiome in a targeted manner by introducing *B. subtilis*. To this end, biofilm communities representative of typical BE microbiome compositions were established in miniaturized foam sponges under controlled laboratory conditions and analysed by metagenome sequencing. The capability of *B. subtilis* to be included and persist as a member of such biofilm communities was assessed by applying spores and tracing the strain's presence over the course of several weeks. Metagenome analysis of replicate biofilm communities without and with *B. subtilis* revealed the absence of *Moraxella* in the latter without affecting the overall microbiome diversity. Thus, application of *B. subtilis* in the context of BE microbiome modulation can offer new opportunities for safe and environmentally friendly sanitation applications.

P-24

Effect of restriction/modification on plasmid transfer in *Bacillus subtilis* natto

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There are many useful bacterial species in the genus *Bacillus* and its relatives from the viewpoint of academic and industrial applications. Functional modification and analysis are necessary to promote the study of these useful bacteria, but many of these species are difficult to transform by conventional methods. We have introduced a DNA transfer system by conjugation using *B. subtilis* as a donor. A conjugative transfer plasmid, pLS20, derived from *B. subtilis* natto, is known to possess transfer ability among various Bacillus species.

Gram-positive broad host range plasmid, pUB110, which is transferred between *B. subtilis* using pLS20 as a helper. The pCJ plasmid was constructed by fusing a plasmid pBR322 with a portion of pUB110.We observed the transfer of the pCJ plasmid into *B. subtilis* natto, but pLS20 was not. It was suggested that the deletion of the genome of *B. subtilis* natto allows the transfer of pLS20.Since this deleted region contains the restriction-modification system genes of *B. subtilis* natto, we hypothesize that the restriction enzymes of the recipient *B. subtilis* natto inhibit the transfer of pLS20.Therefore, we are performing the gene disruption of restriction-modification system genes using pCJ and analyzing effect of disruption on pLS20 transfer.

P-25

Elucidation of the poly-γ-L-glutamic acid (γ-L-PGA) synthesis mechanism and its mutational impacts in *Bacillus subtilis*

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This study sheds light on the production strategies for poly-γ-L-glutamic acid (PGA), a promising bioplastic. Research started with the development of a cost-effective method for the production of DL-PGA using *Bacillus subtilis*, employing glucose and ammonium salts as the sole sources of carbon and nitrogen. To enhance aeration, we overexpressed PgdS, a PGA-degrading enzyme, leading to a reduction in the viscosity of the colonies, which was originally high due to DL-PGA overproduction. Interestingly, we observed the spontaneous emergence of mutant strains capable of producing L-PGA and restoring viscosity, though infrequently. Importantly, our data revealed that all mutations resided within PgsA, a crucial element of the PGA-synthesizing PgsBCA complex. Our detailed analysis indicated that to form L-PGA producing mutants, alterations, either deletions or point mutations, in the C-terminal epimerase domain region of PgsA are necessary. In contrast, the N-terminal region of PgsA and its adjacent transmembrane region were determined to be essential for L-PGA synthesis. Furthermore, we found that removing portions of varying lengths from the C-terminal region dramatically reduced productivity to between 1/8 and 1/40 of the original yield. Predictive modelling using AlphaFold2 suggested notable interactions between the intracellular N-terminal region of PgsA and PgsB, the transmembrane region and PgsC, as well as the extracellular C-terminal epimerase domain and PgsC. Our study underscores the significant role of these interactions in the synthesis of PGA. In conclusion, this study improves our understanding of the synthesis mechanisms and provides potential pathways for the development of more cost-effective bioplastic production methods.

P-26

Unravelling the potential of thermophilic *Geobacillus* spp. as *chassis* organisms for bioplastic upcycling

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Bio-based plastics are necessary to reduce the carbon footprint of everyday materials, but prominent polymers like polylactide (PLA) currently pose a challenge in traditional recycling strategies. In order to enable efficient and economically viable biorecycling of bioplastics, we envisage a consolidated bioprocess in which the depolymerizing enzymes are produced and secreted by the microbial biocatalyst that simultaneously converts the released monomers into value-added products. Thermostable enzymes and thermophilic microbial hosts are required in order to operate closer to the glass transition temperature of the polymer and thus make it more accessible to the depolymerizing enzymes. In order to identify novel thermostable polylactide-depolymerizing enzymes, we pursued a database-assisted screening of roughly 20 000 proteins within our thermophilic strain library and additionally enriched natural producers that grow on PLA at elevated temperatures. The most promising enzymes are being characterized *in vitro* and compared to benchmark enzymes with regard to PLA degradation activity. The best performing enzymes will be overexpressed in *Parageobacillus thermoglucosidasius* while characterizing the lactic acid monomer consumption kinetics *in vivo*. Since the availability of genome editing methods for *Geobacillus* spp. is limited, such methods are being developed especially with regard to CRISPR-Cas systems. The combination of the proof of principle consolidated bioprocess and CRISPR-Cas genome editing methods will pave the way for engineering genetically stable *chassis* organisms of the genus *Geobacillus*, capable of PLA degradation and funneling the resulting carbon flux towards value-added compounds. This concept will be finally expanded towards other bioplastics such as PBAT and PBSA.

P-27

OSIRIS: Orthogonal Sigma for Internal Resources Implementation towards Synthesis

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Bacillus subtilis is widely used as a cell factory for the production of recombinant proteins due to its highly efficient protein secretion system; of its adaptable metabolism and the tools available to modulate the expression of proteins[1] or modify the genome[2]. However, during the production of recombinant proteins, the question arises of the allocation of resources between the production of the host proteins and the protein of interest.

B. subtilis is able, in response to environmental modifications, to modify its transcriptional profile. The specificity of a DNA-dependent RNA polymerase for its targets is due to the sigma subunit it carries. Multiple sigma proteins, each conferring to RNA polymerase a unique preference for a promoter class, have been characterized and are responsible for much of the diversity of transcription profiles[3].

An efficient protein production system based on the production of an orthogonal sigma factor and its associated promoter were constructed. This system, named OSIRIS, was modified to be autonomous (independent of SigA) and invasive (to take over the SigA-dependent transcription program). OSIRIS has been patented and is able, once induced, to switch off SigA-dependent transcription and to redirect all transcription resources from the host needs to the transcription of a gene of interest. To our knowledge, the protein production levels obtained are higher than that obtained from any known constitutive promoters so far, and will be presented.

1. *Nucleic Acids Res*. 2016;44(15):7495-508
2. *Nucleic Acids Res*. 2023;51(6):2974-2992
3. *Science*. 2012;335(6072):1099-103

P-28

Temperature-dependent gene regulation for environmental adaptation in *Clostridium perfringens*

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Pathogenic bacteria adapt to and survive in the host's internal and external environments. Temperature is one of the important environmental cues for pathogens to recognize inside and outside the hosts. *Clostridium perfringens*, a Gram-positive, obligately anaerobic bacterium, causes gas gangrene and food poisoning. They predominantly produce the proteinous extracellular matrix at the external host temperature (25°C) than the internal host temperature (37°C), leading to temperature-dependent biofilm morphological change. Since biofilm morphology is involved in oxygen tolerance, this is considered an environmental adaptation strategy in *C. perfringens* (Obana et al. 2020. Npj biofilms & microbiomes). We hypothesize that temperature-regulated genes other than that encoding the biofilm matrix have a role in the adaptation. This study aims to identify the genes expressing differentially at different temperatures and elucidate the regulation mechanism.

