A bacterial pathogen sheds its cell wall during the transition into VBNC state

Alessandro Pagliuso

7th Symposium
The Dynamics of Peptidoglycan Structure and Function: New Insights into the 'Great Wall'
EMBO workshop

Hotel Vila Galé Sintra, Portugal
September 18-20th, 2023

The organising Committee

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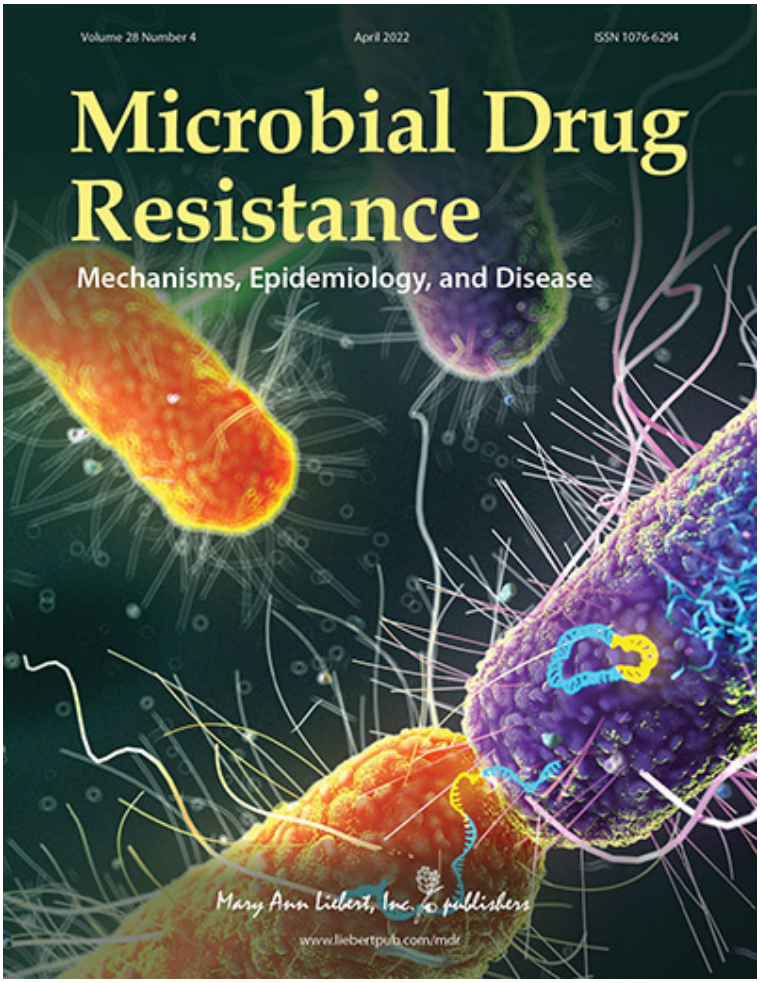

The journal Microbial Drug Resistance has been a privileged partner of the cell wall community even before the creation of the Great Wall Symposium. The former Editor-in-Chief, Alexander Tomasz, a leader in the cell wall and antibiotic field, founded the journal in 1995. At the same time, he co-organized a seminal cell wall meeting occurred in Lago di Garda, Italy. The many participants of the meeting contributed to a special issue of the 1995 meeting promoting the field with many landmark papers. Unfortunately, the meeting did not have any follow-up.

When the Great Wall Symposium was created in 2011 following the 2010 Baeza meeting "The Dynamics of Peptidoglycan Structure and Function: New Insights into the Great Wall" organized by Miguel de Pedro, Joe Dillard and Margaret McFall-Ngai, Alexander Tomasz suggested to have a special issue of the Great Wall Symposium in Microbial Drug Resistance. Since, several special issues have been published on work presented at the Great Wall Symposium.

This year is no exception and there will be a special issue of the journal Microbial Drug Resistance for publication of original work and reviews related to GWS2023. Submission of papers will open in September 2023 for a publication of the special issue first semester of 2024.

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Scientific Program

Sunday, September 17th, 2023

17:00-20:00 Registration

Day 1
Monday, September 18th, 2023

08.00 - 09.00 Registration

Session 1. Building the wall (Chair: Natividad Ruiz, Ohio State Univ, USA)

09.00 - 09.30 Irina Shlosman (Harvard Univ, USA)
Allosteric activation of cell wall synthesis during bacterial growth
09.30 - 10.00 Anke Becker (Philipps-Universität Marburg, Germany)
Unipolar cell wall growth in alphaproteobacterial Hyphomicrobiales: setting the growth zone

10.00 – 10.15 Cécile Morlot (Institut de Biologie Structurale, France)
Click and Collect at high resolution to unlock the secrets of cell wall synthesis
10.15 – 10.30 Nika Pende (Univ Vienna, Austria)
Discovery and characterization of the first two components of the elongasome in archaea with a pseudo-peptidoglycan cell wall

10.30 Coffee break & Posters

11.00 - 11.30 Jeanne Salje (Cambridge Univ, UK)
Peptidoglycan in obligate intracellular bacteria
11.30 - 12.00 Simonetta Gribaldo (Institut Pasteur, France)
One or two membranes? Illuminating the evolution of the cell envelope across the Tree of Bacteria

12.30 Lunch

Afternoon break
15.00 - 16.30 Coffee & Posters

Session 2. Modifying the Wall (Chair: Elitza Tocheva, Univ British Columbia, Canada)

16.30- 17.00 Dominique Missiakas (Univ Chicago, USA)
Peptidoglycan polymerization and cleavage: an enzyme complex to regulate the length of glycan strands in *S. aureus*
17.00 - 17.30 Felipe Cava (Univ Umea, Sweden)
Genome-wide peptidoglycan profiling of *Vibrio cholerae*

17.30 – 17.45 Juan Hermoso (Instituto de Química-Física “Rocasolano”, Spain)
Structural and functional characterization of the Pneumococcal surface protein Spr1875. An unprecedented murein-modifying enzyme
17.45 – 18.00 Quynh Mai Nguyen (Institut de Biologie Structurale, France)

Teichoic acids and peptidoglycan synthesis at the nanoscale

18.00 - 18.30 Tobias Dörr (Cornell Univ, USA)
Regulation of cell wall turnover in Gram-negative pathogens
18.30 - 19.00 Xavier De Bolle (Univ Namur, Belgium)
Structure and growth of the *Brucella abortus* envelope

19.00 Keynote Lecture
Simon Foster (Univ Sheffield, UK)
The Bacterial Cell Wall in Life and Death

Day 2
Tuesday, September 19th, 2022

Session 3. Interactions with the wall (Chair: Sérgio Filipe, Univ Nova Lisboa, Portugal)

09.00 - 09.30 Laure El Chamy (Univ Saint Joseph, Lebanon)
Modulation of innate immune defenses by Bacilli: A *Drosophila melanogaster* perspective
09.30 - 10.00 Ethel Bayer-Santos (Univ São Paulo, Brazil)
Enemy fire: *Salmonella* T6SS antibacterial effectors targeting the cell envelope

10.00 – 10.15 Richard Wheeler (Institut Pasteur, France)
Investigating the intestinal microbiota peptidoglycome and its dissemination across the gut epithelial barrier
10.15 – 10.30 Renata C Matos (Institut de Génomique Fonctionnelle de Lyon, France)
Structure-Function analysis of *Lactiplantibacillus plantarum* DltE reveals D-alanylated lipoteichoic acids as direct cues supporting Drosophila juvenile growth

10.30 Coffee break & Posters

11.00 - 11.30 Qiao Yuan (Nanyang Technological Univ, Singapore)
Impact of microbiota-derived peptidoglycan on infections by *Candida albicans*
11.30 - 12.00 Bavesh Kana (Univ Witwatersand, Sout Africa)
A modified BCG with depletion of enzymes associated with peptidoglycan amidation induces enhanced protection against tuberculosis in mice

12.30 Lunch

Afternoon break
15.00 - 16.30 Coffee & Posters

Session 4. Inhibition of the wall (Chair: Ivo G Boneca, Institut Pasteur, France)

16.30- 17.00 Martin J Loessner (EHT Zurich)
The Great Escape: how L-form conversion and lack of wall saves bacteria from drugs and viruses
17.00 - 17.30 Luiz Pedro Carvalho (Crick Institute, UK)
Mechanistic studies of antibiotic-target engagement

17.30 – 17.45 Kelvin Kho (Institut Pasteur, France)
AI-assisted identification of bacterial cell shape determinants for antibiotic discovery
17.45 – 18.00 Camilla Henriksen (Univ Copenhagen, Denmark)
The role of the PBPs in life and death: new clues to a still unsolved mystery

18.00 - 18.30 Jean-Emmanuel Hugonnet (Sorbonne Univ, France)
Genome wide identification of gene required for alternative peptidoglycan cross-linking in *Escherichia coli* revealed unexpected impacts of beta-lactams
18.30 - 19.00 David Sychantha (McMaster Univ, Canada)
New glycopeptide antibiotics that block cell wall synthesis and degradation

20.00 Cocktail and Gala Diner

Day 3
Wednesday, September 21st, 2023

Session 5. Regulating the Wall (Chair: Orietta Massidda, Univ Trento, Italy)

09.00 - 09.30 Dennis Claessen (Leiden Univ, The Netherlands)
Regulation of cell wall formation in filamentous actinobacteria under hyperosmotic stress conditions
09.30 - 10.00 Beate Henrichfreise (Univ Bonn, Germany)
Tightly controlled - the septal peptidoglycan ring in *Chlamydia*

10.00 – 10.15 Tyler Sisley (Harvard Univ, USA)
A Staphylococcal protein controls global phosphorylation in response to cell envelope stress
10.15 – 10.30 Andrea Dessen (Brazilian Biosciences National Laboratory, Brazil)
Architecture and genomic arrangement of the MurE-MurF bacterial cell wall biosynthesis complex

10.30 Coffee break & Posters

11.00 - 11.30 Malcolm Winkler (Univ Indiana, USA)
Regulation of peptidoglycan synthesis at the pathway and protein interaction levels in ovoid-shaped *Streptococcus pneumoniae*
11.30 - 12.00 Sven Halbedel (Robert Koch Institute, Germany)
Regulation of *Listeria monoctyogenes* peptidoglycan biosynthesis by an essential protein phosphorylation

12.30 Lunch

Afternoon break
15.00 - 16.30 Coffee & Posters

Session 6. Coordinating the Wall (with the cell cycle) (Chair: David Roper, Univ Warwick, UK)

16.30 - 17.00 Natalia Luisa Hiller (Carnagie Mellon, Univ, USA)
Much More Than a Wall: The role of the Gram-Positive peptidoglycan in stress response

17.00 - 17.30 Pamela Brown (Univ Missouri, USA)
Interactome of PBP1a reveals a putative elongasome in *Agrobacterium tumefaciens*

17.30 – 17.45 Helena Veiga (Instituto de Tecnologia Química e Biológica António Xavier, Portugal)
Cell division protein FtsK coordinates bacterial chromosome segregation and daughter cell separation
17.45 – 18.00 Lok-To Sham (National Univ Singapore , Singapore)
The divisome but not the elongasome organizes capsule synthesis in *Streptococcus pneumoniae*

18.00 - 18.30 Seamus Holden (Univ Warwick, UK)
Elucidating the role of the bacterial cytoskeleton in cell division: FtsZ treadmilling drives cell division by promoting Z-ring condensation and septal constriction initiation
18.30 - 19.00 Allister Crow (Univ Warwick, UK)
Structural basis for peptidoglycan hydrolase activation during bacterial cell division

19.00 Closing of the meeting and selection of the next venue

Keynot Lecture

Bacterial Cell Wall Architecture and Dynamics: A Matter of Life and Death

Simon J. Foster

University of Sheffield, Sheffield, UK

The shape and integrity of bacteria are determined by cell wall peptidoglycan, a single macromolecule that surrounds the cell. The synthesis of peptidoglycan is also the site of action of important antibiotics such as penicillin and vancomycin. Thus, bacterial viability, growth and division are dependent on the architecture and dynamics of this essential polymer. We use a range of high-resolution microscopy approaches, coupled with biochemical, genetic and physiological approaches to reveal peptidoglycan architecture, and its dynamics, using the major human pathogen *Staphylococcus aureus* as our primary organism. Atomic force microscopy has demonstrated a complex, nanoscale peptidoglycan architecture in diverse species, which meets the challenges of maintaining viability and growth within their environmental niches, by exploiting the bioengineering versatility of the polymer. The application of super-resolution fluorescence microscopy, coupled with new chemical probes has begun to reveal how the polymer is synthesized and hydrolysed during growth and division. We have also used these approaches to determine the mode of action of antibiotics and molecular mechanisms of antimicrobial resistance.

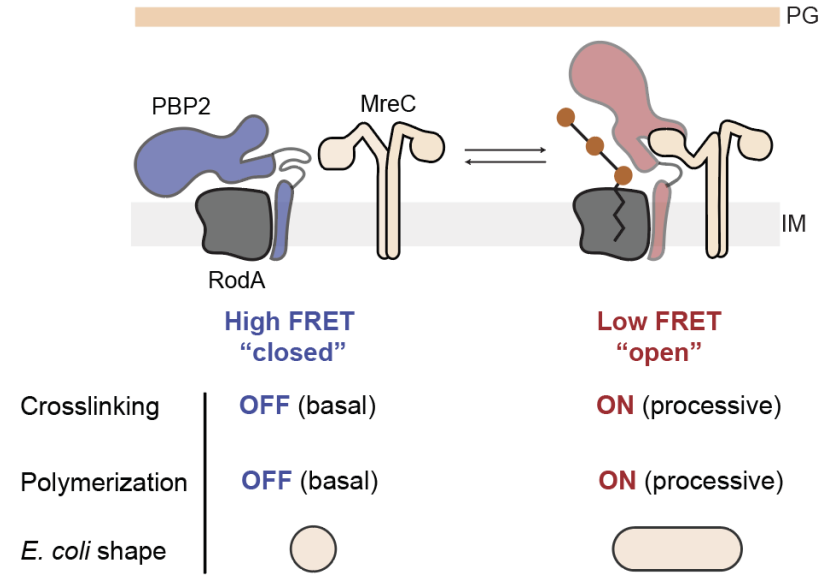
Speakers Abstracts

Allosteric activation of cell wall synthesis during bacterial growth

Irina Shlosman[1], Elayne M. Fivenson[2], Morgan S.A. Gilman[1], Tyler A. Sisley[2], Suzanne Walker[2], Thomas G. Bernhardt[2], Andrew C. Kruse[1] (andrew_kruse@hms.harvard.edu), Joseph J. Loparo[1] (joseph_loparo@hms.harvard.edu)

[1]Department of Biological Chemistry and Molecular Pharmacology, Blavatnik Institute, Harvard Medical School, Boston, Massachusetts 02115, USA.
[2]Department of Microbiology, Blavatnik Institute, Harvard Medical School, Boston, Massachusetts 02115, USA.

The peptidoglycan (PG) cell wall protects bacteria against osmotic lysis and determines cell shape, making this structure a key antibiotic target. Peptidoglycan is a polymer of glycan chains connected by peptide crosslinks, and its synthesis requires precise spatiotemporal coordination between glycan polymerization and crosslinking. However, the molecular mechanism by which these reactions are initiated and coupled is unclear. Here we use single-molecule FRET and cryo-EM to show that an essential PG synthase (RodA-PBP2) responsible for bacterial elongation undergoes dynamic exchange between open and closed states. Our findings reveal that structural opening acts as an allosteric switch, simultaneously activating polymerization and elevating the active site of PBP2 to enable crosslinking. Using mutants that preferentially adopt either the open or the closed state, we demonstrate that bacterial viability and shape can be precisely tuned by altering PBP2 dynamics and that MreC, an essential physiological partner of PBP2, is the likely regulator of this motion. Collectively, this work highlights the central role that PBP2 dynamics play in the initiation and regulation of PG synthesis. Given the high conservation of SEDS-bPBP synthases, this conformational switch likely represents a conserved regulatory mechanism that controls activation of PG synthesis during other cellular processes, including cell division.



MreC regulates PG synthesis through structural dynamics. Schematic view of the proposed model of PG synthesis, in which PBP2 structural dynamics act as an allosteric switch, coupling polymerization and crosslinking. MreC recruits the PG synthase and promotes structural opening, activating synthesis.

Unipolar cell wall growth in alphaproteobacterial Hyphomicrobiales: setting the growth zone

Anke Becker

Center for Synthetic Microbiology (SYNMIKRO), Philipps-Universität Marburg, Marburg, Germany

Bacterial proliferation requires the coordination of cell growth and division. Both involve expansion of the peptidoglycan (PG) sacculus by synthesis and incorporation of new PG, driven by the elongasome and the divisome, both protein complexes that contain shared but also distinct components. Cell elongation of most rod-shaped bacteria takes place in a dispersed manner along the sidewall, using filaments of the actin homolog MreB as scaffold for the PG biosynthesis machinery. However, some rod-shaped bacteria show cell elongation restricted to the new cell pole generated by cell division. In the alphaproteobacterial Hyphomicrobiales this property correlates with absence of MreB and presence of Rgs (rhizobial growth and septation) proteins essential for cell growth and localizing to the septal site and/or the new cell pole (Krol et al. 2020). These bacteria include e.g. *Sinorhizobium meliloti*, *Agrobacterium fabrum*, and *Brucella abortus.* We investigated the role of Rgs and other cell pole organizing proteins in determining cell polarity and PG growth zone localization in *S. meliloti*. Previous (Krol et al. 2021) and more recent insights into this process will be presented.

Krol E, Yau HCL, Lechner M, Schäper S, Bange G, Vollmer W, Becker A (2020) Tol-Pal system and Rgs proteins interact to promote unipolar growth and cell division in *Sinorhizobium meliloti*. mBio 11: e00306-20.
Krol E, Stuckenschneider L, Kästle J, Graumann PL, Becker A (2021) Stable inheritance of *Sinorhizobium meliloti* cell growth polarity requires an FtsN-like protein and an amidase. Nat Commun 12: 545.

Peptidoglycan in obligate intracellular bacteria

Sharanjeet Atwal[a], Suthida Chuenklin[a], Edward M. Bonder[b], Juan Flores[b], Joseph J. Gillespie[c], Timothy Driscoll[d] and Jeanne Salje[a,e,f]

Public Health Research Institute, Rutgers the State University of New Jersey, Newark, USA[a]
Department of Biological Sciences, Rutgers University, Newark, NJ 07102[b]
Department of Microbiology and Immunology, School of Medicine, University of Maryland Baltimore, MD 21201[c]
Department of Biology, West Virginia University, Morgantown, West Virginia, USA[d]
Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand[e]
Cambridge University, Department of Pathology, Department of Biochemistry, Cambridge Institute for Medical Research, United Kingdom[f]

The peptidoglycan sacculus is essential in almost all bacterial species. It protects cells from high internal turgor pressure and maintains cell shape. However, it is also a potent microbe-associated molecular pattern (MAMP) recognised by peptidoglycan recognition proteins across the plant and animal kingdom. Obligate intracellular bacteria are under selective pressure to evolve strategies to avoid being detected by intracellular peptidoglycan recognition proteins, or to block the downstream anti-bacterial pathways triggered by them.