RNA-seq analysis showed that the expression of 38 genes was significantly increased at 25°C compared to 37°C. These include 25 genes involved in utilizing host-derived nutrients, such as sialic acid. Of these temperature-regulated genes, the temperature did not influence the promoter activity of four gene clusters (*nanI*, *nanP*, *iol*, *cit*), while the half-life of these mRNA was notably increased at 25°C. These suggest that these genes are post-transcriptionally regulated via the stabilization of mRNA in response to temperature.

This study suggests that temperature-dependent post-transcriptional regulation of genes related to the utilization of host-derived compounds allows *C. perfringens* to optimize metabolic activities in response to the surrounding environmental changes, such as host death and excretion.

P-29

Regulatory mechanism of the operon containing genes encoding 5-oxoprolinase and manganese importer

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The presenter has recently identified a new gene encoding a manganese influx transporter, *ycsG* (*mntG)* (J Biol Chem, 2023, 299:105069). *mntG* is in an operon composed of seven ORFs. Two genes in the operon were initially identified as the regulatory gene *kipI* for sporulation and the transcription factor *kipR*, which represses this operon expression (Genes and Dev, 1997, 11:2569). At that time, it was found that the expression of operon was induced by glucose and further activated by the transcription factor TnrA. Further genome-wide analysis also revealed the recognition sequence of global nitrogen metabolism regulator TnrA (MicrobiologyOpen, 2015, 4:423). However, later studies showed that three genes in this operon, including *kipI*, are subunits of 5-oxoprolinase, which is involved in glutamate metabolism (J Biol Chem, 2017, 292:16360). However, the function of *ycsG* was still unknown at that time. The presenter found that glucose induction of this operon is mediated by CcpA, and that the transcription factors MntR, which regulates manganese transporter genes, and AhrC, which regulates arginine metabolism genes, repress this operon, and also observed DNA binding of both factors to the operon regulatory regions. In this presentation, we report cis sequences for AhrC and MntR revealed by expression analysis of deletion mutants of *lacZ* fusions and by DNA-binding analysis of AhrC and MntR using the deletion mutants. We also observed that the expression of this operon is induced by the addition of manganese and report the mechanism of action by regulatory transcription factors including KipR.

P-30

Regulation of bacterial transcription by 6S RNAs

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Bacterial 6S RNA, a non-coding RNA that regulates transcription, has been found to bind to the active site of housekeeping RNA polymerase (RNAP) holoenzymes. 6S RNA is present in the majority of bacteria and typically encoded by a single gene. Although 6S RNAs have been subject of a number of mechanistic studies (1), their general and specific impact on bacterial transcriptomes remains poorly understood.

In the case of Firmicutes, such as *Bacillus subtilis*, there are two paralogs of 6S RNA, namely 6S-1 and 6S-2 RNA, with distinct expression profiles. To shed light on their functions, we created deletion strains lacking either 6S-1, 6S-2, or both RNAs in the wild-type *B. subtilis* NCIB 3610 strain. Surprisingly, we discovered strong phenotypes of the Δ6S-2 and Δ6S-1&2 strains, but the underlying molecular mechanisms are currently unknown (2).

To gain insights into the interaction between 6S-1 and 6S-2 RNAs and RNAP holoenzymes containing different sigma factors (σ^A, σ^B, σ^D or σ^F), we investigated their ability to inhibit transcription at strong and weak DNA promoters. Our findings revealed that 6S-1 RNA can bind to σ^B- and σ^F-RNAP, albeit with weaker affinity compared to σ^A-RNAP. On the other hand, 6S-2 RNA was found to be an effective inhibitor of RNAP only at weak σ^A-dependent DNA promoters.

Interestingly, we also discovered that a truncated form of *B. subtilis* σ^A lacking domain 4 and the C-tail can still initiate DNA transcription and bind to 6S RNAs. This suggests that these structural elements of the sigma factor are functionally less important in *B. subtilis* compared with the *E. coli* system.

To identify specific contact sites between 6S-1/2 RNA and σ^A-RNAP, we are employing iCLIP/PAR-CLIP techniques. These methods allow us to map the precise locations where the RNA molecules interact with the RNAP holoenzyme, providing valuable insight into the mechanistic basis of how *B. subtilis* RNAP interacts with and is regulated by 6S RNAs. Additionally, we will utilize ChIP-Seq to examine the impact of single and double knockouts of the 6S RNA genes on the distribution of σ^A-RNAP across the *B. subtilis* genome. This approach will allow us to gain a comprehensive understanding of the

influence of 6S RNAs on σ^A-RNAP dynamics and gene regulation in *B. subtilis*.
Overall, our study contributes to the understanding of the roles and interactions of 6S-1 and 6S-2 RNAs in bacterial transcription, also shedding light on their potential significance in the broader context of biotechnology and biomedicine.

(1) Wassarman KM. 2018. 6S RNA, a global regulator of transcription. Microbiol Spectrum 6(3): RWR-0019-2018. doi: 10.1128/microbiolspec.RWR-0019-2018.
(2) Thüring M, Ganapathy S, Schlüter MAC, Lechner M, Hartmann RK. 6S-2 RNA deletion in the undomesticated *B. subtilis* strain NCIB 3610 causes a biofilm derepression phenotype. RNA Biol. 2021; 18:79-92.

P-31

Novel transcription factors in *Bacillus subtilis*

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Bacteria have evolved a broad range of mechanisms to regulate gene expression, allowing them to adapt to changing environments. A fundamental role in this regulation play transcription factors (TFs). An important group of TFs binds to regulatory regions of DNA and either prevents or promotes RNA polymerase association with promoter DNA. Many of these TFs remain unknown.

Bacillus subtilis, despite being one of the best-characterized model organisms, contains more than 30 % of genes with unknown functions. Using a deep-learning approach, putative TFs were predicted in *B. subtilis* and other organisms. From the list of predicted TFs, we selected 15 top candidates containing the helix-turn-helix motif, known as the most widely used DNA-binding motif in the prokaryotic world.

This project aims to unravel the functions of the 15 selected uncharacterized putative TFs. To get first insights into the cellular roles of these proteins, deletion strains were prepared and evaluated in phenotypic assays followed by proteomic and transcriptomic analyses. We also prepared strains with FLAG-tagged versions of these TFs for ChIPseq experiments to identify their binding sites in the genome. Additionally, immunoprecipitation of these FLAG-tagged TFs will be performed to identify their interacting partners. Finally, the 3D structures of these proteins will be determined.

Taken together, this project will provide a better understanding of how an organism responds to varying environmental conditions through transcriptional regulation.

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P-32

Analysis of *sigI* regulation via lipoteichoic acid synthase in *Bacillus subtilis*

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SigI is an alternative sigma factor in *Bacillus subtilis* and is involved in cell wall homeostasis in concert with a two-component system WalKR. SigI is also regulated by WalKR. *B. subtilis* has four lipoteichoic acid (LTA) synthase genes, *ltaS, yfnI, yqgS*, and *yvgJ.* Of the four LTA synthase gene-deficient strains, only a *yfnI*-deficient strain is reported to have half the promoter activity of *sigI* (Matsuoka et al., 2022). In this study, we analyzed how YfnI was involved in the transcriptional regulation of *sigI*. A interaction test using BACTH method in *E coli* cells was used to evaluate the interaction between the four LTA synthases and WalK and RsgI, which are known as its cognate anti-sigma factor. The results showed that only the combination of WalK and YfnI had statistically significant values compared to other combinations and controls. Based on these results, we hypothesize that YfnI affects the regulation of *sigI* expression through WalK .We are currently using the BiFC (bimolecular fluorescence complementation) method to analyze the interaction between WalK and YfnI in *B. subtilis* cells and attempting to identify the critical site of YfnI in SigI regulation.

P-33

Global regulatory role of ParB through *parS*-mediated autoregulation and gene silencing

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Chromosomal ParABS systems have multiple global roles in chromosome dynamics, including replication initiation, origin separation, and chromosome segregation. ParB proteins usually bind to a small number of specific *parS* sites mostly located close to the origin of chromosome replication, from where they spread locally to provide a recruitment site for SMC / Condensin complexes that promote chromosome organization and segregation. Here we show that loss of a *parS* site within the *B. subtilis spo0J* (*parB*) gene results in overexpression of the protein and a lethal phenotype. We show that this phenotype results from silencing of several essential genes located near *parS* sites. The genes affect translation (*metS*), cell wall dynamics (*walKR*) and phosphate metabolism (*ppaC*). Given that ParB protein accumulation around *parS* sites must be reset as replication forks pass through the origin region following each new round of chromosome replication, we speculate that *parS* sites impose cell cycle regulation on adjacent genes.

P-34

Cell wall composition and dynamics during growth of *B. subtilis*

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Gram-positive bacterial growth and division rely on a delicate balance of peptidoglycan synthesis and controlled degradation to maintain the structural integrity of the cell wall and cell morphology while permitting cell enlargement. The process of growth involves synthesizing new wall material underneath the existing cell wall on the surface of the cell membrane combined with controlled processing and ultimate degradation of the old cell wall on the outer surface. Consequently, the enzymes functioning in cell wall cleavage to either be restricted to the periphery of the cell wall, away from newly synthesised peptidoglycan or their activity is in some way regulated by so mechanism.

Using systematic deletion of the autolytic genes it was found that growth was possible in strains lacking all of the vegetatively expressed autolytic genes except for either CwlO or LytE (consistent with Hashimoto et al. and an independent study by Wilson et al.). Closer analysis of these mutants has shown that there are characteristic phenotypes associated with these deletion strains that indicate that CwlO and LytE are not entirely functionally redundant and have roles in other processes.

Hashimoto M, Ooiwa S, Sekiguchi J
Synthetic lethality of the lytE cwlO genotype in *Bacillus subtilis* is caused by lack of D,L-endopeptidase activity at the lateral cell wall.
Journal of bacteriology. 2012 Feb; 194(4):796-803

S. Wilson, R. Tank, J. Hobbs, S. Foster, E. Garner
An Exhaustive Multiple Knockout Approach to Understanding Cell Wall Hydrolase Function in *Bacillus subtilis*
bioRxiv 2021.02.18.431929

P-35

(p)ppGpp sets the level of tRNA charging through continuous regulation of translation initiation

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Bacteria use RelA/SpoT Homologue proteins to adapt to changing environments. These proteins synthesize (p)ppGpp when amino acids are scarce, leading to a reduction in protein synthesis and growth arrest, known as the stringent response. Using custom-made translational frameshift error reporter systems in *Bacillus subtilis* to monitor limitation in charged tRNA, we show that translation error rates increase in a strain unable to synthesize (p)ppGpp, and peaks in the transition to stationary phase. Although the error rate is positively correlated with GTP abundance, synthetic tuning of GTP abundance is not sufficient to reduce the error rate to that of the wild-type strain. However, adding sublethal concentrations of drugs that allow for fine-tuning of translation initiation eliminates the error rate peak. Consistently, a mutant of the essential initiation factor 2, insensitive to (p)ppGpp, shows a considerable increase in the error rate, demonstrating that (p)ppGpp regulates tRNA charging through two feedback regulations. One regulation quickly acts on the translation initiation rate, while the other has a slower, more permanent effect on ribosome synthesis. This (p)ppGpp-mediated regulation of aa-tRNA pools may be conserved across all living systems, as RelA/SpoT Homologue proteins and (p)ppGpp are found in all kingdoms of life.

P-36

Greedy reduction of *Bacillus subtilis* genome yields emergent phenotypes of high resistance to a DNA damaging agent and low evolvability

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Genome-scale engineering methods enable the construction of synthetic biological systems, that can be used to address fundamental questions about evolution and improve our understanding of living systems. For example, genome-scale engineering allows the rational removal of dispensable genes in chassis genomes. Deviating from this approach, we applied greedy accumulation of deletions of large dispensable regions in the *Bacillus subtilis* genome, yielding a library of 298 strains with genomes reduced in size up to 1.48 Mb. High-throughput physiological phenotyping of these strains confirmed that genome reduction is associated with a substantial loss of cell fitness and an accumulation of synthetic-sick interactions. Transcriptome analysis revealed that less than 15% of the conserved genes in our genome-reduced strains exhibited a twofold or higher differential expression and revealed a thiol-oxidative stress response. Most of the transcriptional changes can be explained by loss of known functions and by aberrant transcription at deletion boundaries. Genome-reduced strains exhibited striking new phenotypes compared to wild type, including a very high resistance (increased >300-fold) to the DNA-damaging agent mitomycin C and a very low spontaneous mutagenesis (reduced 100-fold). Adaptive laboratory evolution failed to restore cell fitness, except when coupled with a synthetic increase of the mutation rate, confirming low evolvability. Although the mechanisms underlying this emergent phenotype are not understood, we

propose that low evolvability can be exploited in an engineering strategy involving a synergistic interplay of reductive and evolutive cycles under induced mutagenesis.

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P-37

Isolation of Novel Translation Arrest Peptides with RAPP and RGPP sequence Motifs

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Proteins that exert physiological functions through regulated translation arrest have recently been discovered. For instance, SecM, found in *Escherichia coli*, utilizes translation arrest to monitor the cellular capacity of the protein translocation pathway to feedback-regulate the expression of the downstream *secA*, which encodes a component of protein localization machinery. *Bacillus subtilis* MifM and *Vibrio alginolyticus* VemP also monitor the YidC-dependent membrane protein insertion and Sec-dependent secretory pathways, respectively, to regulate the level of YidC or SecDF encoded downstream of *mifM* or *vemP*. The amino acid sequences responsible for translation arrest share no appreciable sequence similarity, making it challenging to predict arrest-inducing sequences or to identify novel arrest peptides. However, we have recently identified three novel arrest peptides that turned out to share a RAPP-like sequence within their arrest motifs (Sakiyama 2022).

In this study, we searched for proteins having a RAPP-like motif through the proteome of *Streptomyces lividans* which belongs to Actinobacteria, and *Alteromonas macleodii* which belongs to Gammaproteobacteria. Our subsequent *in-vitro* translation assay allowed us to identify five novel translation arrest peptides with the RAPP or RGPP motif from *S. lividans*. In addition, we identified three novel translation arrest peptides with the RAPP or RGPP motif from *A. macleodii*. In most cases, these amino acid motifs are located near the C-terminus, suggesting that they are involved in regulation of their downstream genes. However,these arrest peptides are not encoded upstream of either *sec* or *yidC* genes, suggesting that their functions are distinct from the regulation of protein localization pathways.