We have sought to understand how obligate intracellular bacteria solve the challenge of building a peptidoglycan cell wall – required for growth and division – whilst evading a lethal response to this MAMP. In answering this question, we have also studied the minimal requirements for peptidoglycan synthesis, with implications for our broader understanding of peptidoglycan biology.

First, we have carried out extensive comparative genomics analyses and found that class A penicillin binding proteins (aPBPs) – normally a major driver of peptidoglycan polymerisation – have been independently lost in multiple lineages of obligate intracellular or host associated bacteria. This led to the hypothesis that building a sacculus in the absence of aPBPs results in a cell wall that benefits the host associated lifestyle, potentially through reduced immunogenicity. Second, we have carried out experimental studies on several obligate intracellular bacteria species within the order Rickettsiales. Natural variation within this lineage on the presence or absence of aPBPs and other peptidoglycan related genes have allowed us to determine the relationship between peptidoglycan synthesis genes and the presence, abundance, and immunogenicity of peptidoglycan.

One or two membranes? Illuminating the evolution of the cell envelope across the Tree of Bacteria

Simonetta Gribaldo

Institut Pasteur, France, Paris

The envelope is one of the oldest and most fundamental cell components, and the first interface between an organism and its environment. Bacteria are known to have either one membrane (Gram-positives or monoderms) or two membranes (Gram-negatives or diderms). How the transition between these two radically different envelopes occurred has been one of the most intriguing mysteries in evolutionary biology. We have recently tackled this question by merging large-scale phylogenomics approaches with the development of a new experimental diderm model belonging to the classical monoderm Firmicutes. Our results shake longstanding assumptions and show the power of continuous exploration of ever larger fractions of microbial diversity.

The lengths of LTA and peptidoglycan strands impact protein secretion and anchoring into the septal compartment of *S. aureus*

Dominique Missiakas

The University of Chicago, Department of Microbiology

Sortase A cleaves the C-terminal end of secreted surface protein precursors such as Staphylococcal protein A (SpA) and attaches these polypeptides to the peptidoglycan of *Staphylococcus aureus*. Sortase A binds LPXTG motif sorting signals, cleaves between threonine (T) and glycine (G) residues, and forms an acyl enzyme between its active-site cysteine thiol and the carboxyl group of threonine (T). Sortase A acyl enzyme intermediates are relieved by the nucleophilic attack of the cross bridge amino group within lipid II, thereby generating surface protein linked to peptidoglycan precursor. Such products are subsequently incorporated into the cell wall envelope by enzymes of the peptidoglycan synthesis pathway. Some of these preproteins, including SpA, also carry an unusually long signal sequence with a conserved YSIRK motif that directs secretion into the septal compartment of dividing cells. Earlier, we performed genetic and pulldown experiments that identified new factors involved in the secretion of preproteins with YSIRK and LPXTG sequences. We will discuss here how SagB and LyrA/SpdC, cleave polymerized glycan chains to their physiological length and impact the incorporation of sortase-anchored proteins into the cell wall. Further, we will discuss how LtaS restricts LTA assembly and secretion of YSIRK preproteins at the septum.

Genome-wide peptidoglycan profiling of *Vibrio cholerae*.

Sara B. Hernandez[1, #], Laura Alvarez[1, #], Barbara Ritzl-Rinkenberger[1], Clara Lambert[1], Emilio Bueno, Bastian Schiffthaler[2], Alonso Serrano[3] and Felipe Cava[1,4] .

1 The laboratory for Molecular Infection Medicine Sweden (MIMS), Department of Molecular Biology, Umeå University, Umeå, Sweden.
2 Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, Umeå, Sweden.
3 Codon Consulting AB
4 SciLifeLab, Sweden
# Contributed equally

Most bacteria cells are protected by a peptidoglycan cell wall. Defining the chemical structure of the peptidoglycan has been instrumental to characterize cell wall associated proteins and to illuminate the mode of action of cell wall-acting antibiotics. However, a major roadblock for a comprehensive understanding of peptidoglycan homeostasis has been the lack of methods to conduct large-scale, systematic studies. Here we have developed and applied an innovative high throughput peptidoglycan analytical pipeline to analyze the entire non-essential, arrayed mutant library of *Vibrio cholerae*. The unprecedented breadth of these analyses revealed that peptidoglycan homeostasis is preserved by a large percentage of the genome organized in complex networks that functionally link peptidoglycan features with genetic determinants. As an example, we discovered a novel bifunctional penicillin-binding protein in *V. cholerae*. Collectively, genome-wide peptidoglycan profiling provides a fast, easy, and unbiased method for systematic identification of the genetic determinants of peptidoglycan synthesis and remodelling.

Peptidoglycan endopeptidase regulation in Gram-negative bacteria

Tobias Dörr

Cornell University, Ithaca, New York

Most free-living bacteria surround themselves with a rigid cell wall composed mostly of peptidoglycan (PG). In order to grow, bacteria must be able to not only synthesize new PG material, but also cleave the existing PG meshwork to promote insertion of new material. PG cleavage is effected by a group of enzymes collectively termed "autolysins". Among the autolysins, the endopeptidases (EPs) play an important role in cell elongation. Intuitively, EP activity must be tightly controlled in order to prevent erroneous and potentially lethal PG cleavage, but how this is accomplished is poorly-understood. We have found that the major EP in *Vibrio cholerae* is controlled at three levels, including transcriptional and post-translational activation and processing. Using a genetic screen for factors mitigating toxicity of a permanently activated enzyme, we show that in addition to these three modes of regulation, ShyA is also controlled at the level of substrate availability, via a preference for PG tetrapeptide vs. pentapeptide. Taken together, our data reveal complex regulatory pathways for EPs, underscoring the importance of keeping autolysins in check to properly balance PG synthesis and degradation.

Structure and growth of the *Brucella abortus* envelope

Caroline Servais[1], Pierre Godessart[1], Victoria Vassen[1], Angéline Reboul[1], Marine Lacritick[2], Stéphane Vincent[2], Xavier De Bolle[1*]

[1]URBM, Dept. of Biology, University of Namur, Namur, Belgium
[2]CBO, Dept. of Chemistry, University of Namur, Namur, Belgium

Abstract
Bacteria of the *Brucella* genus are collectively responsible for a worldwide zoonosis called brucellosis. *Brucella abortus* is a Rhizobiale, part of the alpha-proteobacteria. We found that the most abundant proteins of the outer membrane, Omp2b and Omp25, are attached to the peptidoglycan through a N-terminal extension, with a short conserved motif crucial for the covalent linkage to *meso*-diaminopimelate. In the absence of such linkages, the outer membrane is blebbing. We identify a major enzyme required for these linkages, a L,D-transpeptidase called Ldt4.
The outermost structure of the envelope is the lipopolysaccharide (LPS). In *B. abortus* LPS is known to be poorly immunogenic and to be characterized by a branched structure in the core. The presence of a O-chain is crucial to avoid brutal phagocytosis by macrophages, and we identified the O-chain ligase as WadA, an original enzyme with two active sites on opposite sides on the inner membrane. Indeed, WadA can modify the core in the cytoplasm (as previously shown) and add the O-chain in the periplasm. Our data show that LPS is inserted at the new pole and then at the division site during the cell cycle. Since LPS is translocated from the inner membrane to the outer membrane through a Lpt (LPS transport) system, we localized components of this complex and we found that focalized insertion of LPS correlates with the position of Lpt proteins anchored in the inner membrane.
Localized incorporation of LPS can be demonstrated by the addition of 2-deoxy-D-manno-octulosonic acid (KDO) conjugated at an azido group at position 8 (KDO-N_3). We also show that mannose-N_3 (with an azido group at position 6) can be incorporated at a specific position of the LPS, in the lateral branch of the core. We also identified the enzymatic pathway allowing incorporation of mannose-N_3.
A better understanding of fundamental processes allowing the assembly of the *B. abortus* envelope will lead to new opportunities to fight this nasty but successful pathogen.

Modulation of innate immune defenses by *Bacilli*: A *Drosophila melanogaster* perspective

Zaynoun Attieh[1,2], Mireille Kallassy Awad[1], Aline Rifflet[3], Carine Mouawad[1], Agnès Rejasse[2], Pascal Courtin[2], Marie-Pierre Chapot-Chartier[2], Ivo Gomperts Boneca[3], Vincent Sanchis Borja[2] and Laure El Chamy[1]

1- UR-EGP, Faculté des sciences, Université Saint-Joseph de Beyrouth- Liban
2- Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350 Jouy-en-Josas, France.
3- Institut Pasteur, Université Paris Cité, CNRS UMR6047, INSERM U1306, Unité de Biologie et génétique de la paroi bactérienne F-75015, Paris, France.

At the interface of host-microbe interactions, host pattern recognition receptors patrol microbial signature molecules to trigger the expression of immune regulators and effectors. Prominent among these are potent cationic antimicrobial peptides acting at the frontline of host defenses to counter microbial survival and invasion. At each stage, microorganisms may engage intricate strategies to subvert host defenses thus acquiring the capacity to develop or persist in appropriate niches. This dynamic interplay between host and microbes defines the states of health or disease.

Using the genetically tractable model, *Drosophila melanogaster*, we aim at characterizing the strategies deployed by *Bacillus thuringiensis* to elude innate immune defenses. Our data emphasize several mechanisms used by this bacterium to resist antimicrobial peptides. Of these, D-alanylation of teichoic acids stands out with an additional capacity of hampering the sensing of peptidoglycan by cognate peptidoglycan recognition receptors. We further show that this strategy is also adopted by *Lactobacillus plantarum* for its persistence facing cationic effectors in the gut but also for the modulation of its immune-stimulatory potential as a core component of the Drosophila microbiota. Our data support a model whereby D-alanylation of teichoic acids impedes the accessibility of cationic lysozyme to peptidoglycan thus limiting the release of peptidoglycan immunostimulatory moieties. Altogether, our data pinpoint D-alanylation of teichoic acids as a dual microbial strategy employed by a pathogen and a commensal alike to elude and resist immune defenses. These data further underline the complex interplay between microbe and host factors that influences microbe sensing and the ensuing immune reactions.

Enemy fire: Salmonella T6SS antibacterial effectors targeting the cell envelope

Ethel Bayer-Santos

Universidade de São Paulo, Brazil

The colonization of the gastrointestinal tract of mammalian hosts by Salmonella is an excellent model to study (micro)biological conflicts, with examples of interactions between microbes and microbes and host cells. The antibacterial effectors secreted via the T6SS nanoweapon are important for the pathogen to overcome colonization resistance and establish an infection. These effectors comprise a pool of proteins with interesting activities that can reveal exciting new biology. The group of Dr. Bayer-Santos contributed to the discovery of novel antibacterial molecules with unique biochemical activities affecting the cell wall and some of these examples will be presented.

Impact of microbiota-derived peptidoglycan on infections by *Candida albicans*

Qiao Yuan

Nanyang Technological Univ, Singapore

Candida albicans is the major opportunistic fungal pathogen in humans. Normally residing in the host gut niche as a benign yeast state, *C. albicans* can transform into long filamentous hyphae that facilitate its mucosal penetration and bloodstream dissemination, causing deadly infections in the host. Among the numerous signals that trigger *C. albicans* hyphal growth, bacterial peptidoglycan fragments (PGNs) represent the most potent inducers. Thus, addressing the mechanistic details of PGN-induced *C. albicans* invasive growth may lead to novel insights to combat this deadly fungus.

In this talk, I will present our recent series of studies on gut microbiota-derived PGNs in triggering *C. albicans* hyphal growth. Using the mouse model, we showed that upon the oral intake of beta-lactam antibiotics, the host's gut microbiota suddenly releases a massive amount of PGNs, termed 'the PGN storm' in the gut niche, which in turn contributes to *C. albicans* invasive growth and systemic infections *in vivo*. Our work established bacterial PGNs as the underlying mechanistic link that explains the clinical observations of broad-spectrum beta-lactam drugs as a common risk factor for *C. albicans* infections. To further elucidate the PGN recognition and uptake mechanisms in *C. albicans*, we developed versatile chemoenzymatic PGN probes that bear photoaffinity, and bio-orthogonal or fluorescent functionality, respectively. Using molecular docking and biochemical assays, we identified the putative PGN binding site on the sensor protein adenylyl cyclase (Cyr1) in *C. albicans*, shedding key structural insights for the development of potential antagonists against hyphal growth. Furthermore, we demonstrated that fluorescent PGN probes enter *C. albicans* cells via a receptor-mediated endocytic process. We are now pursuing the identities of the sought-after receptors/transporters in *C. albicans* responsible for bacterial PGN uptake, which could represent a novel class of anti-virulent targets in *C. albicans*.

A modified BCG with depletion of enzymes associated with peptidoglycan amidation induces enhanced protection against tuberculosis in mice

Moagi T. Shaku[1], Peter Um[2], Karl L. Ocius[3], Alexis J. Apostolos[3], Marcos M. Pires[3], William R. Bishai[2] and Bavesh D. Kana[1,4,]

[1] DST/NRF Centre of Excellence for Biomedical TB Research, Faculty of Health Sciences, University of the Witwatersrand, National Health Laboratory Service, Johannesburg, 2001, South Africa
[2] Johns Hopkins School of Medicine, Center for Tuberculosis Research, Department of Medicine, Division of Infectious Diseases, Baltimore, Maryland, United States
[3] Department of Chemistry, Lehigh University, Bethlehem, Pennsylvania 18015, United States
[4] MRC-CAPRISA HIV-TB Pathogenesis and Treatment Research Unit, Centre for the AIDS Programme of Research in South Africa, CAPRISA, Durban, South Africa

Abstract

Mechanisms by which *Mycobacterium tuberculosis* (Mtb) evades pathogen recognition receptor activation during infection may offer insights for the development of improved tuberculosis (TB) vaccines. Whilst Mtb elicits NOD-2 activation through host recognition of its peptidoglycan-derived muramyl dipeptide (MDP), it masks the endogenous NOD-1 ligand through amidation of glutamate at the second position in peptidoglycan sidechains. As the current BCG vaccine is derived from pathogenic mycobacteria, a similar situation prevails. To alleviate this masking ability and to potentially improve efficacy of the BCG vaccine, we used CRISPRi to inhibit expression of the essential enzyme pair, MurT-GatD, implicated in amidation of peptidoglycan sidechains. We demonstrate that depletion of these enzymes results in reduced growth, cell wall defects, increased susceptibility to antibiotics and altered spatial localization of new peptidoglycan. In cell culture experiments, training of monocytes with this recombinant BCG yielded improved control of Mtb growth. In the murine model of TB infection, we demonstrate that depletion of MurT-GatD in BCG, resulting in unmasking of the D-glutamate diaminopimelate (iE-DAP) NOD-1 ligand, yields superior prevention of TB disease compared to the standard BCG vaccine. This work demonstrates the feasibility of gene regulation platforms such as CRISPRi to alter antigen presentation in BCG in a bespoke manner that tunes immunity towards more effective protection against TB disease.

The great escape: how L-form conversion and lack of a wall saves bacteria from drugs and viruses

Martin J. Loessner

Institute for Food, Nutrition and Health, ETH Zurich,

Bacteriophages are known to kill bacteria through osmotic lysis during the lytic cycle. However, in the case of Gram-positive bacteria, the release of peptidoglycan-degrading endolysins at the end of the infection cycle not only leads to explosive cell lysis of the infected host but can also affect neighboring non-infected bystander cells. We investigate how *Listeria monocytogenes* can evade phage predation by undergoing a transient conversion to a cell wall-deficient L-form state in osmotically stabilized environments. The triggering factor for this L-form escape is not phage itself, but the release and accumulation of endolysins during repeated phage infection cycles, which disintegrate the cell wall from the outside. This process induces lesions in the cell walls of non-infected bystander cells, resulting in the turgor-driven extrusion of wall-deficient, yet viable L-form cells. Our findings demonstrate that phage infection and cell lysis can induce L-form conversion among phage-sensitive bacterial populations, providing resistance to further infection.
Remarkably, in the absence of phage predation and endolysin-based selective pressure, we observed a rapid reversion of L-forms to the walled state. This suggests that L-form conversion also serves as a population-level persistence mechanism to evade complete eradication by phage attacks. Furthermore, we extend our observations beyond *L. monocytogenes* to include *Enterococcus faecalis*, a urinary tract pathogen, where we also demonstrate phage-mediated L-form switching during culture in human urine
Collectively, our results indicate that Gram-positive bacteria can evade phage predation at the population level through the transient switching of subpopulations to the L-form state. This escape mechanism may be widespread and has important implications for the development of phage- and endolysin-based therapeutic interventions.

Mechanistic studies of antibiotic-target engagement

Luiz Pedro Carvalho[1,2]

[1] Chemistry Department, The Herbert Wertheim UF Scripps Institute for Biomedical Innovation and Technology, Jupiter, Florida, United States
[2] Mycobacterial Metabolism and Antibiotic Research Laboratory, The Francis Crick Institute, London, United Kingdom

The bacterial cell wall continues to represent a key target for the discovery of novel antibacterial agents, including in mycobacteria such as *Mycobacterium tuberculosis* and *Mycobacterium abscessus*. The same is true for the enzymes that synthesize the building blocks required for peptidoglycan synthesis and remodeling. This talk will cover our decade long mechanistic investigation to understand the interaction of the antibiotic D-cycloserine with its two targets alanine racemase and D-Ala:D-Ala ligase, and how and why *M. tuberculosis* fails to develop resistance to this antibiotic. These efforts led to the discovery of target-mediate antibiotic resistance, which has now been observed in another cell wall-related enzyme. Together, these findings challenge the concept of irreversible inhibition, often used in drug discovery. Finally, I will briefly describe new avenues my groups are taking in these areas.

Genome wide identification of gene required for alternative peptidoglycan cross-linking in *Escherichia coli* revealed unexpected impacts of beta-lactams

Jean-Emmanuel Hugonnet, Michel Arthur, Henri Voedts.

Centre de Recherche des Cordeliers (Sorbonne Université, INSERM, Université Paris-Cité), 75006 Paris FRANCE.

Penicillin Binding Proteins (PBPs) have been extensively studied for the past decades. However, the last step of peptidoglycan precursors polymerization can also be performed by another family of enzymes, the L,D-transpeptidases (LDTs). These enzymes have an important role in the maturation of the cell-wall of mycobacteria and they are targets for pathogen-specific drug-development. Unlike PBPs, LDTs are not inhibited by beta-lactams and can lead to resistance to these antibiotics in laboratory mutants of *Enterococcus faecium* and *Escherichia coli* by bypassing the β-lactam sensitive PBPs. A Tn-Seq approach identified 179 genes that were selectively essential for LDT-mediated β-lactam resistance in *E. coli*. The functions of these genes extend far beyond LDT partners to include proteins involved in stress responses and in the assembly of outer membrane polymers. These genes are essential to mitigate the effects of PBP inactivation by β-lactams. In spite of expression of resistance to β-lactams, these drugs inhibit the anchoring of the Braun Lipoprotein to the peptidoglycan, destabilize the envelope, and increase the permeability of the outer membrane. The latter effect implies that the mode of action of β-lactams involves self-promoted penetration through the outer membrane.