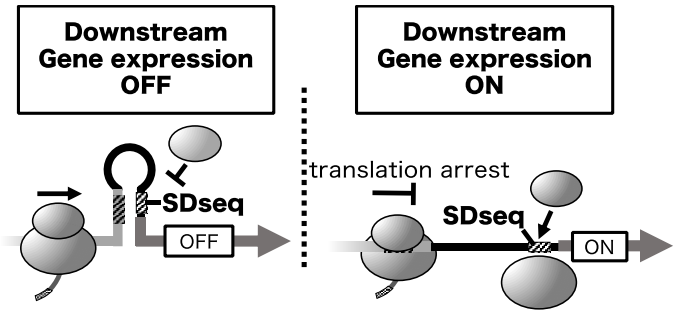

Figure: An example of a gene expression regulation system utilizing translation arrest

P-38

Analysis of the suppressor strain, which recovered the survivability of *Bacillus subtilis* sigma factor minimizing strain.

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In bacterial transcription, sigma factors in the RNA polymerase holoenzyme complex determines which genes are transcribed. Bacteria possess multiple sigma factors, and each sigma factor control the expression of a specific group of genes in response to different conditions. Among them, the major sigma factor SigA is the essential sigma factor required for transcription of housekeeping genes.

Since sigma factors form a highly conserved protein family, they may have diversified through gene duplication and mutation within bacterial species and horizontal gene transfer from other bacterial species, but this has not yet been experimentally elucidated. To analyse the evolution of diversified sigma factors, we constructed a *Bacillus subtilis* sigma factor minimized strain [SigA only] in which all sigma factors except SigA were inactivated.

This strain grew well but lysis when it entered the stationary phase. The suppressor mutant strain that could maintain the stationary phase was emerged from the prolonged cultivation on solid medium. Some base substitutions and deletions were occurred in the ribosomal protein gene, *rpsU*. We tested whether other non-essential ribosomal protein genes can also suppress the phenotype of SigA only strain.

P-39

In vivo comparative analysis of SigA /RpoD family in *Bacillus subtilis*

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The bacterial RNA polymerase holoenzyme consists of a core enzyme with four subunits and a sigma factor. Sigma factors are important transcriptional regulators responsible for promoter recognition and transcription initiation. Among them, the major sigma factor, also called RpoD/SigA family is an essential transcriptional factor conserved in all bacteria, and the motif of recognition DNA sequences within a promoter are thought to be conserved in a wide range of bacterial species. Although the major sigma factor encodes four conserved protein domains, some domains of amino acid sequences are varied among species. This observation suggests that the major sigma factors may be evolved in each species. In this study, we analysed conservation and difference of the major sigma factors to elucidate their biological significance using *Bacillus subtilis* as a host.

The major sigma factors are essential and indispensable. Therefore, when the heterogenous sigma factor was artificially induced in *B. subtilis*, intrinsic *sigA* was knocked down by means of CRISPRi technology. The more evolutionarily distant major sigma factors are, the lower growth complementation is. However, it was possible to completely replace the SigA of *B. subtilis*, with that of a unculturable enterobacteria, Segmented Filamentous Bacteria (SFB) though this strain showed significantly slow growth. We obtained the mutant from this strain, which restored growth rate, by successive culturing, and found the mutation in the region 1 of the major sigma factor of SFB. We also report an attempt to establish a method to facilitate the major sigma factor evolution.

P-40

Analysis of regulatory mechanism of RNA polymerase expression under nutrient starvation conditions in *Bacillus subtilis*

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Bacteria synthesize and accumulate (p)ppGpp, which is a warning molecule called an alarmone in response to amino acid starvation. Accumulation of (p)ppGpp decreases the intracellular ATP/GTP ratio and leads to down-regulation of protein synthesis and up-regulation of amino acids metabolism resulting in growth posing. A (p)ppGpp synthase genes-deficient strain of *Bacillus subtilis* exhibits growth defect on minimal medium. Many mutations that suppress this growth defect have been identified and found to be located within the coding regions resulted in alterations of enzymatic activity. Some of the mutations were occurred in *rpoB* gene, which codes for RNA polymerase beta subunit, however, a mutation was also identified in the 5'UTR upstream of *rpoB* coding region. We assumed that suppression mechanism of this mutation is different from that of other suppressor mutants reported to date and decided to analyse further. Namely, the 5'UTR, in which suppressor mutation was found and stem loop structure is predicted, is related to post-transcriptional regulation of *rpoB*. We constructed a strain lacking the 5'UTR stem loop structure in the (p)ppGpp synthase genes-deficient strain, together with suppressor mutation. In addition, assuming that this suppression is caused by the increase or decrease of RNA polymerase, we constructed a (p)ppGpp synthase gene-deficient strain with overexpressing *rpoA*, the gene coding for RNA polymerase alpha subunit. We are currently testing their growth on amino acid starvation conditions.

P 41

Effect of the acetylation state of 2-oxoglutarate dehydrogenase complex on sporulation in *Bacillus subtilis*.

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In response to shifts of available carbon sources, intracellular metabolic flows dynamically change in every organism. When bacteria exhaust the carbon source such as glucose necessary for growth, they enter stationary phase to survive by switching central carbon metabolism, glycolysis, and the citric acid cycle. A Gram-positive soil bacterium, *Bacillus subtilis*, forms metabolically inactive dormant endospores in such metabolic shifts. Although many molecular mechanisms for switching metabolic phases have been proposed, its precise mechanisms are largely unknown.

The gene, *odhB*, codes for one of the components of 2-oxoglutarate dehydrogenase complex, which catalyses the formation of succinyl CoA from 2-oxoglutarate in the citric acid cycle. Inactivation of *B. subtilis* OdhB caused cell lysis at the stationary phase in LB medium and sporulation deficiency in sporulation medium. We assumed that OdhB is one of the key factors, which involved in the metabolic switch. On the other hand, growing of the cells on glucose reflects the metabolic states that preferentially produce acetyl-CoA, representative acyl-group donor, resulting in acetylation of many metabolic enzymes. Some lysine residues resided in OdhB is shown to be acetylated in glucose consumption.

To examine the effect of the state of acetylation on growth and sporulation, we introduced acetylation-mimic (K to Q), and non-acylation mimic (K to R) mutations at each acylation site of OdhB. In some of the mutants, the spore formation frequency was slightly decreased. We are trying to verify this phenomenon by measuring the promoter activity of spo0A by *lacZ* reporter assay.

P-42

Analysis of Arg phosphorylation site of SigA in *Bacillus subtilis* sporulation initiation.

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In bacteria, sigma factors, which bind to RNA polymerase core enzyme, determine the genes to be transcribed by recognizing specific regulatory DNA sequence, so-called promoter. The major sigma factors (SigA in *Bacillus subtilis*) are responsible for transcription of essential genes for growth and proliferation. In a previous study (Zhou et al. 2019, PNAS), it was found that dephosphorylation of the phosphorylated 365th Arg of SigA facilitates spore germination in *B. subtilis*. This suggests that dephosphorylation makes SigA available for binding to RNA polymerase, thereby restarting transcription. However, it is not clear how SigA Arg365 is phosphorylated during sporulation. Based on these models, we assumed that Arg365 is phosphorylated at the initiation of sporulation and aimed to unravel the biological significance of Arg365 and the phosphorylation mechanism of SigA.

In this study, we created a system for comprehensive analysis of alteration of SigA in the amino acid residue at position 365 during sporulation. First, the *sigA* genes, in which Arg365 was substituted with other 19 amino acids, was placed at *thrC* locus, and expressed heterotropically inducing by addition of IPTG. Next, the intrinsic *sigA* was knocked down by means of the CRISPRi system. By using these strains, we tested effects of alteration of SigA Arg365 on growth rate at log phase and sporulation efficiency in *B. subtilis*. Comparison of the 365th amino acid residue of SigA with the corresponding residue of bacterial species of other phylum revealed its amino acid conservation and diversity.

P-43

Comparative Analysis of Thioflavin T and other Fluorescent Dyes for Fluorescent Staining of Bacillus subtilis Vegetative Cell, Sporulating Cell, and Mature Spore

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Thioflavin T, a cationic benzothiazole dye, is typically used to detect the amyloid fibrils. In this study, we analyzed the staining properties of *B. subtilis* cells using several fluorescent dyes, including Thioflavin T analogues, 2-(4'-Methylaminophenyl) benzothiazole (BTA-1), and 2-(4-aminophenyl) benzothiazole (APBT). Thioflavin T stained vegetative cells in the early log phase and outer layer structures of forespores and mature spores. The inner parts of forespores and heat-killed mature spores were also stained with Thioflavin T. Congo red, auramine O, and rhodamine B stained forespores and mature spores similar to Thioflavin T. In contrast, APBT and BTA-1 fluorescence was detected in the outer layers of vegetative cells, mother cells, forespores, and mature spores, indicating that they bind to the cell membrane and/or cell wall. The combination of the fluorescent dyes used in this study will help analyze morphogenetic processes during sporulation and the damage mechanisms of vegetative cells and spores.

P-44

A novel conserved protein complex controls sporulation in *Clostridium*

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Pathogenic *Bacillus* and *Clostridium* bacteria form spores to acquire a high tolerance to heat, oxygen, and antibiotics, causing infectious diseases such as food poisoning. *Clostridium perfringens* is a causative bacterium of food poisoning and gas gangrene, and sporulation is recognized as an essential process for the pathogenesis of this bacteria. However, the detailed regulatory mechanisms of sporulation by *Clostridium* are poorly understood compared to *Bacillus*. This study identifies *ctrA* and *ctrB* genes, highly conserved in *Clostridium*, as novel sporulation regulators.

CtrA and CtrB proteins form stable heteromeric protein complexes within the cells. These genes are indispensable for sporulation, biofilm formation, and membrane vesicle production. The *ctrB*-disrupted mutant of the food poisoning strain of *C. perfringens* cannot sporulate. The cell morphological change of the *ctrB* mutant in the sporulation medium is arrested at the early sporulation stage (stage I), suggesting that CtrB regulates an initial step of sporulation. Transcriptome analysis of the mutants of *ctrB*, and *spo0A*, a gene for the sporulation master regulator in *Bacillus* and *Clostridium*, shows that more than 200 *ctrB* regulons overlap with *spo0A* regulons. We show that intracellular phosphorylated Spo0A is reduced in the ctrB mutant strain, suggesting that Spo0A phosphorylation mediates sporulation regulation by CtrAB. Furthermore, disrupting the *ctrB* gene in another *Clostridium* sp. also displays sporulation deficiency. These results demonstrate that the CtrAB protein complex is a crucial regulator of sporulation initiation in *Clostridium*.

P-45

Non-secreted intercellular signal transduction during sporulation in *Bacillus subtilis*

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Bacillus subtilis cell is divided into a forespore and a mother cell during sporulation. SpoIIR is expressed in the forespore and activates SpoIIGA protease in the mother cell to process pro-σ^E into the mature σ^E. SpoIIR, SpoIIGA and pro-σ^E were known to be necessary and sufficient to induce pro-σ^E processing, and thus SpoIIR is thought to be an intercellular signaling protein. However, how SpoIIR transduces the signal is poorly understood.

Here we demonstrated that the strain lacking *spoIIR* but normally expressing SpoIIGA and pro-σ^E in the mother cell was induced to sporulate by the co-cultivating with the strain lacking *spoIIGA* but normally expressing SpoIIR in the forespore on the background to prevent genetic transformation. This signaling required expression of SpoIIT, a protein known to support SpoIIR signaling in the forespore, suggesting that SpoIIR is able to transduce intercellular signal between the co-cultivating cells by a common mechanism during sporulation. However, the N-terminal signal peptide-like domain of SpoIIR was interchangeable with transmembrane domains, indicating that SpoIIR is not necessarily secreted. Culture supernatant or crude extract from cells expressing SpoIIR did not activate SpoIIGA, suggesting that SpoIIR is not transferred by extracellular materials including membrane vesicles. SpoIIR signaling between co-cultured cells was significantly enhanced by stopping the culture shaking. Structural modeling suggested a possible interaction of SpoIIR with the cytoplasmic domain of SpoIIGA in the mother cell. These results suggested the possibility that SpoIIR moves from the forespore into the mother cell without secretion through membranous nanotubes to transduce the forespore signal.

P-46

Identification of CgeA as a glycoprotein that anchors polysaccharides to the spore surface in *Bacillus subtilis*

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The *Bacillus subtilis* spore is composed of a core, containing chromosomal DNA, surrounded by a cortex layer made of peptidoglycan, and a coat composed of concentric proteinaceous layers. A polysaccharide layer is added to the spore surface, and likely anchored to the crust, the coat outermost layer. However, the identity of the coat protein(s) to which the spore polysaccharides (SPS) are attached is uncertain. The SPS contributes to the diffusion of spores in water-rich environments by making the spore surface more hydrophilic.

First, we showed that the crust proteins CotVWXYZ and CgeA were all contained in the peeled SPS layer obtained from a strain lacking CotE, the outer coat morphogenetic protein, suggesting that the SPS is indeed bound to at least one of the spore surface proteins. Second, CgeA is known to be located at the most downstream position in the crust assembly pathway. An analysis of truncated variants of CgeA suggested that its N-terminal half is required for localization to the spore surface, while its C-terminal half (CTH) is necessary for SPS addition. Third, glycosylation occurs at the T112 residue in the CTH of CgeA. Structural models suggest that T112 is located at the center of a projecting loop in the CTH. Our results indicated that CgeA is a glycoprotein required to initiate SPS assembly and serves as an anchor protein linking the crust and SPS layers.