New glycopeptide antibiotics that block cell wall synthesis and degradation

David Sychantha

McMaster Univ, Canada

Type V glycopeptide antibiotics (GPA) are microbial natural products that act as bacteriostatic agents and target the mature peptidoglycan (PG) layer of Gram-positive bacteria. By binding to the outer layers of PG, these agents perturb the remodelling of the cell wall and preclude cell division. Several new members of the type V GPA family have recently been identified; still, their precise PG binding site and biochemical impact on specific enzymes involved in PG hydrolysis and biosynthesis are unknown. To test hypotheses relating to type V GPA binding and specificity, we generated soluble PG ligands for biochemical studies, including enzyme inhibition assays and isothermal titration calorimetry. Our work using these approaches indicates that the glycan chain of PG is the primary motif recognized and bound by type V GPAs. The interaction with the glycan chain of PG provides critical insight into the mode of action of type V GPAs and how they evade existing antibiotic resistance mechanisms, such as PG stem peptide modification (D-Lac substitutions in vancomycin-resistant bacteria). It supports a general model for PG dysregulation through broad autolysin inhibition via steric hindrance of mature PG polymers.

Regulation of cell wall formation in filamentous actinobacteria under hyperosmotic stress conditions

Xiaobo Zhong, Shraddha Shitut, Erik Vijgenboom, Dennis Claessen

Molecular Biotechnology, Institute of Biology, Leiden University, Leiden, The Netherlands

Filamentous actinobacteria are ubiquitous in almost all soil environments, where they are frequently exposed to environmental insults. Recent work indicates that some actinobacteria have a natural ability to shed their cell wall under influence of hyperosmotic stress, while others, such as the model organism *Streptomyces coelicolor*, appear unaffected. We here identify the stomatin-like protein StlP as a crucial factor for growth under hyperosmotic stress conditions. StlP localizes at hyphal tips, where it is important to spatially confine growth. In the absence of StlP, filaments start to branch frequently coinciding with a delocalized pattern of cell wall synthesis and a decreased membrane fluidity at hyphal tips. Surprisingly, filaments of the *S. coelicolor* Δ*stlP* mutant extrude cell-wall-deficient cells, while the constitutive expression of StlP in actinobacteria that naturally form such cells blocks their extrusion. Consistent with other stomatin proteins, we show that StlP oligomerizes and interacts with several proteins involved in tip growth. Altogether, these data indicate that StlP plays a central role in coordinating tip growth by organizing cell wall synthesis in localized microdomains and imply that stomatin-like proteins provide competitive advantage to actinobacteria that are frequently exposed to hyperosmotic stress.

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

Séamus Holden

University of Warwick, UK

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. We also found evidence that elongasome processivity and tug-of-war regulate *B. subtilis* cell size and shape. Our data thus demonstrate that molecular motor tug-of-war is a key regulator of elongasome activity in *B. subtilis*.

Dynamics of elongasome protein movement in ovoid-shaped *Streptococcus pneumoniae* D39

Amilcar J. Perez[1†], Melissa M. Lamanna[1†], Kevin E. Bruce[1†], Marc A. Touraev[1], John D. Richardson[1], Julia E. Page[2], Sidney L. Shaw[1], Ho-Ching Tiffany Tsui[1], and Malcolm E. Winkler[1].

[1]Department of Biology, Indiana University Bloomington, Bloomington, IN 47405; [2]Department of Microbiology, Blavatnik Institute, Harvard University Medical School, Boston, MA 02115. [†]Contributed equally.

Ovoid-shaped bacteria, such as *Streptococcus pneumoniae* (pneumococcus), have two spatially separated peptidoglycan (PG) synthase nanomachines that locate zonally to the midcell of dividing cells. The septal PG synthase bPBP2x:FtsW closes the septum of dividing pneumococcal cells, whereas the elongasome located on the outer edge of the septal annulus synthesizes peripheral PG outward. We showed previously by sm-TIRFm that the septal PG synthase moves circumferentially at midcell, driven by PG synthesis and not by FtsZ treadmilling. The pneumococcal elongasome consists of the PG synthase bPBP2b:RodA, regulators MreC, MreD, and RodZ, but not MreB, and genetically associated proteins Class A aPBP1a and muramidase MpgA. Given its zonal location separate from FtsZ, it was of considerable interest to determine the dynamics of proteins in the pneumococcal elongasome. We found that bPBP2b, RodA, and MreC move circumferentially with the same velocities and track durations at midcell, driven by PG synthesis. However, outside of the midcell zone, the majority of these elongasome proteins move diffusively over the entire surface of cells. Depletion of MreC resulted in loss of circumferential movement of bPBP2b, and bPBP2b and RodA require each other for localization and circumferential movement. Notably, a fraction of aPBP1a also moved circumferentially at midcell with a faster velocity and shorter track duration than core elongasome proteins. Other aPBP1a molecules were stationary at midcell or were diffusing over cell bodies. Last, MpgA displayed short bursts of fast circumferential movement and diffusive motion that was largely confined to the midcell region and not over the cell body.

Together these results demonstrate that unlike in rod-shaped bacteria, the pneumococcal core elongasome exhibits zonal circumferential motion that is independent of FtsZ treadmilling and the presence of MreB filaments and that is separate from the circumferential motion of the septal PG synthase closing the septal annulus. Also unlike in rod-shaped bacteria, a Class A PBP moves circumferentially at midcell, but separately from the core PG elongasome or septal PG synthase. What directs these linear, circumferential motions remains to be determined, but may involve ordered structure in the existing PG itself. In contrast, the MpgA muramidase displays a different kind of confined diffusive motion that is located at the midcell region by a mechanism that is also currently unknown.

PASTA kinase-dependent phosphorylation of ReoM controls bacterial peptidoglycan homeostasis

Sabrina Wamp[1], Patricia Rothe[1], Daniel Stern[2], Gudrun Holland[3], Jörg Döllinger[4], Janina Döhling[1], Sven Halbedel[1,5]

[1] FG11 - Division of Enteropathogenic bacteria and *Legionella*, Robert Koch Institute, Burgstrasse 37, 38855 Wernigerode, Germany;
[2] ZBS3 - Biological Toxins, Robert Koch Institute, Seestraße 10, 13353 Berlin, Germany;
[3] ZBS4 - Advanced Light and Electron Microscopy, Robert Koch Institute, Seestraße 10, 13353 Berlin, Germany;
[4] ZBS6 – Proteomics and Spectroscopy, Robert Koch Institute, Seestraße 10, 13353 Berlin, Germany;
[5] Institute for Medical Microbiology and Hospital Hygiene, Otto von Guericke University Magdeburg, 39120 Magdeburg, Germany;

Biosynthesis of the peptidoglycan (PG) mesh consumes substantial amounts of resources and energy and therefore needs to be harmonized with nutrient supply and growth conditions. In Gram-positive bacteria, PG biosynthesis is controlled by PASTA domain containing protein serine/threonine kinases, such as PrkA of the foodborne pathogen *Listeria monocytogenes*. Recently, it was shown that PrkA-dependent control of PG biosynthesis is exerted through phosphorylation of the small cytosolic protein ReoM. Phosphorylation of ReoM influenced the proteolytic stability of *L. monocytogenes* MurA, the first committed step enzyme in PG biosynthesis, in a ClpCP-dependent manner, and was linked to growth, PG biosynthesis and β-lactam resistance (1,2). However, many details on the mechanisms explaining how ReoM controls proteolytic degradation of MurA, how the ~20 other known PrkA targets contribute to PG biosynthesis regulation and how ReoM phosphorylation itself is controlled stayed unclear. We have isolated *murA* mutants that escape ClpCP-dependent proteolytic degradation in suppressor screens. The *murA* escape mutations fully suppressed otherwise essential *prkA*-dependent phenotypes as well as the toxicity of a phosphoablative *reoM* mutation, suggesting that MurA stability control through regulation of ReoM phosphorylation is the key purpose of PASTA kinase signaling in *L. monocytogenes*. ReoM interacted directly with MurA and this interaction was sensitive to phosphorylation. Phosphorylation of ReoM was found to occur constitutively *in vivo*, but genetic constellations were discovered, under which ReoM was not phosphorylated. Our results support a model according to which PrkA-dependent phosphorylation of ReoM activates PG production in response to signals related to cell cycle progression and/or metabolic workload in PG biosynthesis. They also have important implications for the high intrinsic β-lactam resistance levels of *L. monocytogenes* and related Gram-positive bacteria.

1 – Wamp et al. 2020. ***Elife***. doi: 10.7554/eLife.56048.
2 – Wamp et al. 2022. ***PLOS Pathog***. doi: 10.1371/journal.ppat.1010406.

A Molecular Link between Cell Wall Biosynthesis, Translation Fidelity, and Stringent Response in *Streptococcus pneumoniae*

N. Luisa Hiller[1*], Surya D. Aggarwal[1#], Adrian J. Lloyd[2*], Saigopalakrishna S. Yerneni[3], Ana Rita Narciso[4,5], Jennifer Shepherd[2], David I. Roper[2], Christopher G. Dowson[2], Sergio R. Filipe[4,5*]

[1]Dept. of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, USA
[2]School of Life Sciences, University of Warwick, Coventry, West Midlands, UK
[3]Dept. of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA, USA
[4] Laboratory of Bacterial Cell Surfaces and Pathogenesis, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Oeiras, Portugal
[5]UCIBIO-REQUIMTE, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Caparica, Portugal
[#]Current Address: Dept. of Microbiology, NYU Grossman School of Medicine, New York, NY, USA

Survival in the human host requires bacteria to respond to unfavorable conditions. In the important Gram-positive pathogen *Streptococcus pneumoniae,* cell wall biosynthesis proteins MurM and MurN are tRNA-dependent amino acyl transferases, which lead to the production of branched muropeptides. We demonstrate that wild-type cells experience optimal growth under mildly acidic stressed conditions, but a Δ*murMN* strain displays growth arrest and extensive lysis. Further, these stress conditions compromise the efficiency with which alanyl-tRNA^{Ala} synthetase can avoid non-cognate mischarging of tRNA^{Ala} with serine, which is toxic to cells. The observed growth defects are rescued by inhibition of the stringent response pathway, or by overexpression of the editing domain of alanyl-tRNA^{Ala} synthetase that enables detoxification of tRNA misacylation. Further, MurM can incorporate seryl groups from mischarged Seryl-tRNA$^{Ala}_{UGC}$ into cell wall precursors with exquisite specificity. We conclude that MurM contributes to the fidelity of translation control and modulates the stress response by decreasing the pool of mischarged tRNAs. Finally, we show that enhanced lysis of □*murMN* pneumococci is caused by LytA, and the *murMN* operon influences macrophage phagocytosis in a LytA-dependent manner. Thus, MurMN attenuates stress responses with consequences for host-pathogen interactions. Our data suggest a causal link between misaminoacylated tRNA accumulation and activation of the stringent response. In order to prevent potential corruption of translation, consumption of seryl-tRNA^{Ala} by MurM may represent a first line of defense. When this mechanism is overwhelmed or absent (□*murMN*), the stringent response shuts down translation to avoid toxic generation of mistranslated/misfolded proteins.

Interactome of PBP1a reveals a putative elongasome in *Agrobacterium tumefaciens*

Jacob Bouchier[1,2], Robert Kazmierczak[1], Laurie Agosto[1], and Pamela Brown[1]

[1]Division of Biological Sciences, University of Missouri – Columbia, USA
[2]Current address: Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, USA

In contrast to many rod-shaped bacteria, *Agrobacterium tumefaciens* lacks a canonical elongasome comprised of MreB-RodA-PBP2. Instead, *A. tumefaciens* uses a bifunctional penicillin binding protein, PBP1a, as the primary driver of elongation. To identify proteins that interact with PBP1a, we used PBP1a as a bait protein and identified proteins that complex with PBP1a. These efforts have revealed several proteins involved in the synthesis and regulation of peptidoglycan metabolism, including RgsS. RgsS is an FtsN-like protein and has a well conserved SPOR-domain suggesting that it may be recruited to mid-cell in late predivisional cells where the peptidoglycan is denuded. Alphafold predictions suggest that the periplasmic domain of RgsS likely binds to the active site of the glycosyltransferase domain of PBP1a hinting at a regulatory role similar to FtsN binding to PBP1b in *E. coli*. Surprisingly, depletion of RgsS does not inhibit polar growth, but rather cell division is blocked and the elongation rate accelerates, suggesting that RgsS may function as a negative regulator of PBP1a activity at the growth pole. Given the importance of amidase activity in establishing growth polarity in *A. tumefaciens*, we hypothesize that RgsS may recruit elongation machinery to the site of cell division to prime the new poles. Indeed, we find that RgsS is necessary to recruit the polar organizing protein, PopZ, to mid-cell. Furthermore, RgsS interacts with GPR, the growth pole ring, in the cytoplasm. Together these data suggest that RgsS plays a critical role in establishment of growth poles in *A. tumefaciens*. Overall, this work has improved our understanding of the *A. tumefaciens* elongasome and its regulation.

Tightly controlled – the septal peptidoglycan ring in *Chlamydia*

Beate Henrichfreise

University of Bonn, Germany

Chlamydia are human pathogens of major public health concern that replicate exclusively inside eukaryotic host cells. Inside their intracellular niche, the diderm *Chlamydia* are protected from osmotic challenges and the reductive adaptation to their host is reflected by the loss of an energy cost-intensive peptidoglycan-based cell wall. Instead, the minimalist organisms synthesize a transient peptidoglycan ring to support cell division. The unusual process of chlamydial cell division is initiated by a budding/blebbing mechanism similar to that in the FtsZ-lacking *Planctomycetes* and the asymmetric cell poles then mature into two approximately equally-sized daughter cells that are separated by a MreB-controlled septum. The entire process is supported by a peptidoglycan ring that expands during the early stages, constricts during the later stages, and is degraded during septation. Moreover, the otherwise bactericidal penicillin does not kill *Chlamydia* but blocks cell division. The underlying mechanisms of this reversible phenomenon are not fully understood.
We explored the biological function of the chlamydial peptidoglycan biosynthesis pathway and linked conservation of peptidoglycan precursor lipid II cycling to cell division. Here, we provide evidence that AmiA, the only cell division amidase retained in *Chlamydia*, is involved in peptidoglycan ring remodeling in *Chlamydia trachomatis* and comparatively analyze enzymatic functioning and inhibition of chlamydial AmiA homologs that lack autoregulatory domains as characterized in free-living model bacteria. In addition to new findings on the role of NlpC/P60 proteins in the underexplored process of peptidoglycan ring recycling, a summary on our recent research on cellular effects and molecular targets of cell wall antibiotics and modulation of NOD factor activation, will be given.
Our studies may help to define functioning of (new) players within the unique peptidoglycan ring and cell division machineries in *Chlamydia* and provide insight into deviations from canonical septal peptidoglycan and cell division control mechanisms in free-living model bacteria.

Breaking the wall: Peptidoglycan hydrolase activation during bacterial cell division

Allister Crow

University of Warwick, UK

The peptidoglycan layer confers strength and shape to the bacterial cell envelope and protects against osmotic shock. In Gram negative bacteria, the peptidoglycan layer also serves as an anchoring point for the outer membrane. During cell division, the peptidoglycan layer must be partially broken to accommodate the insertion of growing strands, and ultimately, to allow separation of daughter cells. In this talk, I discuss the role of peptidoglycan amidases in hydrolysing peptidoglycan crosslinks at the division site with a focus on the FtsEX-EnvC-AmiA/B system. Available data from our lab, and others, supports a remarkable mechanism for amidase activation in which cytoplasmic ATP binding and hydrolysis drive conformational changes in FtsEX-EnvC complex to activate AmiA or AmiB in the periplasm. Understanding amidase activation via FtsEX-EnvC may offer new opportunities to design antimicrobial drugs that interfere with cell division or promote bacterial autolysis.

Short Talk Abstracts

From: Cecile Morlot <cecile.morlot@ibs.fr>

Title: Click and Collect at high resolution to unlock the secrets of cell wall synthesis

Text: The peptidoglycan is a three-dimensional sugar and peptide network that surrounds the bacterial cell. It confers a cell shape adapted to the ecological niche of the bacterium and protects it against mechanical stresses. Peptidoglycan synthesis and integrity are thus essential for bacterial proliferation and survival. Despite the importance of these fundamental processes, which constitute sources of antibiotic targets, we still poorly understand how the peptidoglycan is assembled and remodeled in space and time to ensure proper cell division, shape and integrity. This is particularly true for ovoid-shaped bacteria such as streptococci and enterococci, in which two different modes of peptidoglycan synthesis, dedicated to cell division and elongation, are confined to an annular region with nanometric dimensions at midcell.
Fluorescence microscopy is a method of choice to investigate cell wall assembly, but it suffers from two major drawbacks. First, the newly synthesized material must be labeled with a probe that will not perturb the physiological process. Second, the physical properties of light limit the resolution to about 250 nm, which approximates the dimensions of the cell wall synthesis region. We have met these two challenges by combining metabolic peptidoglycan labeling (using click chemistry) and super-resolution fluorescence microscopy (dSTORM) in the ovoid-shaped human pathogen *Streptococcus pneumoniae*. Our nanoscale-resolution data (about 30 nm) unravel unprecedented spatio-temporal features of peptidoglycan assembly and fate along the cell cycle. It provides geometrical and kinetic parameters of peptidoglycan synthesis that we further use to simulate the morphogenesis of the ovoid cell in silico.
I will present our methodological strategy and the major insights that our experimental and modeling analyses can reveal into peptidoglycan synthesis and morphogenesis in ovococci.