P-47

Disruption of phage integration gene leads to unexpected changes in *Bacillus subtilis*

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Certain temperate phages use functional loci as integration sites in bacterial genomes, causing gene inactivation and potentially altering bacterial phenotype. The SPβ phage, that targets *Bacillus subtilis*, integrates into *spsM* gene, previously identified as a biofilm-associated gene and shown to be involved in spore formation. Integration of SPβ into *spsM* gene can therefore have interesting implications for phage transmission and phage-host interactions. Through plaque assays, growth curves, and macrocolony morphology tests on solid media, we assessed changes in lytic phage susceptibility and biofilm morphology using non-lysogenic *B. subtilis* strains. No significant changes in susceptibility to lytic phages were observed after disrupting the *spsM* gene in *B. subtilis* laboratory strain. Consistent with previous findings, disruption of the *spsM* gene in *B. subtilis* clinical isolate resulted in significant biofilm morphology changes. While in *B. subtilis* soil isolate, the changes caused by *spsM* deletion were less pronounced. In *B. subtilis* laboratory strain, no phenotypic changes were observed. Complementing the *spsM* gene disruption restored wild-type morphology only in clinical isolate. Disruption of the *spsM* gene also caused spontaneous diversification of macrocolonies in soil isolate. Full genome sequencing and further genetic engineering revealed that the observed diversification is caused by a single, frame-shift mutation, leading to inactivation of a gene important for swarming and motility. We showed that disruption of phage attachment gene has different consequences for host development and evolution, depending on host genetic background. Therefore, next to introduction of new genetic arsenal, prophages impact host physiology and evolution through interruption of attachment loci.

P-48

The sporulation specific 3' exoribonuclease KapD is involved in the spore crust and outer coat formation in *B. subtilis*

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Bacterial RNA degradation is both important for the recycling ribonucleotides controlling gene expression through a variety of cleavage mechanisms (1, 2). The activity of some ribonucleases and the accessibility of their RNA substrates are regulated under specific stress conditions, such as nutrient starvation or amino-acid depletion, to allow a tight control of RNA decay and maturation (1, 3). In *B. subtilis*, high stress conditions such as nutrient deprivation, heat, desiccation or radiation leads to the induction of an irreversible pathway to sporulation and ultimately to the formation of an endospore, a highly resistant and dormant bacterial state allowing the bacteria to survive to extreme environmental conditions (4, 5). Although the pathway to sporulation is generally well-characterized, the function of some of the genes expressed during this process are still unknown.

Here, we present KapD, a ribonuclease exhibiting a 3'-exonuclease activity and specifically expressed during sporulation of *B. subtilis.* KapD is regulated by sigma factors E and K, allowing expression within the mother cell at the early and late stage of sporulation (5). Deletion of the *kapD* locus or inactivation of its catalytic site strongly decreases the adhesiveness of the coat and crust layers of the endospore, suggesting that its ribonuclease activity is essential for the correct formation of the spore morphological structure. Using KapD-GFP fusions, we show a dynamic assembly of the protein into the spore crust layer in a similar way to outer spore coat proteins. Using a yeast two-hybrid assay, we showed that KapD interacts with the crust protein CotY, accounting for its localization in the outermost spore layer. KapD also interacts with RNase Y and the DNA helicase Mfd, suggesting potential functions in a degradosome-like complex or in the rescue of stalled RNA polymerases in the mother cell. RNA-seq and proteomic studies have identified potential mRNA substrates, but these have proven

frustratingly difficult to validate. New approaches are currently being developed to unravel the role of KapD RNase activity in endospore formation.

Keywords: Sporulation, Ribonuclease, *B. subtilis*, Spore coat

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P-49

Investigation of a physiological role of the ferredoxin-NADP$^+$ oxidoreductase paralog found in *Bacillus subtilis*

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In *Bacillus subtilis* genome one FNR paralog *ycgT* as well as one genuine ferredoxin-NADP$^+$ oxidoreductase (FNR) gene *yumC* have been annotated. Although the identity of the amino acid sequence is rather high (~0.5), the residues indispensable for the FNR activity are substituted in YcgT. Our previous works using recombinant YcgT protein revealed that the activity as FNR was drastically decreased compared to YumC, suggesting YcgT possess different physiological function in *B. subtilis* cells. Here we report a potent physiological role of YcgT examined by biochemical approaches using purified YcgT protein.

On B. subtilis Expression Data Browser provided by INRA, the expression of *ycgT* is enhanced under the iron limiting conditions. In other bacteria such as Staphylococci and *E. coli*, FNR and its homologues have been reported to participate in the reduction of Fe^{3+} bound to the siderophore molecules. Based on this information, we assessed the reduction activity of Fe^{3+} bound to some iron-chelating molecules. Obtained results indicated that YcgT can reduce Fe^{3+} bound to dihydroxy benzoil-Gly, suggesting YcgT can function as a Fe^{3+} reductase. Interestingly YcgT is photosensitive in the presence of surfactants such as EDTA. We will also report the photochemistry of *B. subtilis* FNR and YcgT.

P-50

Application of a High-Throughput Colony Growth Measurement System to *Bacillus subtilis* under Different pH Circumstances

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Quantitative growth measurement is important for bacterial research. The commonly used turbidity method for growth measurement is not suitable for comprehensive research due to its low throughput. Therefore, a new bacterial growth measurement system, Colony-live, was developed for *Escherichia coli*. This system automatically measures growth dynamics on agar media with a high throughput rate at 74,000 colonies per a batch. The system also enables us to observe colony morphology. In our previous studies on intracellular pH, we applied this system to *E. coli* library of comprehensive single-gene deletion. As a result, we could determine candidate genes for intracellular pH regulation of *E. coli*. Even though *Bacillus subtilis* is another important model microorganism, the intracellular pH regulation system is not as clear as *E. coli*. Therefore, we applied our high-throughput colony growth measurement system on *B. subtilis*.

In this study, we aimed to identify candidate genes for intracellular pH regulation of *B. subtilis*. We used comprehensive single-gene deletion library of *B. subtilis* (BKE collection) and the Colony-live system. Each strain of the BKE collection was grown on medium of different pH conditions, and the growth kinetics were quantified by the Colony-live system.

P-51

Bacillus subtilis tRNA^{Arg} is indispensable for the competence development

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Competence of *B. subtilis* 168 develops at the early stationary phase in poor media; it involves DNA uptake and homologous recombination that are regulated by a complex and sophisticated signal transduction network. We found that the transformation activity was remarkably decreased when a certain tRNA gene responsible for decoding arginine codons was deleted. This result suggested that this tRNA^{Arg} contributes to the development of competence in *B. subtilis*. No significant inhibition of growth and translational activity was observed in the tRNA^{Arg}-deletion mutant strain. To understand the detailed mechanism for this phenomenon, we performed a reporter assay to monitor the expression of *comK* and *srfA* that play important roles in competence development. Activity of the beta−galactosidase under the control of *comK* regulatory sequence (P_{comK}-*lacZ*) was significantly decreased in the tRNA^{Arg}-deletion mutant. Our analysis implied that the inhibitory effect of the tRNA^{Arg} -deletion seems attributable to the instability of ComK protein other than the defect of its synthesis (i.e., translation).

P-52

Divergent cell shapes by a mutation in the IIA domain of MreB

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Bacteria actin MreB is widely conserved among rod-shaped bacteria. MreB plays an important role for regulating the peptidoglycan (PG) synthesis. Deficiencies of MreB create various morphological changes. We found that a single amino acid substitution mutant, D285G, in the IIA domain of MreB is shaped like a teardrop. The IIA domain is involved in interaction with both FtsZ, a bacteria tubulin, and RodZ, a key component for cell elongation. The teardrop cell elongated and divided, producing a cone and a needle-like cell. Immunofluorescence analysis showed that the $MreB^{D285G}$ helical structure distributed throughout a whole cell. Live-cell imaging revealed that the PG synthesis/degradation occurred at the cap region of the $MreB^{D285G}$ mutant. These results strongly suggest that the $MreB^{D285G}$ lost the proper localization pattern and regulation of the PG synthesis/degradation. Furthermore, we found that changing the gene dosage of the $mreB^{D285G}$ or the co-expression of $mreB^{D285G}$ with $mreB^{wt}$ led to the various shaped cells. It seems that the cell morphology of bacteria rigidly keeps under a fine balance between cell elongation of MreB and cell division of FtsZ. The $MreB^{D285G}$ mutant indicates that MreB alone can create various cell shapes due to tuning elongasome and divisome simultaneously

P-53

Adaptive evolution of *oriC* through *in vitro* propagation in RCR

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Escherichia coli replicates its chromosome from a compact replication origin (*oriC*), which is 245-bp in length. In the *oriC* region, the initiator protein DnaA forms homo-multimeric complexes, leading to the opening the duplex DNA at the duplex unwinding element (DUE) and initiating bidirectional replication. We previously achieved exponential amplification of circular DNA containing *oriC* using the reconstituted *E. coli* replication cycle reaction (RCR). By combining the RCR with an enzymatic multi-fragment assembly reaction (RA), named RA-RCR, circular DNA containing *oriC* can be amplified from multiple DNA fragments. During the amplification of various DNA sequences using RA-RCR, we encountered difficulties in amplifying low GC content (24%) DNA. After several attempts to amplify low GC% DNAs, we coincidentally obtained amplification products with mutations in *oriC*. These *oriC* mutations occurred within the DUE and facilitated the RCR amplification of low GC% circular DNA. Subsequently, we attempted to isolate *oriC* mutants that enhance RCR amplification efficiency using evolutionary molecular engineering. By creating a library of *oriC* mutants through random mutagenesis using error-prone PCR, we successfully obtained amplified DNA products containing evolved *oriC* from low GC% DNA fragments. Next-generation sequencing (NGS) analysis of the amplified DNAs revealed common mutations in the evolved *oriC*s, which were found not only within the DUE but also at ATP-bound DnaA (ATP-DnaA) specific binding sites. We would like to discuss the implications of these mutations in enhancing low GC% DNA amplification in the RCR.

P-54

Expression analysis of genes involved in propidium iodine permeability in *Bacillus subtilis*

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The cell membrane of *Bacillus subtilis* contains glucolipids, and these syntheses are mediated by UgtP. In our previous study, propidium iodide (PI), a membrane-impermeable nucleic acid staining dye, was found to be permeable in a *ugtP* mutant (Kawakami and Matsuoka, 2022). This is thought to be due to a glucolipid deficiency that alters the function of membrane proteins, causing them to be permeable to PI. To identify genes involved in PI permeability, we screened 432 candidate gene disruption strains in which genes encoding transporters, channels and membrane proteins. We obtained seven candidate genes that reduced the cell membrane permeability of PI in the *ugtP* mutant to a relative ratio of 1.5 or less compared to the wild-type strain. Transcriptional analysis of two candidate genes showed that the transcript levels of the candidate genes varied in the *ugtP* mutant compared to the wild-type strain. The fact that the transcript levels of these genes vary in the *ugtP* mutant suggests that the changes may be involved in PI permeability. We are analyzing transcriptional analysis of other candidate genes. We plan to clone candidate genes into the *ugtP* mutant to measure the variation in PI permeability.

P-55

Spo0A-dependent membrane vesicle production in *Bacillus subtilis*

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Bacteria release small membranous spherical structures called “membrane vesicles (MVs)”, which are derived from the cellular membrane components. MVs contain and convey various biomolecules including nucleic acids, proteins, metabolites, and signal molecules, thereby participating in many biological processes. Yet, the molecular mechanism of formation of MVs still remains to be elucidated, in particular, in Gram-positive bacteria. To date, it is known that *Bacillus subtilis* can be induced to produce abundant MVs by treatment with a DNA-damaging agent, mitomycin C, or by long-term cultivation in Brain-Heart Infusion (BHI) medium. We have previously reported that PBSX prophage-encoded lytic enzymes trigger cell lysis and the MV production upon DNA damage (Toyofuku *et al*., 2017). In this study, we aim to clarify the mechanism of the MV production in the BHI medium. In *Clostridium perfringens*, MV production depends on Spo0A (Obana *et al*., 2017), which is the highly conserved master regulator of sporulation and biofilm formation in Gram-positive spore formers. We confirmed the involvement of Spo0A in the MV production in *B. subtilis*. Moreover, we show that a global transcriptional regulator AbrB, which acts downstream of Spo0A, negatively regulates the MV production. Out of a number of genes controlled by the Spo0A-AbrB regulatory cascade, we found that the biosynthesis genes for sublancin is required for the MV production. Because sublancin has a bactericidal activity, the MV production is inferred to be associated with sibling killing by the antimicrobial and the consequent cell lysis. We will also discuss the mechanism of action of sublancin.

P-56

YabG is a novel arginine-specific cysteine protease

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In order to characterize the probable protease gene *yabG* found in the genomes of spore-forming bacteria, *Bacillus subtilis yabG* was expressed as a 35 kDa His- tagged protein (BsYabG) in *Escherichia coli* cells. During purification using Ni-affinity chromatography, the 35 kDa protein was degraded via several intermediates to form a 24 kDa protein. Furthermore, it was degraded after an extended incubation period. The effect of protease inhibitors, including certain chemical modification reagents, on the conversion of the 35 kDa protein to the 24 kDa protein was investigated. Reagents reacting with sulfhydryl groups exerted significant effects strongly suggesting that the *yabG* gene product is a cysteine protease with autolytic activity. Site-directed mutagenesis of the conserved Cys and His residues indicated that Cys218 and His172 are active site residues. No degradation was observed in the C218A/S and H172A mutants. In addition to the chemical modification reagents, benzamidine inhibited the degradation of the 24 kDa protein. Determination of the N-terminal amino acid sequences of the intermediates revealed trypsin-like specificity for YabG protease. Based on the relative positions of His172 and Cys218 and their surrounding sequences, we propose the classification of YabG as a new family of clan CD in the MEROPS peptidase database.

P-57

Exploration of D-amino acid producing lactic acid bacteria by bioassay using *Bacillus subtilis* mutants.

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Most naturally occurring D-amino acids are L-amino acids, but D-amino acids are known to occur in fermented foods. The D-amino acids in them are produced by bacteria, particularly lactic acid bacteria, which have been reported to produce free D-amino acids extracellularly. We constructed a bioassay system using *Bacillus subtilis* to search for lactic acid bacteria that produce D-amino acids that can be used in fermented milk such as yogurt. First, a D-alanine (Ala)-requiring mutant was obtained by disrupting the alanine racemase gene *alr* in *B*. *subtilis*. We then tested whether the mutant could grow in culture supernatants from lactic acid bacteria grown in MRS broth. We found that 5 strains of *Lactiplantibacillus plantarum* produced D-Ala in the supernatant of MRS broth. These five strains were cultured on reconstituted skim milk, and the D-amino acids in the culture supernatant were quantified by UHPLC and found to produce not only D-Ala but also D-leucine and D-arginine. The genetic lineage of these D-amino acid producing strains was assessed by MLSA and found not to be restricted to any particular lineages.