Authors names: Jennyfer Trouve, Andre Zapun, Claire Durmort, Christophe Grangeasse, Yung-Sing Wong and Cecile Morlot

Affiliations: Institut de Biologie Structurale, CNRS/CEA/UGA, Grenoble, FRANCE

From: Nika Pende <nika.pende@univie.ac.at>

Title: Discovery and characterization of the first two components of the elongasome in archaea with a pseudo-peptidoglycan cell wall

Text: Archaeal cell biology is an emerging field of research. Most Archaea divide by binary fission using an FtsZ-based system similar to Bacteria - however, they lack most of the proteins that form the bacterial divisome and elongasome.
Methanobacteriales and Methanopyrales have two unique features compared to other more established archaeal models: they are anaerobes with a pseudo-peptidoglycan (pPG) cell wall and have only one FtsZ homologue. Using the human gut-associated archaeon *Methanobrevibacter smithii* as a model organism, we have recently identified and characterized the first two components of the divisome, the ring-forming protein FtsZ and its anchor SepF. Interestingly, all pPG-bearing archaea, including *M. smithii*, possess bona fide homologues of the bacterial cell shape determining protein MreB, whose function is unknown, and whose genomic context shows a conserved protein of unknown function, which we have named MbaP (MreB associated protein). However, pPG-bearing archaea lack all other components of the bacterial elongasome, suggesting the existence of a novel machinery for guiding pPG synthesis during growth.
Here, we combined structural, cell biological and evolutionary approaches, to investigate the function of MreB and MbaP in *M. smithii*. We show that, like its bacterial counterpart, M. smithii MreB polymerizes into anti-parallel double filaments. Immunolabeling experiments reveal that MreB and MbaP mostly co-localize at different stages of the life cycle and around the septation plane. Consistently, we show by Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy (15N-NMR) that MreB and MbaP interact in a salt concentration-dependent manner through their ordered protein regions. Finally, we obtained the crystal structure of MbaP, and are now performing Hydrogen-Deuterium exchange Mass Spectrometry (HDX-MS) on MbaP and MreB proteins in complex. This will allow us to map the residues involved in the interaction onto the MbaP structure and propose a model of the complex.
Taken together, our results strongly suggest that MreB and MbaP belong to a new type of elongasome in walled archaea, whose full composition and mode of function remains to be elucidated. More broadly, this study paves the way for a better understanding of the biology of methanogens in the human microbiome.

Authors names: Nika Pende[1,2], Minoli Doshi[1], Iñaki Guijarro[3], Najwa Taib[1], Ariel Mechaly[4], Ahmed Haouz[4], Simonetta Gribaldo[1]

Affiliations: 1. Evolutionary Biology of the Microbial Cell Unit, Department of Microbiology, Institut Pasteur, Paris, France; 2. Archaea Biology and Ecogenomics Division, University of Vienna, Austria; 3. Biological NMR and HDX-MS Technological Platform, Institut Pasteur, Paris, France; 4. Crystallography Platform, Institut Pasteur, Paris, France

From: Juan A. Hermoso <xjuan@iqfr.csic.es>

Title: Structural and Functional Characterization of the Pneumococcal Surface Protein Spr1875. An Unprecedented Murein-Modifying Enzyme

Text: The bacterial cell wall is a dynamic structure whose synthesis and remodeling proceed in concert during bacterial growth and division or to ensure survival under stress conditions such as exposure to β-lactams antibiotics. The gene spr1875 has been identified in the VicRK regulon that is up-regulated in the absence of PcsB, the essential pneumococcal lytic cell-division protein, and highly overexpressed under antibiotics exposure.
The protein Spr1875 is predicted as a LysM, cell-wall binding domain, and an MT3 putative hydrolytic domain, Spr1875MT3, connected by a long-disordered linker. Here we present a multidimensional study using X-ray crystallography combined with biochemical, computational, and cellular approaches. Five high-resolution crystallographic structures and MD simulations reveal that Spr1875MT3 alternates between two different states, one of them non-hydrolytic acting as a zinc scavenger, and an active hydrolytic state. Spr1875MT3 demonstrates an unprecedented conformation in its inactive state, with the ability to host one to four in-line Zn cations. However, after refolding, Spr1875MT3 showcases its active site and catalytic machinery surrounding a single catalytic Zn cation. Liquid chromatography and mass spectrometry analysis with different synthetic peptidoglycan fragments prepared for this study, revealed Spr1875MT3 presents muralytic activity with L-Ala D-Glu endopeptidase activity. We also provide evidence that this protein is directed to the septum, and its activity is essential for pneumococcal growth under stress conditions. A potential new approach to enhance the susceptibility of bacteria to antibiotics could be to inhibit the dual function of this protein.

Authors names: Vega Miguel-Ruano[1], Iván Acebrón[1], Mijoon Lee[2], Antonio J. Martín-Galiano[3], Celine Freon[4], Adrien Ducret[4], Balajee Ramachandran[2], Daniel Straume[5], Christophe Grangeasse[4], Leiv Sigve Håvarstein[5], Shahriar Mobashery2 and Juan A. Hermoso[1]

Affiliations: [1]Department of Crystallography and Structural Biology, Instituto de Química-Física “Rocasolano”, CSIC, Madrid, Spain. [2] Department of Chemistry and Biochemistry, University of Notre Dame, Indiana 46556. [3]Intrahospital Infections Laboratory, Carlos III Health Institute, National Centre for Microbiology, Madrid, Spain. [4]Molecular Microbiology and Structural Biochemistry, CNRS UMR, Université de Lyon, Lyon, France. [5]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway.

From: Quynh Mai NGUYEN <quynh-mai.nguyen@univ-grenoble-alpes.fr>

Title: Teichoic Acids and Peptidoglycan Synthesis at the Nanoscale

Text: Gram-positive bacteria such as *Streptococcus pneumoniae* possess a thick layer of peptidoglycan (PG), a 3D network of glycan chains cross-linked by short peptide chains, which maintains cell morphology and structural strength to resist osmotic pressure. Anchored to this layer are teichoic acids (TA), complex anionic polymers that play important roles in morphogenesis and virulence processes. While both components are essential for a healthy cell wall, TA are still poorly understood compared to PG, and little is known about their assembly during cell growth. Past studies have showed that in *S. pneumoniae*, PG and TA are seemingly synthesized concomitantly in a mid-cell annular region of only 100 nm width-wise; however, since the resolution of conventional fluorescence microscopy is limited to 250 nm due to the diffraction of light, it remains a great challenge to investigate the area of active cell wall synthesis in details. To elucidate the relative dynamics of TA insertion into the PG layer, we employ a labelling method using chemically modified metabolites probes and observe labelled cells with the single-molecule localization microscopy technique called dSTORM (direct STochastic Optical Reconstruction Microscopy), which permits a resolution of about 30 nm. Labelled regions in wild-type and mutant cells are then analysed and compared at the nanoscale, demonstrating interesting variations as the cell cycle progresses.

Authors names: Mai Nguyen, Claire Durmort, Andre Zapun, Christophe Grangeasse, Yung-Sing Wong and Cecile Morlot

Affiliations : Institut de Biologie Structurale, CNRS/CEA/UGA, Grenoble, FR

From: Richard Wheeler <rwheeler@pasteur.fr>

Title: Investigating the intestinal microbiota peptidoglycome and its dissemination across the gut epithelial barrier

Text: The intestinal microbiota is a symbiotic community that drives physiological processes critical for the development and health of the host (1). Although the composition of the gut microbiota is frequently linked to health and disease, the precise mechanisms through which microbiota collectively affect their host are poorly understood. Peptidoglycan, the cell wall network of bacteria, is emerging as a major player in signaling between the microbiota and host. Peptidoglycan fragments continually cross the host intestinal epithelial barrier and enter the host system, where they regulate diverse physiological effects ranging from immune maturation and homeostasis to developmental and even behavioral effects (2-5). Conversely, microbiota peptidoglycan can also be a driver of chronic inflammatory disease and is therefore a potential therapeutic target (6,7). A major question is how such diverse physiological effects can be driven by the microbiota peptidoglycan?
To gain insight into the diverse effects of gut microbiota peptidoglycan on the host, our research aims to understand the nature of peptidoglycan produced by microbiota, and the mechanisms underlying its absorption, dissemination, and metabolism in mammalian hosts (8). We have extensively characterized the absorption of peptidoglycan across the gut barrier, and dissemination to organs and tissues, using radio-tracking and fluorescence microscopy. We show that uptake of peptidoglycan occurs through subsets of intestinal epithelial cells, and that absorption via the gut favors the dissemination and accumulation of peptidoglycan to organs including the brain. We further demonstrate that uptake of peptidoglycan across the gut epithelial barrier is an active process that is regulated by members of the intestinal microbiota. Finally, we explore the diversity of the intestinal peptidoglycome – the peptidoglycan moieties generated collectively by the intestinal microbiota – in mice and humans. We show how peptidoglycomics can be applied to study dysbiosis of the microbiota peptidoglycan in the context of arthritis.

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Authors names: Richard Wheeler (1), Paulo André Dias Bastos (1), Olivier Disson (2), Aline Rifflet (2), Marc Lecuit (2,3,4), and Ivo Gomperts Boneca (1)

Affiliations: 1) Institut Pasteur, Université Paris Cité, CNRS UMR6047, INSERM U1306, Unité de Biologie et génétique de la paroi bactérienne, F-75015, Paris, France; 2) Institut Pasteur, Université Paris Cité, Inserm U1117, Biology of infection unit, F-75015, Paris, France; 3) Institut Pasteur, National Reference Centre and WHO Collaborating Centre Listeria, F-75015, Paris, France; 4) Necker-Enfants Malades University Hospital, Division of Infectious Diseases and Tropical Medicine, APHP, Institut Imagine, F-75006, Paris, France

From: Renata C. Matos <renata.matos@ens-lyon.fr>

Title: Structure-Function analysis of *Lactiplantibacillus plantarum* DltE reveals D-alanylated lipoteichoic acids as direct cues supporting Drosophila juvenile growth

Text: Metazoans establish complex interactions with their resident microorganisms for mutual benefits. When in homeostasis, these interactions contribute to different aspects of host physiology. In the gut, microbial communities enhance digestive efficiency by providing enzymatic functions that help their hosts optimize extraction of dietary energy and nutrients. Despite the renewed interest in understanding the functional impact of gut microbiota on host physiology, a clear view of the molecular dialogue engaged upon host/microbiota interaction remains elusive. Therefore, the use of simple animal models, such as Drosophila, may help unravel the evolutionarily conserved mechanisms underlying the impact of intestinal bacteria in their host physiology, since it combines genetic and experimental tractability with a cultivable microbiota.
Previously, we showed that upon chronic undernutrition, strains of L. plantarum, a major commensal partner of Drosophila, promote host juvenile growth and maturation partly via enhanced expression of intestinal peptidases. This effect is in part mediated by the action of the proteins encoded by the *dltEXABCD* operon. Those proteins are involved in the D-alanylation of lipoteichoic acids (LTA), a major component of gram-positive cell envelope. Following an integrative structural biology approach, our studies revealed an atypical role for DltE (former PbpX2) which presents a carboxyesterase activity on teichoic acids by removing D-alanines from LTA. Furthermore, we demonstrate that the carboxy-esterase activity of DltE is required to sustain Drosophila juvenile growth and that D-alanylated LTAs are symbiotic cues supporting Drosophila intestinal response and juvenile growth.

Authors names: Nikos Nikolopoulos[1*], Renata C. Matos[2*], Stéphanie Ravaud[1*], Pascal Courtin[3*], Houssam Akherraz[2], Simon Palussière[3], Virginie Gueguen-Chaignon[4], Marie Salomon-Mallet[3], Alain Guillot[3], Yann Guerardel[5,6], Marie-Pierre Chapot-Chartier[3#], Christophe Grangeasse[1#] and François Leulier[2#]

Affiliations: [1] Molecular Microbiology and Structural Biochemistry, CNRS UMR 5086, Université Claude Bernard Lyon 1, Lyon, France. [2] Institut de Génomique Fonctionnelle de Lyon, Ecole Normale Supérieure de Lyon, CNRS UMR 5242, Université Claude Bernard Lyon 1, Lyon, France. [3] Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en-Josas, France [4] Protein Science Facility, CNRS UAR3444, INSERM US8, Université Claude Bernard Lyon 1, Ecole Normale Supérieur de Lyon, Lyon, France. [5] Univ. Lille, CNRS, UMR 8576 - UGSF - Unité de Glycobiologie Structurale et Fonctionnelle, F-59000 Lille, France. [6] Institute for Glyco-core Research (iGCORE), Gifu University, Gifu, Japan

From: Kelvin Kho <kkho@pasteur.fr>

Title: AI-assisted identification of bacterial cell shape determinants for antibiotic discovery

Text: Bacterial cell shapes are very diverse, ranging from simple coccoids and short rods to filaments and helices. These shapes are adapted to their lifestyle and can even be plastic depending on genetic factors and external stressors. However, cell shape analysis necessitates microscopy thus screens for shape determinants can yield unwieldy datasets. To overcome these limitations, we are developing an AI-assisted image analysis pipeline for rapid and unbiased bacterial cell shape classification. We determined the feasibility of this approach by cross-training and testing the pipeline on *Escherichia coli* and the WHO-designated high priority target, *Helicobacter pylori*. Then, we are using it to cluster cell shapes upon treatment with chemical libraries as well as with transposon mutant libraries. By correlating these datasets, we aim to obtain the complete network of morphological determinants whilst identifying the mechanisms of actions and molecular targets of potential antibiotics.

Authors names: Kelvin Kho[1], Daniel Krentzel[2], Nassim Mahtal[3], Spencer Shorte[3], Nathalie Aulner3, Christophe Zimmer[2], Ivo Gomperts Boneca[1]

Affiliations: [1] Institut Pasteur, University Paris Cité, CNRS UMR6047, INSERM U1306, Biology and Genetics of the Bacterial Cell Wall Unit; [2] Institut Pasteur, University Paris Cité, Imaging and modeling Unit; [3] Institut Pasteur, University Paris Cité, Center for Technological Resources and Research, UTechS Photonic BioImaging, Paris, France

From: Camilla Henriksen (maiden name Camilla Jensen)

Title: The role of the PBPs in life and death: new clues to a still unsolved mystery

Text: Since the discovery of penicillin, β-lactams have become the most widely used class of antibiotics. β-lactams inhibit cross-linking of cell wall peptidoglycan by binding irreversibly to the transpeptidase (TP) domain of penicillin-binding proteins (PBPs). According to the current dogma, bacterial killing is associated with lysis due to unsynchronized activation of autolysins, however, relatively little is known about the checkpoints that safeguard bacteria from the detrimental activity of cell wall hydrolases.
To identify factors involved in controlling separation of *S. aureus* daughter cells, we here characterized spontaneous suppressor mutations that rescue growth of cells that accumulate the Sle1 cell wall amidase. The accumulation of Sle1 puts cells at risk for initiating daughter cell separation prior to septum completion resulting in cell lysis.
Interestingly, a large deletion in *pbpA*, encoding the essential PBP1, was repeatedly identified. The deletion removed 60 aa of the TP active site, thereby preventing PBP1 from binding Bocillin-FL, a structural analog of the natural TP substrate. PBP1 is essential for cell division and expression of PBP1Δ60 resulted in increased cell size, aberrant septa, and delayed daughter cell separation. These changes were phenocopied by exposing wild type cells to β-lactams. Electron microscopy demonstrated that the septal cross-wall was not separated at the midline suggesting that TP activity of PBP1 is required for the characteristic architecture of the septum allowing separation of the septal walls. We have identified several autolysins that might be involved in separation of the septum. Autolytic activity in the septal wall is determined through incorporation of teichoic acids. Interestingly, the most frequently selected suppressor mutations were loss-of-function in *ltaS* encoding the lipoteichoic acid (LTA) synthetase. This prompted us to determine if PBP1 controls autolysis by regulating LTA synthesis. Indeed, the amount of active LtaS was clearly reduced in cell walls of cells with the deletion in the TP domain supporting that PBP1 plays a role in activation of LTA synthesis.
We propose that the TP domain of PBP1 is a sensor of septal completion and an activator of LtaS activity. By controlling incorporation of LTA at the septal site, the TP domain of PBP1 functions as a checkpoint coordinating PG incorporation and autolytic splitting in the septal walls. Importantly, our results suggest that β-lactam induced killing of *S. aureus* involves inhibition of the septal autolysins.

Authors names: Camilla Henriksen (1), Viktor H. Mebus (1), Jesper Larsen (2), Lijuan Xu (1), and Dorte Frees (1)

Affiliations: (1) Department of Veterinary and Animal Disease, University of Copenhagen, Frederiksberg, Denmark. (2) Statens Serum Institut, Copenhagen S, Denmark

From: Tyler Sisley <tsisley@g.harvard.edu>

Title: A Staphylococcal protein controls global phosphorylation in response to cell envelope stress

Text: Bacterial growth requires cells to sense the extracellular environment and modify cell envelope synthesis in response to stress. To do so, cells must transduce stimuli across the membrane and relay back responses to balance synthesis, installation, and decoration of numerous cell envelope polymers. *Staphylococcus aureus* employs a series of histidine and tyrosine kinases to do this. Interestingly, it also contains only one serine/threonine kinase, PknB, that senses disruptions in the peptidoglycan cell wall and initiates a phosphor-relay to modify expression of cell wall remodeling genes. How the cell regulates PknB activity is unknown. We have discovered a protein regulator of PknB that represses its activity. This regulator forms a physical complex with PknB to repress autophosphorylation in its activation loop. Failure to form this complex results in hyperactive PknB and sensitizes cells to compounds that target cell envelope synthesis. Similarly, cells that overexpress PknB are also highly sensitized to inhibitors of cell wall synthesis and teichoic acid modification. Our findings show that, while too little PknB is indeed toxic under some conditions, too much PknB activity is toxic under a variety of other conditions. We conclude that PknB activity needs to be carefully controlled in an appropriate range to support stable proliferation in *S. aureus*.

Authors names: Tyler A. Sisley (1), Youngseon Park (1), Thomas M. Bartlett (1), Wanassa Beroual (1), Ace G. Santiago (1), Joao A. Paolo (2), Isabella A. Sobolewski (1), Thomas G. Bernhardt (1), Daniel Kahne (3), Suzanne Walker (1)

Affiliations: (1) Department of Microbiology, Blavatnik Institute, Harvard Medical School, Boston, Massachusetts, USA (2) Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, USA (3) Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts, USA

From: Andrea Dessen <andrea.dessen@ibs.fr>

Title: Architecture and genomic arrangement of the MurE-MurF bacterial cell wall biosynthesis complex

Text: Peptidoglycan is a central component of the bacterial cell wall, and the disruption of its biosynthetic pathway has been a successful antibacterial strategy for decades. Peptidoglycan biosynthesis is initiated in the cytoplasm through sequential reactions catalyzed by Mur enzymes that have been suggested to associate into a multi-membered complex. This idea is supported by the observation that in many eubacteria, *mur* genes are present in a single operon within the well conserved *dcw* cluster, and in some cases pairs of *mur* genes are fused to encode a single, chimeric polypeptide able to catalyze successive reactions. We performed a vast genomic analysis using > 140 bacterial genomes and mapped Mur chimeras in numerous phyla, with Proteobacteria carrying the highest number. MurE-MurF, the most prevalent chimera, exists in forms that are either directly associated or separated by a linker. The crystal structure of the MurE-MurF chimera from Bordetella pertussis reveals a head-to-tail, elongated architecture supported by an interconnecting hydrophobic patch that stabilizes the positions of the two proteins. Fluorescence polarization assays reveal that MurE-MurF interacts with other Mur ligases via its central domains with KDs in the high nanomolar range, backing the existence of a Mur complex in the cytoplasm. These data support the idea of stronger evolutionary constraints on gene order when encoded proteins are intended for association, establish a link between Mur ligase interaction, complex assembly, and genome evolution, and shed light on regulatory mechanisms of protein production and stability in pathways of critical importance for bacterial cell survival.