P-58

Isolation of the suppressor mutations that restore the growth of *zwf* mutant in *B. subtilis*

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Pentose phosphate (PP) pathway is one of the major sources of reducing power NADPH and metabolic intermediates for biosynthetic processes. The disruption of *zwf* gene encoding glucose-6-phosphate dehydrogenase (the first enzyme in PP pathway) causes a significant decrease in the intracellular NADPH pool. In *B. subtilis*, a *zwf* mutant showed inoculum-dose-dependent growth. Although the *zwf* mutant grew in LB medium with 1% (vol/vol) of the inoculum, a lower-dose inoculum (below 0.1%) caused a decrease in the growth frequency. This observation suggests that cell growth starting from a lower cell density might require much more NADPH for anabolism. To investigate the correlation between intracellular NADPH and cell growth, I attempted to isolate the spontaneous suppressor mutant from a *zwf* mutant.

When the overnight culture of *zwf* mutant was diluted 1:1000 or 1:10000 and plated onto LB agar plate, the possible suppressor mutants appeared. Eighty-two colonies were further tested for the growth with 0.1% of the inoculum. Out of 82 colonies, five mutants were selected and used for genome sequencing analysis. Then, seven mutations were found in *adeR*, *relA*, *yqeZ*, *lysP*, *ccpA*, *trmF*, or *thrS* gene. Transformation analysis revealed that these mutations except for *adeR* mutation can restore the growth of *zwf* mutant. Therefore, six mutation were identified as the suppressor mutation of *zwf* mutation.

P-59

Dual-wield NTPases: An uncharacterized protein family mined from AlphaFold Protein Database conserved among *Bacilli* and *Clostridia*

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AlphaFold protein structure database (AlphaFold DB) archives a vast number of predicted models. We conducted systematic data mining against AlphaFold DB and discovered an uncharacterized P-loop NTPase family among *Bacilli* and *Clostridia*. The structure of the protein family was surprisingly novel, showing an atypical topology for P-loop NTPases, noticeable two-fold symmetry, and two pairs of independent putative active sites. However, the biological function of dwNTPases remains completely unknown although their highly novel structure suggests they might possess unique functions. We would like to discuss the potential molecular functions of dwNTPases implied by their unique structures.

P-60

Identification of a minimum set of tRNA repertoire in *B. subtilis*

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According to the orthodox base-pairing and wobbling theory, it is generally assumed that 35-50 species of anticodon are involved in the translation of non-parasitic bacteria. On the other hand, knowledge of tRNA repertoires that are essential for translation and viability is limited to some specific organisms and systems. To identify the minimum set of tRNA species and to understand the functional role of individual tRNA genes in bacteria, we attempted to construct tRNA deletion mutants of *B. subtilis*, a model gram-positive bacterium.

The *B. subtilis* 168 genome encodes tRNA genes for 35 species of the anticodon. By replacing each tRNA gene with an antibiotic-resistance gene, single-deletion mutants for eight species of the anticodon were constructed. The growth of the majority of the mutants was not affected under the typical laboratory growth condition. The beta-galactosidase reporter assay revealed that the translational activity was not impaired by the tRNA deletions. We also constructed multi-gene deletion mutants by combining selected tRNA and modification enzymes responsible for the wobble position of the anticodon; the resulting strains were viable and showed efficient beta-gal activities. These results suggested the robustness of the *B. subtilis* cells to the limited repertoire of the anticodon.

P-61

Comparative Analysis of Growth Retardation Effects on *Bacillus subtilis* Spores via Diverse Microbial Control Methods: Heat, Gamma-Rays, UV, and Essential Oils from Spices for the Development of Optimal Combined Processes

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The growth of bacterial spores involves distinct stages, including germination, post-germination growth, and subsequent nutrient proliferation, which can be monitored through turbidity measurements during liquid cultivation. In this study, we investigated the effects of sterilization treatments on each stage of spore development using *Bacillus subtilis* 168. Specifically, we examined the impact of heat treatment and the co-presence of Carvacrol or thymol on spore germination, post-germination growth, and nutrient proliferation. Heat treatment was found to significantly suppress the germination process, while Carvacrol and thymol exhibited inhibitory effects on both germination and subsequent nutrient proliferation. Conversely, gamma radiation did not exhibit significant inhibitory effects on spore germination but showed suppression during post-germination growth and nutrient proliferation stages. Furthermore, the combined treatment of gamma radiation and ultraviolet (UV-C) radiation, known for inducing genomic DNA damage and cellular lethality, displayed the potential for synergistic effects.

P-62

Novel approach to the genome (giant DNA) synthesis

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In this century, with the advent of genome editing technologies, we are moving towards an era where we can "modify and utilize " genomes and even "freely design and synthesize'' them. Genetic engineering developed using plasmid vectors in *Escherichia coli* focusing on a few kb-sized DNA. However, manipulation for large-sized DNA like genome, which are much larger in size, requires unconventional approaches. This is because large sized DNAs are more susceptible to physical damage, and large-sized DNAs are not suitable for *in vitro* manipulations involving pipetting. The double-helix structure of DNA forms long polymers, and as they become longer, they are more prone to shear stress from fluid flow in solution, making it challenging to handle long intact DNA in solution. Experience has shown that when the size exceeds 200 kb, even a single pipetting may cause damage. For this reason, the method of joining DNA fragments of a several kb "in the cells of the microorganism" has been commonly used for genome synthesis. A typical example is the budding yeast Gap repair cloning (GRC) method, which takes advantage of the excellent homologous recombination ability of budding yeast to efficiently and easily assemble multiple fragments. However, it is becoming clear in our studies that this method is not necessarily universal. Therefore, we reevaluated the GRC method using budding yeast and devised a novel approach using *Bacillus subtilis*. In this report, we describe the advantages and possibilities of using *B. subtilis* compared to the GRC method using budding yeast.

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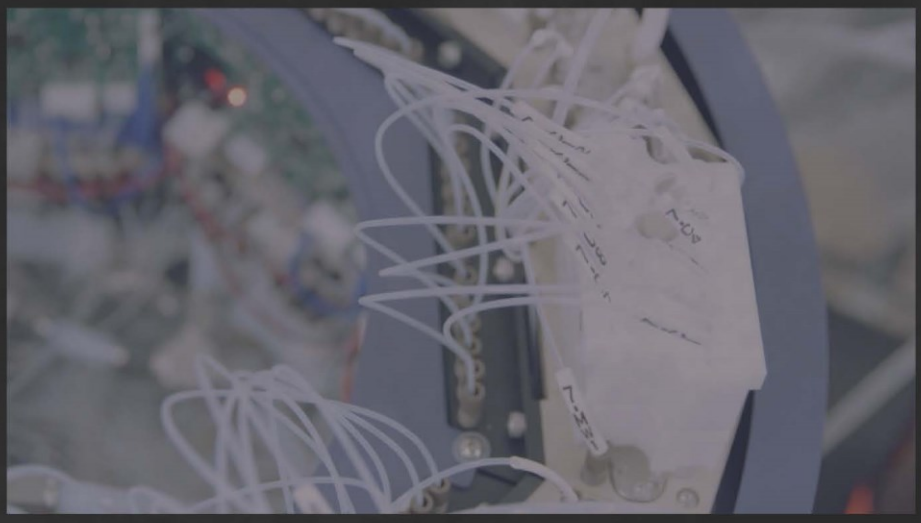
Tsuge, K. et al. Sci. Rep., 5, 10655 (2015), Tsuge K. et al. Nucleic Acids Res., 31:e133 (2003)



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