Authors names: Karina T. Shirakawa [1], Fernanda Angélica Sala [1], Mayara M. Miyachiro [2], Viviana Job [2], Daniel Maragno Trindade [1] and Andréa Dessen [1,2]

Affiliations: [1] Brazilian Biosciences National Laboratory (LNBio), CNPEM, Campinas 13084-971, São Paulo, Brazil [2] Univ. Grenoble Alpes, CNRS, CEA, Institut de Biologie Structurale (IBS), F-38044 Grenoble, France

From: Helena Veiga <hveiga@itqb.unl.pt>

Title: Cell division protein FtsK coordinates bacterial chromosome segregation and daughter cell separation

Text: Unregulated cell cycle progression may have lethal consequences and therefore bacteria have various mechanisms in place for the precise spatiotemporal control of cell cycle events. We have uncovered a new link between chromosome replication/segregation and splitting of the division septum. We show that the divisome protein FtsK, with a DNA translocase domain, regulates cellular levels of a peptidoglycan hydrolase, Sle1, involved in cell separation in the bacterial pathogen *Staphylococcus aureus*. FtsK interacts with a chaperone (trigger factor, TF) causing it to establish a FtsK-dependent concentration gradient, higher in the septal region. TF binds Sle1 and promotes its preferential export at the septal region, while also preventing Sle1 degradation by the ClpXP proteolytic machinery. Upon DNA damage or impaired DNA replication/segregation, conditions that lead to paused septum synthesis, TF gradient is dissipated and Sle1 levels are reduced, halting premature septum splitting.

Authors names: Helena Veiga[1], Ambre Jousselin[1], Simon Schäper[1], Bruno M. Saraiva[1], Leonor B. Marques[1], Patricia Reed[1], Joana Wilton[1], Pedro M. Pereira[1], Sérgio R. Filipe[1,2], Mariana G. Pinho[1]

Affiliations: [1] Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa; Oeiras, Portugal. [2] UCIBIO-REQUIMTE, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa; Caparica, Portugal.

From: (Chris) Lok-To Sham <miclts@nus.edu.sg>

Title: The divisome but not the elongasome organizes capsule synthesis in *Streptococcus pneumoniae*.

Text: The bacterial cell envelope is a complex, multi-layered structure. Precise coordination of its synthesis is required to ensure every layer is faithfully produced. Many gram-positive bacteria conceal peptidoglycan (PG) and underlying antigens with capsular polysaccharides (CPS). Yet, how CPS synthesis integrates with PG synthesis remains unclear. In *Streptococcus pneumoniae*, the peripheral and septal PG is produced respectively by the elongasome and the divisome. We show that CPS synthesis initiates from the division septum and propagates along the long axis of the cell, organized by the bacterial tyrosine kinase system CpsCD. CpsC and the rest of the CPS complex are recruited to the septum by proteins associated with the divisome but not the elongasome. The CPS complex assembly starts with CpsCD, then CpsA and CpsH, the glycosyltransferases, and finally CpsJ. Remarkably, targeting CpsC to the cell pole is sufficient to reposition CPS synthesis, leading to diplococci that lack CPS at the septum. We propose that septal CPS synthesis is important for chain formation and complement evasion, thereby promoting survival inside the host.

Authors names: Rei Nakamoto[1], Sarp Bamyaci[2], Karin Blomqvist[2,3], Staffan Normark[2,3], Birgitta Henriques Normark[2,3], Lok-To Sham[1]

Affiliations: 1. Infectious Diseases Translational Research Programme and Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore, 117545, Singapore; 2. Department of Microbiology, Tumor and Cell biology (MTC), Karolinska Institutet, Stockholm SE-17177, Sweden; 3. Department of Clinical Microbiology, Karolinska University hospital Solna, SE-17176, Stockholm, Sweden

Poster Abstracts

1

From: Gyanu Lamichhane <gyanu@jhu.edu>

Title: Basis of activities of a β-lactam and Dual β-lactams: evidence that challenges the historical model

The historical model of activity of β-lactam antibiotics teaches that they inhibit only one enzyme class, namely D,D-transpeptidases (also known as penicillin-binding proteins, PBPs). This model instructs to treat an infection with a β-lactam most specific to the causative bacterium. D,D-transpeptidases catalyze the final step in bacterial cell wall peptidoglycan synthesis. In Mycobacterium tuberculosis, we identified an enzyme class required for it to cause disease. This enzyme class, L,D-transpeptidases, is evolutionarily unrelated to the D,D-transpeptidases as the amino acid sequences and structures of these two protein classes are distinct. While D,D-transpeptidases generate 4-3 linkages, L,D-transpeptidases generate 3-3 linkages in the peptidoglycan. M. tuberculosis simultaneously uses D,D- and L,D-transpeptidases to generate peptidoglycan. Moreover, L,D-transpeptidases generate most of the linkages in the peptidoglycan of M. tuberculosis. M. tuberculosis lacking a L,D-transpeptidase cannot sustain growth in the lungs of mice and is avirulent. We observed that D,D- and L,D-transpeptidases of mycobacteria are differentially inhibited by β-lactams. We tested the hypothesis that combining two β-lactams may optimally inhibit peptidoglycan synthesis and exhibit synergy in killing mycobacteria. Select dual β-lactams exhibited synergy against *Mycobacterium abscessus* both in vitro and in vivo and provided evidence for using β-lactam combinations to treat this disease. The historical model not only does not anticipate synergy between β-lactams but considers it counterintuitive since it considers D,D-transpeptidases as the only target of β-lactams. Emerging evidence indicates that dual β-lactams may be effective against a broader spectrum of bacteria.

Authors names: Pankaj Kumar*, Elizabeth Story-Roller*, Emily C. Maggioncalda, Gyanu Lamichhane.

Affiliations: Johns Hopkins University

2

From: Barbara Walenkiewicz <bwalenk@iu.edu> phone : 2182690755

Title: Fluorescent D-amino acid-based approach enabling fast and reliable measure of antibiotic susceptibility in bacterial cells

The threat of multidrug resistant bacteria has been increasing steadily in the past century, posing a major health risk to the public. Even though every year, 226 million antibiotics are prescribed in the United States alone, 50% of the prescriptions are inappropriate for the patient's condition. The increasing abuse of antibiotics in healthcare, as well as agriculture, has resulted in the rise of antibiotic resistance at an alarming rate. Traditional susceptibility testing requires extended cell incubation, delaying timely treatment response to the infection. Herein, we report that a short-pulse fluorescent D-amino acid (FDAA)-based approach provides insight not only into bacterial antibiotic susceptibility, but also into the mechanism of action of the antibiotic. In a clinical setting, fast and accurate recognition of the pathogen allows for the most effective choice of treatment, highlighting the need for a novel, fast, and accurate antibiotic susceptibility testing. Using FDAA-labeling signal as a reflection of peptidoglycan (PG) integrity after treatment with corresponding antibiotics, antibiotics targeting PG biosynthesis resulted in a significant decrease in fluorescence, while antibiotics affecting other cellular targets resulted in no fluorescence changes. This method was tested through fluorescent microscopy and spectrofluorometry, providing fast and accurate results and expanding FDAAs application in the antibiotic susceptibility studies.

Authors names: Barbara Walenkiewicz

Affiliations: VanNieuwenhze lab, Indiana University Bloomington

3

From: Elisa Pierre <elisa.pierre@uclouvain.be>

Title: Elucidating the specific functions of LdtA, LdtB and LdtC, three periplasmic L,D-transpeptidases

In *E. coli*, stabilization of the cell envelope mainly relies on tethering the peptidoglycan layer (PG) to the outer membrane (OM) via the lipoprotein Lpp. The cross-linking of Lpp to the PG is mediated by three enzymes of the L,D-transpeptidases (LDTs) family: LdtA, LdtB, and LdtC. While these appear to be redundant, non-essential homologous enzymes, the physiological distinction between them is still unknown. Why *E. coli* expresses three different LDTs with a seemingly redundant activity remains unclear and suggests that these enzymes may have specific roles to play, especially under various stress conditions.
Here, we aim to understand how LdtA, LdtB, and LdtC contribute to PG synthesis and remodeling during bacterial cell life, how they are regulated and how they enable bacteria to cope with environmental stresses.
Since PG synthesis is intimately related to bacterial growth and division it is likely that LDTs are part of protein complexes responsible for such processes. Preliminary data from pull-down experiments seem to support this hypothesis as we could identify promising candidates as partners.
Because *E. coli* possesses many stress response systems, LDTs might also be part of stress-related regulatory networks. Preliminary data suggest that LDT's expression profiles greatly differ from one another, supporting this hypothesis.
The maintenance of a structural link between the OM and the PG is essential to preserve the cell envelope integrity. In this context, strains with an impaired connection between the OM and the PG have been engineered to study the effects of osmotic changes on bacterial survival, morphology, cell composition and sensitivity to antimicrobial agents. While some of these strains displayed morphological defects, deletion of LdtA, LdtB or LdtC had strikingly different impacts on the cell morphology, hinting at the fact that additional levels of functional specialization might exist between those LDTs.

Authors names: Elisa S. Pierre, Michael Deghelt, Jean-François Collet

Affiliations: 1 de Duve Institute, Université catholique de Louvain, B-1200 Brussels, Belgium. 2 WELBIO, B-1200 Brussels, Belgium

4

From: Randy Morgenstein <randy.morgenstein@okstate.edu>

Title: MreB has distinct roles in regulating cell shape and viability

The actin-homolog MreB is a crucial component of the rod-machinery that adds new peptidoglycan material to the cell wall for lateral elongation during growth. It is necessary for maintaining the rod shape of *Escherichia coli* and many other rod-shaped bacteria. It is functionally active as polymeric nanofilaments localized beneath the inner membrane along the long axis of the cell. As expected, cells lacking MreB or in the presence of the depolymerizing agent A22 suffer loss of rod shape that ultimately leads to cell death. Previous studies looking at random mutations in MreB observed varying degrees of alteration in cell shape and size implying the effect of each amino acid residue for rod-shape dynamics is independent. Here, we have taken a systematic approach by creating an alanine-scanning library to better understand the role of each residue in MreB. Of the 346 amino acids in MreB we have successfully created 330-point mutants and quantitatively analyzed cell shape data for 253-point mutants. Surprisingly, fourteen mutants have a round shape similar to A22 treated cells. The fact, they are viable when grown at 37°C in rich medium despite this shape defect suggests MreB has vital functions related to growth in addition to shape maintenance. In support of this, cell shape suppressor analysis of mutants able to restore rod shape only revealed reversions or two intragenic mutations in MreB suggesting that MreB is absolutely necessary for rod shape. Additionally, one of these intragenic mreB mutations can suppress the shape defect in many of the round mutants.

Authors names: Suman Maharjan, Ryan Sloan, Jada Lusk, Rose Bevienguevarr, Jacob Surber, and Randy Morgenstein

Affiliations: Oklahoma State University

5

From: Stephane Mesnage <s.mesnage@sheffield.ac.uk> phone : 00 44 781 678 3984

Title: Software tools for peptidoglycomics

The structural analysis of peptidoglycan (PG) is a challenging task due to the unusual composition and crosslinking of this molecule. Liquid chromatography coupled to tandem mass spectrometry is routinely used for PG analysis but 'omics tools are unable to handle the corresponding datasets. As a result, data analysis remains a manual process that is time-consuming and prone to errors. Combined with the lack of information on the search strategy, there is a pressing need to establish consistent and reproducible approaches for PG structural analysis.
We have developed a series of open-source software and a standard pipeline to analyze PG structure by LC-MS/MS. A step-by-step methodology was established using *Rhizobium leguminosarum* peptidoglycan as a proof of concept. Our analysis revealed the exquisite complexity of *R. leguminosarum* PG structure, providing a framework to explore the dynamics of this molecule in the context of legume symbiosis.

Authors names: Marcel G. Alaman-Zarate [1*], Brooks Rady [1*], Raphael Ledermann [2], Neil Shephard [3], Robert Turner [3], Aline Rifflet [4], Ankur Patel [1], Ivo Gomperts Boneca [4], Philip S. Poole [2], Stéphane Mesnage [1]

Affiliations: [1] School of Biosciences, University of Sheffield, Sheffield, UK [2] Department of Plant Sciences, University of Oxford, Oxford, UK. [3] Research Software Engineer team, University of Sheffield, Sheffield, UK [4] Institut Pasteur, Université Paris Cité, CNRS UMR6047, INSERM U1306, Unité Biologie et Génétique de la Paroi Bactérienne, Paris, France

6

From: Marie-Pierre Chapot-Chartier <marie-pierre.chapot-chartier@inrae.fr>

Title: Molecular mechanisms underlying the structural diversity of complex rhamnose-rich cell wall polysaccharides in lactococci

In Gram-positive bacteria, cell wall polysaccharides (CWPS) play critical roles in bacterial growth, and in interactions between bacteria and their nearby environment. In lactococci, CWPS comprise two components: a conserved rhamnan embedded inside the peptidoglycan layer and a surface-exposed polysaccharide pellicle (PSP), which are linked together to form a large rhamnose-rich CWPS (Rha-CWPS). Notably, the structurally diverse PSP has an important function as a receptor for many bacteriophages infecting lactococci. We have proposed a model biosynthesis scheme in which rhamnan and PSP are synthesized independently, with rhamnan being anchored onto peptidoglycan after synthesis and export, and PSP constituting a decoration added extracellularly onto rhamnan. Here, using in vitro enzymatic tests with lipid acceptor substrates combined with LC-MS analysis, modeling of protein 3D-structure, complementation experiments and phage assays, we examined the first two steps of PSP biosynthesis. We showed that PSP is synthesized on an undecaprenyl-monophosphate (C55P) lipid carrier. Synthesis is initiated by WpsA/WpsB complex with C55P-GlcNAc synthase activity, and the second step is performed by a glycosyltransferase (WpsC) with variable specificity among lactococcal strains, thus resulting in the synthesis of PSP with different structures. Moreover, we engineered the PSP biosynthesis pathway in lactococci to obtain a chimeric PSP structure, which allowed us to pinpoint the importance of a single residue of the PSP subunit in phage recognition. In conclusion, our results validate the biosynthesis scheme of PSP on a lipid-monophosphate carrier as an extracellular modification of rhamnan and shed light on the molecular mechanisms supporting the structural diversity of complex Rha-CWPS in lactococci.

Authors names: Hugo Guérin[1], Pascal Courtin[1], Alain Guillot[1], Christine Péchoux[2], Chris Whitfield[3], Jennifer Mahony[4], Douwe van Sinderen[4], Saulius Kulakauskas[1], Christian Cambillau[4,5], Thierry Touzé[6] and Marie-Pierre Chapot-Chartier[1]

Affiliations: [1]Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en-Josas, France; [2]Université Paris-Saclay INRAE, AgroParisTech, GABI, Jouy-en-Josas, France; [3]Department of Molecular and Cellular Biology, University of Guelph, 50 Stone Road E., Guelph, ON, N1G 2W1, Canada; [4]School of Microbiology and APC Microbiome Ireland, University College Cork, Western Road, Cork, Ireland; 5AlphaGraphix, 24 Carrer d'Amont, 66210 Formiguères, France; [6]Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC), Gif-sur-Yvette, France.

7

From: Alix Dachsbeck <alix.dachsbeck@uclouvain.be>

Title: Why is the outer membrane attached to the peptidoglycan in the envelope of Gram-negative bacteria?

Although the multilayered architecture of the cell envelope of Gram-negative bacteria was described in the 1960s, we are still unravelling the links between the structure of this cellular component and its functions in the cell. In *Escherichia coli*, tethering of the outer membrane (OM) to the peptidoglycan (PG) is carried out by the lipoprotein Lpp. Two additional OM proteins, the lipoprotein Pal and the β-barrel OmpA participate in OM-PG attachment, but non-covalently. Excitingly, a series of exploratory experiments revealed that cells expressing OmpA and Lpp variants with altered PG-binding properties have a substantially decreased ability to resist sudden drops in osmotic pressure. This was unexpected because the general view is that the PG alone allows bacteria to resist changes in the osmotic environment, not the OM. It is therefore unclear why tethering the OM to the PG is important for osmoprotection. I want to address this question here by establishing the specific contribution of Lpp, Pal and OmpA in connecting the OM to the PG and determining the functional importance of these proteins in allowing *E. coli* to resist osmotic shocks.

Authors names: Alix Dachsbeck, Michaël Deghelt, Jean-François Collet

Affiliations: De Duve Institute, UCLouvain

8

From: Jessica L Davis jldavis2@sheffield.ac.uk

Title: Solving the structure of the enterococcal polysaccharide antigen (EPA)

Enterococcus faecalis is an opportunistic pathogen, commonly found in the healthy human gut, which can also cause life-threatening diseases such as meningitis and septicaemia. The enterococcal polysaccharide antigen (EPA) is a surface exposed polymer produced by all *E. faecalis* strains, and is a major virulence factor, being essential for host immune evasion during infection. It is comprised of a repeating rhamnose backbone which is decorated with strain specific teichoic acid decoration subunits. Work by our group (Smith R et al. (2019) PLoS Pathog 15, e1007730) has shown that these strain specific decoration subunits are responsible for the biological activity of EPA, rather than the highly conserved polyrhamnose backbone. Structural characterisation of EPA decoration subunits could therefore provide key insights into strain-strain phenotype variation and pathogenicity. Recently, the structure of EPA produced by a clinical isolate (V583; Guérardel Y et al. (2020) mBio 11, e00277-20) has been solved, which has paved the way for more detailed analysis of EPA structural diversity and molecular activity. Here, for the first time, we have solved the complete structure of the EPA decoration subunits in the model clinical isolate, OG1RF. EPA was purified enzymatically from the cell wall of OG1RF, then broken into three uniform fragments, corresponding to the polyrhamnose backbone and two decoration subunits, using HF acid treatment. A specialised pipeline of 2D NMR experiments (HSQC, COSY, TOCSY, ROESY and HMBC) was then employed to allow the assignment of each sugar residue, and subsequently solve the structures of the purified decoration subunits. OG1RF decoration subunits were found to differ in size, composition, and level of branching, when compared to V583. This variant structure may help to explain the increased pathogenicity of OG1RF, as compared to V583, and is hopefully one of the first steps towards understanding how EPA impacts *E. faecalis* pathogenesis.

Authors names: Jessica L. Davis[1], Robert E. Smith[1], Emmanuel Maës[2], Evgeny Vinogradov[3], Yann Guérardel[2], Stéphane Mesnage[1]

Affiliations: [1]The University of Sheffield, [2]Université de Lille, [3]National Research Council Canada

9

From: Marcel Gustavo Alaman Zarate <mgalamanzarate1@sheffield.ac.uk>

Title: Identification of a novel enzyme catalysing 1-3 peptidoglycan crosslinks

Text: The two most common and best described peptidoglycan crosslinks are the 3-3 crosslinks produced by L,D- transpeptidases and the 3-4 crosslinks produced by D,D-transpeptidases. Other crosslinks (1-3 and 2-4) have been reported in the literature but the enzymes responsible for these activities are unknown.
We sought to identify the enzyme capable of catalysing 1-3 crosslinks in *Gluconobacter oxydans*. The structural analysis of PG from transposon mutants led to the identification of a gene required for the formation of 1-3 crosslinks. Using a combination of in vitro assays and genetic experiments, we explored the enzymatic properties of this novel enzyme.

Authors names: Marcel Gustavo Alaman-Zarate [1], Brooke Pian [2], Sabrina Marecos Ortiz [2], Buz M Barstow [2], Stéphane Mesnage [1]

Affiliations: [1] University of Sheffield, School of Biosciences, S10 2TN Sheffield, UK. [2] Department of Biological and Environmental Engineering, Cornell University, Ithaca, NY 14853 USA

10

From: Camilla Henriksen <camjen@sund.ku.dk>

Title: A clinically selected *Staphylococcus aureus clpP* mutant survives daptomycin treatment by reducing binding of the antibiotic and adapting a rod-shaped morphology

Text: Daptomycin is a last-resort antibiotic used for treatment of infections caused by Gram-positive antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA). Resistance to daptomycin can develop during therapy but the killing mechanism of daptomycin and the mechanisms of resistance remain incompletely understood. Here we show that a mutation selected during daptomycin therapy inactivates the highly conserved ClpP protease resulting in diminished expression of central virulence factors and reduced susceptibility to daptomycin, vancomycin, and β-lactam antibiotics. Super-resolution microscopy demonstrated that inactivation of ClpP reduced binding of daptomycin to the septal site and mitigated the membrane-damaging defects imposed by daptomycin. In both the parental strain and the *clpP* strain, daptomycin inhibited the inward progression of septum synthesis eventually leading to lysis and death in the parental strain while surviving *clpP* cells were able to continue synthesis of the peripheral cell wall in the presence of 10 × MIC daptomycin resulting in a rod-shaped morphology. To our knowledge, this is the first demonstration that synthesis of the outer cell wall continues in the presence of daptomycin. Our data provide novel insight into the killing mechanism of daptomycin and emphasize that treatment with last-line antibiotics is selective for mutations that, like the SNP in *clpP*, favor antibiotic resistance over virulence gene expression.

Authors names: Lijuan Xu[1], Camilla Henriksen[1], Viktor Mebus[1], Romain Guérillot[2], Andreas Petersen[3], Nicolas Jacques[4], Kirsten Leeten[4], Timothy P. Stinear[2], Cécile Oury[4], Anton Peleg[5], Benjamin Howden[2], and Dorte Frees[1]

Affiliations: 1) Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark, 2) Department of Microbiology and Immunology, University of Melbourne at the Doherty Institute for Infection and Immunity, Melbourne, Victoria, Australia. 3) Statens Serum Institute, Copenhagen, Denmark, 4) Laboratory of Cardiology, GIGA Institute, University of Liège Hospital, Liège, Belgium. 5) Department of Infectious Diseases, The Alfred Hospital and Central Clinical School, Monash University, Melbourne, Victoria, 3004, Australia, and the Infection and Immunity Program, Monash Biomedicine Discovery Institute and Department of Microbiology, Monash University, Clayton Campus, Melbourne, Victoria, Australia.

11

From: Damien Devos <damienpdevos@gmail.com>

Title: Essential gene complement of the non-model bacterium Planctomycetes *Planctopirus limnophila* reveals divergent biology

Text: *Planctopirus limnophila* is a reference for the non-model microorganism in the Planctomycetes phylum of the Bacteria, a key lineage in the exploration of biodiversity and cell evolution. Here we report a genome wide identification of the repertoire of genes essential to *P. limnophila* growth in culture. We confirm previous targeted gene knockouts, uncover the non-essentiality of functions assumed to be essential to the Planctomycetes cell, including the peptidoglycan and cell division, and highlight essential genes whose functions are yet to be determined. Our results demonstrate non-canonical peptidoglycan synthesis and division processes in Planctomycetes, revealing the novel biology present in this bacterial phylum. Furthermore, we explore potential stages of evolution of the essential gene repertoire of the planctomycetal cell and associated, *Verrucomicrobia* and *Chlamydia* (from the PVC superphylum).

Authors names: Elena Rivas-Marin, David Moyano-Palazuelo, Valentina Henriques, Enrique Merino, Damien P. Devos

Affiliations: Centro Andaluz de Biología del Desarrollo, Universidad Pablo de Olavide, Consejo Superior de Investigaciones Científicas, Spain

12

From: Yves Brun <yves.brun@umontreal.ca>

Title: Evolution of a novel mode of bacterial cell elongation by modified localization of the peptidoglycan synthesis machinery

Text: Despite decades of study, we know the patterns or modes of cell elongation in very few bacterial species. Because most of the best-studied species are phylogenetically distant from one another, how bacterial elongation mechanisms have evolved remains to be determined. To address this question, we used fluorescent D-amino acid labeling to track the spatio-temporal dynamics of peptidoglycan (PG) biosynthesis in closely related species of the Caulobacterales. We discovered that *Asticcacaulis excentricus* and *Phenylobacterium conjunctum* have a unidirectional-midcell elongation mode that is distinct from the bidirectional elongation mode of their close relative, *Caulobacter crescentus.* The difference in elongation modes is due to repositioning of the PG synthase PBP2. Furthermore, we found that *A. biprosthecum* combines the unidirectional-midcell elongation mode of *A. excentricus* with a polar elongation mode. Thus, repositioning of homologous proteins involved in PG synthesis can result in the evolution of a diversity of elongation modes at close evolutionary distance.

Authors names: Liu Yang [1], Marie Delaby [1], Michael S. VanNieuwenhze [2], Yves V. Brun [1*]

Affiliations: 1. Département de microbiologie, infectiologie et immunologie, Université de Montréal, Montréal, Canada; 2. Department of Chemistry, Indiana University, Bloomington, IN, USA

13

From: Kevin Whitley <kevin.whitley@newcastle.ac.uk>

Title: Watching bacterial cell division one molecule at a time in vertical cells

Text: Cell division is a fundamental need for bacteria and a key antibiotic target. Central to this process in most bacteria are the cytoskeletal protein FtsZ that forms a ring of motile filaments at the division site and the peptidoglycan (PG) synthases that build the cell wall inward. Despite their centrality, exactly how these molecules contribute to cell division—and coordinate their activities with one another—is unclear. In my talk, I will show how we use single-molecule microscopy to understand the mechanism of cell division in the model Gram-positive bacterium *Bacillus subtilis*. I will introduce an imaging method we recently developed where we force rod-shaped cells to stand vertically in nanofabricated arrays of bacteria-shaped holes, forcing their division rings into a single microscope imaging plane. I will show how we use this new method to watch single molecules of the PG synthase PBP2B as they move around the division ring to build the cell wall inward. Finally, I will show how we use division-specific antibiotics to understand how PBP2B coordinates its activity with FtsZ. Our results reveal key new information about the molecular mechanism of cell division in Gram-positive bacteria while demonstrating broadly applicable new methods for bacterial microscopy.

Authors names: Kevin D Whitley, James Grimshaw, Séamus Holden

Affiliations: Centre for Bacterial Cell Biology, Biosciences Institute, Newcastle University; School of Life Sciences, University of Warwick

14

From: Viktor Hundtofte Mebus <vhm@sund.ku.dk>

Title: Control of autolytic activity in *Staphylococcus aureus* is dependent on post-transcriptional and proteolytic regulators.

Text: Bacterial cells encode a wide range of cell wall hydrolases, to allow for the incorporation of new cell wall material and timely daughter cell separation. Given the lethal consequences of dysregulated peptidoglycan degradation, cell wall hydrolase activity must be tightly coordinated with peptidoglycan synthesis. Relatively little is known of the checkpoints that safeguard bacteria, from the detrimental activity of cell wall hydrolases. In *Staphylococcus aureus*, large-scale identification of substrates of the ClpX/P protease revealed abundant trapping of several uncharacterized cell wall hydrolases, suggesting that cell wall hydrolase regulation can be attributed to proteolysis. In my project I have confirmed that the putative autolysin, (SAUSA300_0651) is a substrate of the ClpX/P protease. Additionally, I have demonstrated that an anti-sense RNA transcript controls this putative autolysin. We speculate that the involvement of ClpX in regulating autolytic activity, is conserved for other gram-positive bacteria. Here, we used the naturally autolytic *Streptococcus pneumoniae* as a model organism and in May-June I will conduct large scale trapping of ClpX/P substrates in this bacterium in Greifswald, Germany.

Authors names: Viktor H. Mebus, Dorte Frees, Camilla Henriksen, Tobias Krogh Nielsen.

Affiliations: Copenhagen University

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From: Camilo Perez <camilo.perez@unibas.ch>

Title: Mechanistic basis of choline import and lipid flipping involved in teichoic acids synthesis and modification

Text: Teichoic acid decorated with phosphocholine molecules have fundamental roles in bacteria adhesion to host cells, immune evasion, and persistence. The biosynthesis of teichoic acids is a multistep process that involves several membrane proteins, including transporters of lipids and soluble precursors. *Streptococcus pneumoniae* is a prominent pathogen where phosphocholine decoration plays a fundamental role in virulence. This decoration occurs after choline uptake by LicB, and conversion to phosphocholine by LicA and LicC proteins. Teichoic acids decorated with phosphocholine, are then flipped across the plasma membrane, an essential step that regulates the population of the biopolymer at the cell wall. Cell wall exposed phosphocholine epitopes allow anchoring of choline-binding proteins, which contribute to adherence, colonization, and virulence. Here, we discuss cryo-EM and crystal structures of the membrane proteins involved in choline uptake and lipid flipping during teichoic acid synthesis in *S. pneumoniae*. We describe architectural and mechanistic elements essential to their activity, and together with *in vitro* and *in vivo* functional characterization, we study their role in the adaptation of *S. pneumoniae* to stress conditions. Our results provide previously unknown insights into the molecular mechanism of two transporters involved in bacterial pathogenesis and establish a basis for inhibiting the teichoic acid synthesis pathway in *S. pneumoniae*.

Authors names: Arantza Balsebre, Natalie Bärland, Gonzalo Cebrero, Camilo Perez

Affiliations: Biozentrum, University of Basel

16

From: Nicholas Briggs <n.briggs@warwick.ac.uk>

Title: Structural & Biochemical Investigations of the Pneumococcal FtsEX-PcsB Cell Division Complex

Text: Bacterial cell division as long been considered a promising target for novel antibiotic chemotherapies, since the process is essential and subject to many mechanisms of coordinated regulation required to control the process. Successful daughter cell generation is achieved by the concerted efforts of the "Divisome" protein complex, a highly dynamic collection of sub-complexes that work together to ensure the coordinated remodelling of the bacterial cell wall, whilst retaining cellular shape and rigidity. Disruption of this complex leads to profound morphological defects in model organisms, supporting their candidacy for next-generation antibiotic therapies. Here, we consider the pneumococcal FtsE-FtsX-PcsB subcomplex. FtsEX is predicted to resemble a type 7 ABC transporter, and serves as the structural basis and control mechanism for the putative endopeptidase; PcsB. This peptidoglycan hydrolase co-localises to the peripheral peptidoglycan synthesis ring with FtsEX during division, suggesting it plays a role in Divisome complex formation as well as peripheral peptidoglycan biosynthesis, by creating "holes" in the existing sacculus for new peptidoglycan strands to be inserted. This activity is thought to be driven by the ATPase activity of FtsE providing a conformational change which is transmitted through FtsX to PcsB, thus promoting the activation of the endopeptidase. We have shown through in silico predictions of conformational states & the bioengineering of a disulphide bond within PcsB that the endopeptidase can be locked in an inactive conformation, which subsequently inhibits the activity of FtsE. These findings have major implications for the mechanistic understanding of the complex, and thus how inhibitors to the complex should be designed.

Authors names: Nicholas Briggs, Prof. David Roper

Affiliations: University of Warwick

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From: Jean-Pierre Simorre <jean-pierre.simorre@ibs.fr>

Title: “In-cell” NMR for studying β-lactamase and PBP competition

Text: Rapid antibiotic resistance spread across bacterial pathogens necessities new therapeutic approaches. Overuse, underinvestment and lack of innovation lead to a shortage of effective antimicrobials. During my postdoctoral training, I work in collaborative project aiming to develop new antibiotics to specifically eliminate antibiotic-resistant pathogens. The final drug will comprise of two beta-lactam antibiotics. First part, termed the “sacrificial beta-lactam” is susceptible to cleavage of beta-lactamases, hydrolytic enzymes synthetized by drug-resistant strains. Hydrolysis of sacrificial component releases a second drug, the “warhead beta-lactam”. It binds PBP enzymes and inhibits their action, which leads to bacterial cell death.
To provide molecular understanding of this reaction in atomic resolution and in the native context, we utilised solution nuclear magnetic resonance (NMR) approach. We have first established in vitro conditions to monitor hydrolysis of a set of commercially available beta-lactams by two beta-lactamases (KPC-2 and NMD-1). This allowed to explicitly define antibiotic bonds which are hydrolysed by these enzymes and track the appearance of the reaction products in the time course. To get insights into the competition for beta-lactam between beta-lactamases and the cellular target of the antibiotic, the same reaction was run in the presence of PBP2. This revealed that one of the studied antibiotics binds PBP2 prior to its digestion by beta-lactamase, which indicates it is a good candidate for the “sacrificial” part of the developed drug. In addition, through NMR titrations, we defined which amino acids of beta-lactamase are involved in the antibiotic binding. Finally, building on this, we switched gears to in-cell NMR approach. Immobilising cells in a gel solution allowed us to monitor the beta-lactam hydrolysis in real time directly in bacterial periplasm using specifically designed NMR filtering methods.
With this project, we have developed a combined NMR approach to monitor the hydrolysis of beta-lactam antibiotics (1) in vitro under conditions that closely mimic the native reaction context (competition with PBP2), and (2) in vivo directly in bacterial periplasm. This strategy is suitable for tracking the metabolism of other small molecules in the bacterial periplasm, which opens a door to new applications and we strongly believe will be of great interest to the CellMAP Symposium audience.

Authors names: Razew A., Miyachiro M., Bougault C., Dessen A., Arthur M., Simorre JP

Affiliations: Institut de Biologie Structurale, Grenoble, France

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From: Adria Sogues <asoguesc@vub.be>

Title: S-layer localised proteins play a role in cell wall remodelling

Text: Bacillus anthracis is the etiological agent of anthrax, an ancient and deathly disease that affects humans, livestock and wildlife. It is a spore-forming, Gram-positive bacterium whose genome contains several virulence factors. The surface layer (S-layer) is one of them as it represents the first line of contact with the host. The S-layer is the outermost component of the cell envelope generally composed of a single protein that self-assembles forming a two-dimensional para-crystalline monolayer. In B. anthracis, the main structural components of the S-layer harbour three SLH domains that interact via non-covalent interaction with the secondary cell wall polysaccharide (SWCP) that decorates the peptidoglycan. Interestingly, there are other 22 proteins in B. anthracis that also contain several SLH domains but do not form a crystalline array, these S-layer localized proteins, are known as minor components representing only 10% of the total S-layer composition. BslO is a minor component that shows septal S-layer localization and plays a role in reducing the length of bacillus chains. Here, we have solved the crystal structure of BslO reporting that it contains a β-N-acetylglucosaminidase and an SH3b domain. We used CRISPR/Cas9 to generate the knockout of bslO and we observed that, in addition to the very long chain of cells, the chains are supercoiled indicating that BslO also plays a role in peptidoglycan crosslinking relaxation. Finally, intrigued by the septal localization of an S-layer protein, we constructed several mutants that have helped us to shed light on the key factors responsible for its localisation.

Authors names: Adrià Sogues (1,2), Antonella Fioravanti (1,2) and Han Remaut (1,2)

Affiliations: 1.Structural and Molecular Microbiology, Structural Biology Research Center, VIB, Pleinlaan 2, 1050 Brussels, Belgium 2.Structural Biology Brussels, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium

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From: Quentin Herail <herailquentin1@gmail.com>

Title: Beta-lactamase production in clinical isolates versus laboratory strains of *Escherichia coli*.

Text: My PhD project is related to the design of prodrugs that will be selectively activated by β-lactamases produced by pathogens. For this purpose, two β-lactam antibiotics will be combined within the same molecule to obtain prodrugs with no intrinsic activity. Hydrolysis of a sacrificial β-lactam will liberate an active β-lactam ("warhead") only in the periplasm of the pathogens thereby achieving selectivity and activity. My PhD project will focus on the biological evaluation of these prodrugs in terms of antibacterial activity, penetration, and hydrolysis by β-lactamases. To achieve this goal, I have constructed derivatives of *Escherichia coli* that express prototypical β-lactamases (NDM-1 and KPC-2) at various levels based on the use of various combinations of multi-copy vectors and inducible promoters. A deletion of the *ampC* cephalosporinase gene was introduced in the chromosome of the host strain. These strains will be used to assess the efficacy and selectivity of the prodrugs on strains producing a single β-lactamase. In my poster, I will present correlations between the level of production of the β-lactamases and the phenotype expression of resistance to representatives of the four classes of β-lactams. Multidrug resistant clinical isolates of *E. coli* and *Klebsiella pneumoniae* were similarly characterized. I show that the levels of production of the β-lactamases in clinical isolates are mimicked by high-level production of β-lactamases achieved by the cloning of the corresponding genes in multi-copy plasmid harboring the ColE1 replication origin and under the control of strong promoters such as *ptrC*. The level of production of the β-lactamases required for expression of β-lactam resistance were lower in clinical isolates that in engineered E. coli strains presumably due to a lower permeability of the outer membrane.

Authors names: Herail Quentin and Michel Arthur

Affiliations: UMRS 1138, Centre de Recherche des Cordeliers, Sorbonne Université, INSERM, Université Paris Cité, Paris 75006

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From: Izabela Sabala <isbala@imdik.pan.pl>

Title: Electrostatic interaction with the bacterial cell envelope tunes the lytic activity of peptidoglycan hydrolases

Text: Peptidoglycan (PG) hydrolases play a crucial role in the metabolism of the bacterial cell wall (CW), but many aspects of interactions of the enzymes with bacterial cell walls are not fully revealed. Detailed mechanism of regulation of enzymes activity based on electrostatic interactions between hydrolase molecule and bacterial CW surface remains unknown. To address this question by comprehensive study using as a model two novel PG hydrolases, SpM23_A, and SpM23_B, which although share the same bacterial host, similarities in sequence conservation, domain architecture, and structure, display surprisingly distinct net charges (in 2D electrophoresis, pI, 6.8, and pI 9.7, respectively). We demonstrate a strong correlation between hydrolases surface net charge and the enzymes activity by modulating the charge of both, enzyme molecule and bacterial cell surface. Teichoic acids, anionic polymers present in the bacterial CW, are shown to be involved in the mechanism of enzymes activity regulation by the electrostatics-based interplay between charged bacterial envelope and PG hydrolases. These data serve also as a hint for the future development of chimeric PG hydrolases of desired antimicrobial specificity.

Authors names: Alicja Razew, Łukasz Łezniak, Elzbieta Jagielska, Izabela Sabała

Affiliations: Mossakowski Medical Research Institute Polish Academy of Sciences, Warsaw, Poland

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From: Leonor Teixeira Bismarck Quintas Marques <leonor.bismarck@gmail.com>

Title: Understanding the function and regulation of *Staphylococcus aureus* hydrolases.

Text: Although the structure and synthesis pathway of peptidoglycan are well studied, the process of peptidoglycan (PG) hydrolysis that leads to a mature PG has been less studied. However, maturation of the PG required both processes to be in tight balance. Regulation and activity of PG hydrolases is still under explored. This is due to several facts including that these enzymes are not essential, have redundant roles and participate in different cellular processes.
In this project we have selected to work with classes of two PG hydrolases: glucosaminidases (SagA, SagB and ScaH) and lytic transglycosylases (SceD and IsaA).
We analyzed lytic transglycosylase and glucosaminidase knockout mutants for cell cycle progression, cell morphology and cell wall (CW) structure. We detected small differences for JE2 Δ*sceD* and Δ*isaA* mutants, with more spherical cells and with cells taking slightly longer time to split the septum, indicating a role in cell shape control. In JE2 Δ*sagB,* JE2 Δ*atl,* JE2 Δ*sagA* Δ*sagB,* JE2 Δ*sagB* Δ*scaH,* JE2 Δ*sagB* Δatl_{GL} and JE2 Δ*scaH* Δatl_{GL} mutants, cells took longer to begin septum synthesis. No major differences in muropeptides composition were observed with exception to an increase in low cross-linked species in the JE2 Δ*scaH*, JE2 Δ*sagB* and JE2 Δ*sagB* Δ*scaH* mutants.

Authors names: Leonor B. Marques[1], Helena Veiga[1], Sérgio R. Filipe[2], Mariana G. Pinho[1]

Affiliations: [1] Bacterial Cell Biology Lab, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras Portugal. [2] Bacterial Cell Surfaces and Pathogenesis Lab, Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, Caparica, Portugal.

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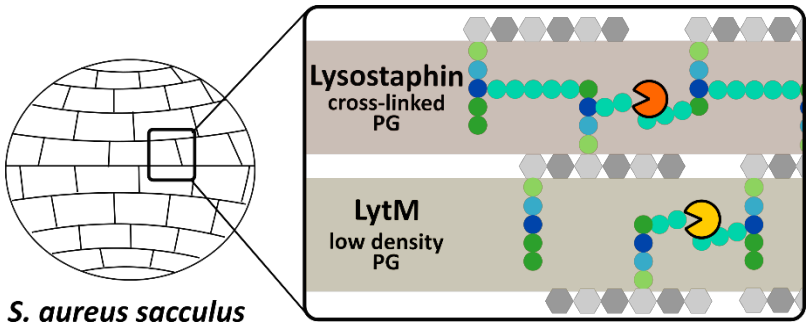
From: Alicja Razew (Wysocka) <alicja.razew@ibs.fr>

Title: *Staphylococcus aureus* sacculus mediates activities of M23 hydrolases

Text: Peptidoglycan (PG) is a giga-dalton polymer constituting scaffold and integrity of bacterial cell wall (CW). PG is metabolised by a large and diverse group of PG hydrolases, guards of bacterial cell growth and division. These enzymes have been the focus of many studies over the years, but molecular understanding of their action within PG mesh is missing. In our recent work we filled this gap by studying the interaction of two evolutionary related peptidases of the M23 family, lysostaphin and LytM, with short PG fragments and the entire sacculus. We integrated solution and solid state nuclear magnetic resonance, information-driven modelling, mass spectrometry, site-directed mutagenesis and biochemical approaches to provide high-resolution details of their interaction with the *Staphylococcus aureus* PG. We determined which bond lysostaphin and LytM hydrolyse in the muropeptide multimers and in the PG sacculus, as well as the residues that form binding interface for each of these ligands. We found that these enzymes form rich network of molecular contacts with PG sacculus, which influences their specificity. Based on that, we proposed a new model in which PG cross-linking affects the activity of these two enzymes differently (**fig. 1**).
Our work sheds new light on the action of PG hydrolyses in the complex mesh of the *S. aureus* cell wall. In a broader perspective, it provides a basis for future research on the development of new antimicrobial agents. Due to the inherent heterogeneity and flexibility of PG, structural studies on this polymer are scarce. The present work paves the way for future research on enzymes acting on bacterial PG using structural and quantitate approaches, which we believe will be of great interest to the GW symposium audience.

Fig. 1 Proposed model of lysostaphin and LytM regulation by PG cross-linking.



Authors names: Razew A.[1,2,3], Laguri C.[1], Vallet A.[1], Bougault C.[1], Sabala I.[2,3#] & Simorre JP[1#]

Affiliations: [1]Univ. Grenoble Alpes, CNRS, CEA, Institut de Biologie Structurale, 38000 Grenoble; [2]International Institute of Molecular and Cell Biology in Warsaw, Poland; [3]Laboratory of Protein Engineering, Mossakowski Medical Research Institute, Polish Academy of Sciences, Warsaw, Poland

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From: Constantin Anoyatis-Pelé <canopele@gmail.com>

Title: Comparison of genome-wide Tn-seq and CRISPRi approaches for the identification of genes selectively essential for L,D-transpeptidase mediated β-lactam resistance.

Text: Peptidoglycan cross-linking in *Escherichia coli* is mainly performed by D,D-transpeptidases belonging to the penicillin-binding protein (PBP) family. Bypass of these PBPs by the YcbB L,D-transpeptidase (LDT) results in broad spectrum resistance to β-lactams. Tn-seq analysis previously identified 179 genes that are selectively essential for LDT-mediated β-lactam resistance. This approach achieved a very low false discovery rate (<5%) but was resource and time-consuming precluding the analyses of a large number of genetic backgrounds and growth conditions. In this report, a genome wide CRISPRi approach was investigated to address these issues. In comparison to Tn-seq, CRISPRi identified a larger number of genes essential for LDT-mediated β-lactam resistance (179 versus 364, respectively). There were 257 genes identified as essential by CRISPRi but not by Tn-seq, which fall in four groups. (i) Genes located upstream of an essential gene in the same operon are misidentified as essential because CRISPRi leads to polar effects (34 genes). (ii) Silencing of certain essential genes results in a more rapid elimination of the bacteria in the presence of the drug than in its absence. CRISPRi data are only analyzed as a fold change between two conditions, i.e. growth in the presence or absence of the β-lactam ceftriaxone in the current experiment. For this reason, genes essential in both conditions but more rapidly eliminated in the presence of the drug are misidentified as selectively essential for resistance by CRISPRi (142 genes). (iii) Genes encoding proteins containing both essential and non-essential domains for resistance are not classified as essential by Tn-seq because the average number of Tn insertions per gene is used for the statistical analysis. CRISPRi identifies these genes as essential and is therefore more accurate for this group of genes (11 genes). Together, the latter three groups account for 187 of the 257 genes that were classified as essential by CRISPRi and as unessential by Tn-seq. The remaining genes, 70 out of 257, are tentatively assigned to the false discovery rate of the CRISPRi approach. Seventy-three genes were identified as essential by Tn-seq but not by CRISPRi. These genes comprise a large set of genes involved in colanic acid synthesis (20 out of 73). It was previously shown that colanic acid is an unessential polymer for growth both in the presence and absence of the drug. However, Tn insertion in any of the colanic biosynthetic genes results in the accumulation of precursors containing the undecaprenyl lipid carrier that is also essential for peptidoglycan synthesis. This is not compatible with growth in the presence of β-lactams because exposure to these drugs incurs a high anabolic demand on the peptidoglycan assembly pathway. For this reason, Tn-seq identified as essential for resistance all colanic acid biosynthesis genes except wcaJ encoding the enzyme committed to the first step of colanic acid production. Due to polar effects, CRISPRi silenced the whole colanic acid pathway. Altogether, 107 genes were identified as selectively essential for YcbB-mediated β-lactam resistance both by CRISPRi and Tn-seq. Thus, in spite of discrepancies partly accounted for by polar effects, these results indicate that CRISPRi is a valuable alternative to Tn-seq that is more adapted to high throughput investigations. In combination, the two approaches also provide complementary information.

Authors names: Constantin Anoyatis-Pelé[1], Henri Voedts[1], David Bikard[2], Michel Arthur[1], Jean-Emmanuel Hugonnet[1]

Affiliations: 1-UMRS 1138 Centre de recherche des Cordeliers- Sorbonne Université, INSERM, Université Paris Cité. 75006 PARIS France. 2- Institut Pasteur, Université de Paris, CNRS UMR 6047, Synthetic Biology, 75015 Paris, France

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From: Yucheng Liang <lycheer42@gmail.com>

Title: The dynamics of the anchoring of the Braun lipoprotein to peptidoglycan in *Escherichia coli*

Text: The envelope of *Escherichia coli* is stabilized by a link between the outer membrane and the peptidoglycan mediated by the Braun lipoprotein (Lpp). Three members of the L,D-transpeptidase (LDT) family (Ybis, ErfK, and YcfS) catalyze formation of a covalent link between the α carbonyl of DAP at the third position of a peptidoglycan tripeptide stem and the side-chain amino group of an L-Lys residue located at the C-terminal position of Lpp. An additional member of the LDT family, YafK, was recently shown to cleave the resulting tripeptide-Lpp amide bond in vitro. However, no phenotype was associated with the deletion of the gene encoding YafK and its role in the envelope homeostatis therefore remains elusive. Here, we explore the mode of insertion of newly synthesized Lpp molecules into the peptidoglycan network and the role of YafK in hydrolyzing existing tripeptide-Lpp links. The experimental design was based on cultures of E. coli in minimal medium containing 13C glucose and 15N ammonium chloride as sole sources of carbon and nitrogen, respectively. Bacteria were pelleted, resuspended in minimal medium containing 12C glucose and 14N ammonium chloride, and the appearance of light isotope-containing peptidoglycan and Lpp molecular species was determined by mass spectrometry at 0, 20, 40, and 60 min after the medium switch. At 20 min, light (neo-synthesized) tripeptide linked to light (neo-synthesized) peptidoglycan was not detected. This result shows that new peptidoglycan subunits and new Lpp are independently inserted into the peptidoglycan. Thus, Lpp is not anchored to lipid II or to newly synthesized glycan strands resulting from the polymerization of this lipid intermediate. In the host producing the functional YafK hydrolase, mass spectrometry analysis revealed abundant hybrid tripeptide-Lpp isotopologues containing a heavy (existing) Lpp moiety and a light (neo-synthesized) tripeptide moiety. The abundance of this hybrid was drastically reduced in the mutant obtained by deletion of the gene encoding YafK indicating that this hydrolase cleaves existing tripeptide-Lpp links thereby providing existing (heavy) Lpp for anchoring to neo-synthesized (light) peptidoglycan stem peptides. These results directly establish that peptidoglycan anchored and free Lpp forms are in a dynamic equilibrium mediated by the amidase activity of YafK and the transpeptidase (anchoring) activity of Ybis, ErfK, and YcfS.

Authors names: Yucheng Liang(1), Jean-Emmanuel Hugonnet(1), Filippo Rusconi (1 and 2), and Michel Arthur(1)

Affiliations: 1-UMRS 1138-Centre de Recherche des Cordeliers, Sorbonne-Université-INSERM-Université Paris Cité; 2-PAPPSO, Université Paris-Saclay, INRAE, CNRS, Paris, France.

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From: Vanessa Becker <s6vabeck@uni-bonn.de>

Title: “Novel points of attack” – Exploiting capsule biosynthesis in *Streptococcus pneumoniae* for antibacterial treatment

Text: Synthesis of a polysaccharide capsule is crucial for pneumococci to resist the immune system during infection. Whereas capsules have been successful targets for vaccines, biosynthetic reactions and mechanisms regulating capsule expression have not been investigated as potential therapeutic targets so far. Most S. pneumoniae serotypes produce capsular polysaccharides (CP) via the Wzx/Wzy-dependent pathway. In this case, CP building blocks are assembled on the C55-P at the inner side of the cytoplasmic membrane, translocated and polymerized in a non-processive manner on the exterior of the cell. Enzymes of the LytR-CpsA-Psr (LCP) family are presumed to catalyze the transfer and the covalent linkage of CP to peptidoglycan under release of the lipid carrier. However, biochemical evidence and molecular details for these reactions are mostly lacking. To get a deeper understanding of these processes, streptococcal capsular biosynthesis reactions are functionally reconstituted in vitro using purified recombinant enzymes and substrates. Furthermore, post-translational regulatory mechanisms, particularly phosphorylation, which allow the orchestration of CP and PGN reactions, are investigated.

Authors names: Vanessa Becker, Melina Arts, Marvin Rausch, Tanja Schneider and Anna Müller

Affiliations: Institute for Pharmaceutical Microbiology, University Hospital Bonn, University of Bonn, Bonn, Germany

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From: Melina Arts <melina.arts@uni-bonn.de>

Title: Differences in mode of action and target recognition of lipid II binding antibiotics

Text: The emerging spread of antimicrobial resistance exerts immense pressure on health care systems worldwide with infections caused by (multi-)drug resistant bacteria being the cause for numerous hospitalizations and deaths. The discovery of new antibiotics targeting various structures of the bacterial cell as well as research on potential target structures are therefore urgently necessary. We compare the mode of action of two structurally similar antibiotic compounds. Both antibiotics were found to target a broad spectrum of gram-positive bacteria, however they exhibit differential antimicrobial activity. Applying a combination of in vitro and in vivo systems, we obtained proof that both compounds target cell wall biosynthesis and bind the ultimate peptidoglycan precursor lipid II. However, we determined substantial differences in the interaction with additional target molecules. We further analyzed the architecture of the compound:lipid complex by crystallization to resolve the characteristics of target recognition.

Authors names: Melina Arts, Ioli Kotsogianni, Philipp Hendricks, Yvonne Thoma, Irina Helmle, Anna Müller, Gregor Hagelüken, Heike Brötz-Oesterhelt, Harald Groß, Hideki Hashizume, Nathaniel I. Martin, Tanja Schneider

Affiliations: 1. Institute for Pharmaceutical Microbiology, University Hospital Bonn, University of Bonn; Bonn, Germany. 2. Biological Chemistry Group, Institute of Biology Leiden, Leiden University; Leiden, the Netherlands. 3. Institute of Structural Biology, University of Bonn, Bonn, Germany. 4. Interfaculty Institute of Microbiology and Infection Medicine, Dept. of Microbial Bioactive Compounds, University of Tuebingen; Tuebingen, Germany. 5. Department of Pharmaceutical Biology, Pharmaceutical Institute, University of Tübingen, Tübingen, Germany. 6. Department of Microbiology, Institute of Microbial Chemistry, Tokyo, Japan.

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From: Chenyu Li <lich0052@e.ntu.edu.sg>

Title: Structural elucidation and functional investigation of gut microbiota-derived peptidoglycan fragments in host

Text: The host gut is the natural habitat of enormous bacteria that proactively participate in host health regulation. Elucidation of molecular mechanisms underlying gut microbiota-host communication not only contributes to better comprehension fundamentally but provides insights into potential therapeutic strategies against certain microbiome-associated diseases. Our latest studies revealed the ubiquitous presence of peptidoglycan fragments (PGNs), essential microbial metabolites, in host systemic circulation and their implication in host autoimmunity. It was also disclosed that increased PGNs in the host gut niche can trigger invasive fungal growth, implying diverse functions of microbial PGNs in the host. Here in the current study, we introduced an HPLC-MS/MS platform for the quantification and structural profiling of naturally released PGNs in the host and demonstrated their engagement in innate or adaptive immunoregulation via NOD1/2-independent pathway. Our progress may significantly advance the current knowledge of commensal bacterial PGNs as an emerging family of effector molecules.

Authors names: Chenyu Li, Yuan Qiao (PI)

Affiliations: Nanyang Technological University

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From: Jie Xiao <xiao@jhmi.edu>

Title: A structural model of the essential E. coli septal cell wall synthesis complex formed by FtsWI, FtsQLB, and FtsN

Text: The bacterial cell wall is a single macromolecule composed of a mesh network of cross-linked peptidoglycans (PG). During cell division, the cell wall provides mechanical stability and protection to the cell and is carefully remodeled and constricted to allow cell division without the risk of lysing. This process is orchestrated by a myriad of cytoskeletal proteins, PG processing enzymes and their associated regulators. Recent studies from our and other groups have discovered that FtsZ, an essential tubulin homolog, uses its GTP hydrolysis to power treadmilling dynamics to distribute the core sPG synthase complex FtsQLBWI (formed by FtsQLB, a tripartite divisome subcomplex, FtsW, the glycosyltransferase, and FtsI, the transpeptidase) evenly along the septum to ensure smooth, symmetric septum formation. We show that the core complex can also exit treadmilling FtsZ polymers to initiate processive septal cell wall synthesis, which requires the presence of FtsN, another essential divisome protein that binds to denuded PG, an intermediate of septal cell wall along the destruction pathway. Using single-molecule imaging, computational modeling, and mutagenesis, we investigate the structure, dynamics, and activation mechanism of the core complex FtsQLBWI and its coordination with septal cell wall degradation.

Authors names: Jie Xiao, Brooke Britton, Remy Yovanno, Xinxing Yang, Jason Liu, Amilcar Perez, Josh McCausland, Albert Lau, Zach Hensel, Jian Liu

Affiliations: Johns Hopkins School of Medicine

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From: Elżbieta Jagielska <ejagielska@imdik.pan.pl>

Title: LytM catalytic domain and LytM_LssSH3b Chimera efficiently remove staphylococci from biofilms

Text: One of the top five major pathogens responsible for food borne illnesses globally is *Staphylococcus aureus*, causing staphylococcal food poisoning. Bacterial strains transmitted in the food chain show both, antibiotic-resistance and tolerance to disinfectants. There is a need for environmentally friendly alternatives to control Staphylococcus spp. and other bacteria in food processing environments that enable sustainable production and consumption of food that is safe. We have been investigating catalytic domain of *Staphylococcus aureus* autolysin LytM (LytM_CD) alone and fused with *Staphylococcus simulans* lysostaphin SH3b domain Chimera. Both enzymes share the same catalytic domain, which belongs to M23 Zn-dependent metallopeptidases family and have been proven to act as specific peptidoglycan hydrolases on planktonic cells, targeting pentaglycine bridges, which can be found only in peptidoglycan structure of *Staphylococcus* spp. cell walls. Here we demonstrate enzymes efficiency also in removal of bacterial cells from various surfaces and biofilms. LytM_CD and LytM_SH3b Chimera reduced 99% of initial number of staphylococcal cells from surfaces, e.g., glass, silicone, metal, but also cells growing in a form of biofilm. Additionally, enzymes were shown to be safe for eukaryotic cells, not toxic and stable, which overall allow to consider them as good candidates for surface disinfectants, especially those, which have contact with food during food processing.

Authors names: Ana Astorga, Michał Zaród, Justyna Czarnecka, Izabela Sabała, Elżbieta Jagielska

Affiliations: Mossakowski Medical Research Institute

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From: Catherine L. Tooke <ct12425@bristol.ac.uk>

Title: Interactions of diazabicyclootanes with β-lactamases revealed by time-resolved crystallography

Text: β-Lactams, the most prescribed antibiotics worldwide, target cell wall biosynthesis. Resistance in Gram-negative pathogens is increasingly prevalent due to the production of β-lactamases (BLAs), enzymes which break them down. Diazabicyclooctanes (DBOs, e.g. avibactam) are reversible BLA inhibitors that are clinically combined with β-lactam antibiotics to potentiate their bactericidal activity. New DBO iterations have activity towards both BLAs and penicillin binding proteins (PBPs) and so could represent a new class of antimicrobials. Structure-activity investigations of how DBOs bind and inactivate their target enzymes are of importance to BLA and PBP inhibitor design and development. Conventional cryo-crystallography approaches largely focus on the formation of late-stage inhibitory complexes. We present new developments in serial, room-temperature crystallography using drop-on-chip mixing at synchrotron and X-ray free electron laser (XFEL) sources to investigate BLA:inhibitor interactions. In particular, we explore the reaction of the DBO avibactam with the clinically important BLA CTX-M-15. Within 2 ms avibactam covalently carbamylates the catalytic Ser70 and accumulates in crystallo at a concentration that permits a second, intact molecule of avibactam to bind. Our data reveal time-resolved movements of avibactam N-6 with respect to the carbamyl ester C-7 that are important for avibactam recyclization. N-6 rapidly (within 2 ms) flips away from C-7 following carbamylation yet equilibrates over time to a conformation that is 'primed' for recyclization. These developments in drop-on-chip sample delivery allow rapid diffusion of ligand microdroplets within microcrystals, enabling high-resolution (1.65 Å) images of covalent ligand binding within 2ms, to date the fastest in crystallo rapid mixing experiment so far reported. The dynamic snapshots of BLA inhibition that we report will inform development of future potent BLA/PBP inhibitor scaffolds.

Authors names: Catherine L. Tooke[1*], Pierre Aller[2*] Philip Hinchliffe[1*], Johan Glerup[2], Agata Butryn[2,3], Anastaysa Shilova[2,3], Tiankun Zhou[2,3], Sam Horrell[2,3], Michael Beer[1], James Beilsten-Edmands[2,3], Pauline A. Lang[4], Patrick Rabe[4], Jos J. A. G. Kamps[2,3], Jaehyun Park[5], Christopher J. Schofield[4], Robin L. Owen[2,3], Allen M. Orville[2,3], and James Spencer[1].
Affiliations: [1]School of Cellular and Molecular Medicine, University Walk, Bristol, UK [2]Diamond Light Source, Harwell Science and Innovation Campus, Didcot, UK [3]Research Complex at Harwell, Rutherford Appleton Laboratory, Didcot, UK [4]Department of Chemistry, Chemistry Research Laboratory, University of Oxford, Oxford, UK [5]PAL-XFEL, 836 Jigok-dong, Nam-gu, Pohang-si, Gyeongsangbuk-do, South Korea
* CLT, PA and PH contributed equally to this work

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From: Jessica Burnier <jessica.burnier@unil.ch>

Title: LadE is a mid-cell localizing adaptor protein required for correct cell morphology in streptococci

Text: Peptidoglycan (PGN) in oval-shaped streptococci is assembled by lateral and septal machineries located at mid-cell. Cell elongation is mainly driven by Pbp2b and associated proteins. However, several streptococci, such as *S. pyogenes*, do not contain Pbp2b. In addition, the molecular mechanisms underlying the coordination of these two machineries in streptococci that do have Pbp2b remain poorly understood. Here, we identified and characterized SPD1429 from *S.pneumoniae* (hereafter called LadE for **L**ysM containing **A**daptor protein involved in **D**ivision and **E**longation). We show that LadE has a conserved mid-cell localization in Streptococci. Mid-cell localization of *S. pneumoniae* LadE depends on its transmembrane spanning domain and not through its LysM PG-bindingdomain. Using immunoprecipitation, split-luciferase assays and fluorescence colocalization, we discovered that *S. pneumoniae* LadE interacts with proteins involved in the regulation (GpsB, DivIVA and StkP) and synthesis and modification of PG (Pbp1a, RodZ, MpgA, and PgdA). Genetic interaction analysis, PG analysis and structural predictions show that LadE is an adaptor protein that is required for proper functioning of Pbp1a and MpgA through GpsBand cells lacking LadE show aberrant cell morphologies. The data suggest a model in which the LadE/Pbp1A/MpgA/RodZ complex constitute an alternative cell elongation complex independent from Pbp2B. In line with this, the LadE-complex is conserved in other streptococci that do not contain Pbp2B and a *ladE* mutant in *S. pyogenes* is severely affected in cell morphology. Finally, we show that LadE is important for bacterial fitness in several animal models of infection suggesting that LadE is an interesting candidate for vaccine or antibiotic discovery projects.

Authors names: Jessica Burnier[*1], Clément Gallay[*1], Louise Martin[1], Johann Mignolet[1], Daniela Vollmer[2], Jacob Biboy[2], Igor H Wierzbicki[3], David J Gonzalez[3], Waldemar Vollmer[2], Jan-Willem Veening[1]

Affiliations: [1]Department of Fundamental Microbiology, Faculty of Biology and Medicine, University of Lausanne, 1015, Lausanne, Switzerland. [2]Centre for Bacterial Cell Biology, Biosciences Institute, Newcastle University, Richardson Road, Newcastle Upon Tyne NE2 4AX, UK. [3]Department of Pharmacology and the Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093, USA.

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From: Beile Gao <gaob@scsio.ac.cn>

Title: The Common Origin and Degenerative Evolution of Flagella in the Actinobacteria phylum Correlates with Terrestrial Adaptation and Species Diversity

Text: Flagellar evolution is a fundamental question to understand the mechanism of self-driven propeller, origin of life and design principles for synthetic biology. Half of the bacterial species have flagella and their flagella display great diversity in both structure and function. Currently, most studies are based on the *E. coli* model or other species with complex motor, lacking information regarding the minimal composition of a motile flagellar nanomachine and why/how 50% species lost it. The *Actinobacteria* phylum has very few species with flagella and some species possess zoospores with the highest swimming speed but very short motile period during their lifecycles. Little to nothing is known about how *Actinobacteria* species acquire their flagellar genes, how their motor structures look like, and why this phylum with diverse species but few are flagellated. Here, we performed a comprehensive evolutionary genomics study of flagellar genes in representative species of the *Actinobacteria* phylum. Our results allow us to resolve the mystery about the origin and evolution of flagella in *Actinobacteria* and its implications for the evolution of bewildering diversity of species in this phylum. Moreover, this study paves the way towards a better understanding of flagellar rotation in thick peptidoglycan layer of gram-positive bacteria and rational design of protein nanomachines.

Authors names: Beile Gao

Affiliations: South China Sea Institute of Oceanology, Chinese Academy of Science

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From: Joshua Sutton <joshua.sutton@sheffield.ac.uk>

Title: A role for GpsB in cell shape determination in *Staphylococcus aureus*

Text: The spheroid bacterium, *S. aureus* is often used as a model of morphogenesis due to its apparent simple cell cycle. *S. aureus* has many cell division proteins that are conserved across many bacteria alluding to common functions. Despite intensive study we still do not know the roles of many of these components. Here we have examined the functions of DivIA and GpsB in the *S. aureus* cell cycle. Cells lacking *gpsB* display a more spherical phenotype than wild type, associated with a decrease in peripheral cell wall peptidoglycan synthesis. This correlates with an increased localisation of penicillin binding proteins at the developing septum, notably PBP3. Our results highlight the role of GpsB as an apparent regulator of cell morphology in *S. aureus*.

Authors names: Joshua A. F. Sutton[1,2,] Mark Cooke[1], Katarzyna Wacnik[1,2], Bartłomiej Salamaga[1,2], Callum Portman-Ross[1,2], Mariana Tinajero-Trejo[1,2], Jamie K. Hobbs[2,3], Simon J. Foster[1,2]

Affiliations: [1]School of Biosciences, University of Sheffield, Sheffield, United Kingdom [2]The Florey Institute for Host-Pathogen Interactions, University of Sheffield, Sheffield, United Kingdom [3]Department of Physics and Astronomy, University of Sheffield, Sheffield, United Kingdom

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From: Anne Marie Wehenkel <anne-marie.wehenkel@pasteur.fr>

Title: Eukaryotic-like gephyrin and cognate membrane receptor coordinate corynebacterial cell division and polar elongation

Text: The order Corynebacteriales includes major industrial and pathogenic actinobacteria such as *Corynebacterium glutamicum* or *Mycobacterium tuberculosis*. Their elaborate multi-layered cell wall, composed primarily of the mycolyl-arabinogalactan-peptidoglycan complex, and their polar growth mode impose a stringent coordination between the septal divisome, organized around the tubulin-like protein FtsZ, and the polar elongasome, assembled around the DivIVA homologue, Wag31. Here, we report the identification of two new divisome members, a gephyrin-like repurposed molybdotransferase (GLP) and its membrane receptor (GLPR). We show that the interplay between the GLPR/GLP module, FtsZ and Wag31 is crucial for orchestrating cell cycle progression. Our results provide a detailed molecular understanding of the crosstalk between two essential machineries, the divisome and elongasome, and reveal that Corynebacteriales have evolved a protein scaffold to control cell division and morphogenesis similar to the gephyrin/GlyR system that in higher eukaryotes mediates synaptic signaling through network organization of membrane receptors and the microtubule cytoskeleton.

Authors names: Martinez[1*], M., Petit[1*], J., Leyva[2*], A., Sogues[1+], A., Megrian[1], D., Rodriguez[2], A., Gaday[1], Q., Ben Assaya[1], M., Portela[2], M., Haouz[3], A., Ducret[4], A., Grangeasse4, C., Alzari[1], P. M., Durán[2#], R., Wehenkel[1#], A.

Affiliations: 1. Structural Microbiology Unit, Institut Pasteur, CNRS UMR 3528, Université Paris Cité, F-75015 Paris, France. 2. Analytical Biochemistry and Proteomics Unit, Institut Pasteur de Montevideo, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay. 3. Plate-forme de cristallographie, C2RT-Institut Pasteur, CNRS, UMR 3528, Université Paris Cité, F-75015 Paris, France. 4. Molecular Microbiology and Structural Biochemistry, CNRS UMR 5086, Université de Lyon, 7, passage du Vercors, 69367 Lyon, France.
*These authors contributed equally
#Correspondance: rduran@pasteur.edu.uy and anne-marie.wehenkel@pasteur.fr

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From: Pedro Alzari <pedro.alzari@pasteur.fr>

Title: Structural basis of FtsEX-independent RipA-mediated cell separation in Corynebacteriales

Text: The bacterial cell wall is a multi-layered mesh, whose major component is peptidoglycan (PG), a sugar polymer cross-linked by short peptide stems. During cell division, a careful balance of PG synthesis and degradation, precisely coordinated both in time and space, is necessary to prevent uncontrolled destruction of the cell wall. In Corynebacteriales, the D,L endopeptidase RipA has emerged as a major PG hydrolase for cell separation, and RipA defaults have major implications for virulence of the human pathogens *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae*. However, the precise mechanisms by which RipA mediates cell separation remain elusive. Here we report phylogenetic, biochemical, and structural analysis of the *Corynebacterium glutamicum* homologue of RipA, Cg1735. The crystal structures of full-length Cg1735 in two different crystal forms revealed the C-terminal NlpC/P60 catalytic domain obtruded by its N-terminal conserved coiled-coil domain, which locks the enzyme in an auto-inhibited state. We show that this auto-inhibition is relieved by the extracellular core domain of the transmembrane septal protein Cg1604. The crystal structure of Cg1604 revealed a (b/a) protein with an overall topology similar to that of receiver domains from response regulator proteins. The atomic model of the Cg1735-Cg1604 complex, based on bioinformatical and mutational analysis, indicates that a conserved, distal-membrane helical insertion in Cg1604 is responsible for Cg1735 activation. The reported data provide important insights into how intracellular cell division signal(s), yet to be identified, control PG hydrolysis during RipA-mediated cell separation in Corynebacteriales.

Authors names: Q. Gaday, D. Megrian, G. Carloni, M. Martinez, B. Sokolova, M. Ben Assaya, P. Legrand, S. Brûlé, A. Haouz, A.M. Wehenkel and P.M. Alzari

Affiliations: Unité de Microbiologie Structurale, Institut Pasteur, CNRS & Université Paris Cité, 75724 Paris, France

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From: Brooks Rady <b.j.rady@gmail.com> and Raj Bahadur <r.bahadur@sheffield.ac.uk>

Title: The Characterisation of 17 Rhizobial LD-Transpeptidases Implicated in Symbiotic Adaptation

Text: Plants belonging to the family of Fabaceae (legumes such as peas, beans, soybeans, or lentils) rely on a mutually beneficial interaction (symbiosis) with a group of bacteria called rhizobia to utilise atmospheric nitrogen. Assimilation of nitrogen by rhizobia is therefore a critical process for plant growth, which in turn supports animal life, with a tremendous economic and environmental impact. The plant-rhizobia symbiosis involves a complex molecular dialog that leads to the bacterial invasion of root tissues and the formation of root nodules. Inside these nodules, rhizobia form specialised cells called bacteroids able to transform atmospheric nitrogen into ammonia used by plants. We have identified several L,D-transpeptidases that are differentially expressed during the life cycle of the model symbiont *Rhizobium leguminosarum*. To get insights into the role of L,D-transpeptidases in the life cycle of Rhizobium, we investigated the enzymatic activity of these enzymes in peptidoglycan remodelling.

Authors names: Brooks Rady[1], Raj Bahadur[1], Marcel Alaman-Zarate[1], Raphael Ledermann[2], Andrew Lovering[3], Philip Poole[2], Stéphane Mesnage[1]

Affiliations: [1] School of Biosciences, University of Sheffield, Sheffield, UK [2] Department of Biology, University of Oxford, Oxford, UK [3] School of Biosciences, University of Birmingham, Birmingham, UK

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From: Thimoro Cheng <thimoro.cheng@pasteur.fr>

Title: Dynamics of Shapeshifting in *Helicobacter pylori*: From a corkscrew to a ball

Text: *Helicobacter pylori* is a microaerophilic ε-proteobacterium known to cause peptic ulcer and stomach cancer that has infected more than half of the world's population. Until today, its route of transmission and all features implicated in its pathogenesis remain poorly understood. As its name implies, this organism possesses a helical shape that aids in its colonization of the stomach by allowing it to bore into the mucus layer in a corkscrew-like motion. Remarkably, *H. pylori* has been observed to live a dimorphic lifestyle and can transform into a spherical coccoid made up of a distinctive peptidoglycan motif that can evade immune recognition. This coccoid form has been further suggested to be its persistent form that can participate in antibiotic tolerance. These pose as immediate concerns and compel us to investigate the dynamics of the morphological transformation in H. pylori and its role in transmission and pathogenicity.
Since bacterial cell shape is dictated by its peptidoglycan cell wall, the key to understanding the morphological transformation in *H. pylori* must lie within this layer. Our group had already identified key cell-shape determinants that implicate in this process such the Csd3/HdpA D,D-carboxypeptidase (1). We are using fluorescence imaging coupled with biochemistry to investigate the spatiotemporal dynamics of these proteins during the transformation. We are also tracking structural changes inside the peptidoglycan layer using molecular probes such as fluorescent D-amino acids (FDAAs). Our preliminary results showed that the cellular poles of *H. pylori* remained inert during the transformation while its lateral body was heavily modified and that penicillin-binding protein 3 (PBP3) and not PBP2 mediated this transition. We have also obtained evidence suggesting that there exist four distinct subpopulations of bacterial cell during the early on-set phase of this phenomenon distinguishable by their ability to regrow which eventually led to two subtypes of coccoid *H. pylori*.

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Authors names: Thimoro CHENG, Ivo GOMPERT BONECA

Affiliations: Institut Pasteur, Université Paris Cité, UMR6047 CNRS, INSERM U1306, Unité Biologie et génétique de la paroi bactérienne, Paris, France

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From: Wolfram Zueckert <wzueckert@kumc.edu>

Title: A *Borrelia burgdorferi* LptD Homolog is Required for Flipping of Surface Lipoproteins Through the Spirochetal Outer Membrane

Text: *Borrelia* spirochetes are unique among diderm bacteria in their lack of lipopolysaccharide (LPS) in the outer membrane (OM) and their abundance of surface-exposed lipoproteins with major roles in transmission, virulence, and pathogenesis. Despite their importance, little is known about how surface lipoproteins are translocated through the periplasm and the OM. Here, we characterized *Borrelia burgdorferi* BB0838, a distant homolog of the OM LPS assembly protein LptD. Using a CRISPR interference approach, we showed that BB0838 is required for cell growth and envelope stability. Upon BB0838 knockdown, surface lipoprotein OspA was retained in the inner leaflet of the OM, as determined by its inaccessibility to in situ proteolysis but its presence in OM vesicles. The topology of the OM porin/adhesin P66 remained unaffected. Quantitative mass spectrometry of the *B. burgdorferi* membrane-associated proteome confirmed the selective periplasmic retention of surface lipoproteins under BB0838 knockdown conditions. Additional analysis identified a single in situ protease-accessible BB0838 peptide that mapped to a predicted beta-barrel surface loop. BB0838 depletion also led to apparent upregulation of BB0323, a periplasmic OM-anchored lipoprotein that might be involved in stabilizing the envelope by binding peptidoglycan via a C-terminal LysM domain. Alphafold Multimer modeled a B. burgdorferi LptB2FGCAD complex spanning the periplasm. Together, this suggests that BB0838/LptDBb facilitates the essential terminal step in spirochetal surface lipoprotein secretion, using an orthologous OM component of a pathway that secretes LPS in proteobacteria.

Authors names: Huan He, Ankita S. Pramanik, Selene K. Swanson, David K. Johnson, Laurence Florens, Wolfram R. Zückert

Affiliations: University of Kansas School of Medicine, Department of Microbiology, Molecular Genetics and Immunology, Kansas City, Kansas, USA; Stowers Institute for Medical Research, Kansas City, Missouri, USA; University of Kansas, Computational Chemical Biology Core, Lawrence, Kansas

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From: Henri Voedts <henri.voedts@gmail.com>

Title: The (p)ppGpp alarmone mediates β-lactam resistance by downregulating ribosomal RNA transcription in *Escherichia coli*

Text: Bacteria respond to nutrient deprivation through the stringent response, which is mediated by the nucleotides tetra- or penta-phosphate (p)ppGpp. Among its pleiotropic effects, this alarmone alters the promoter selectivity of RNA polymerase, resulting in the downregulation of DNA replication and translation. While the role of (p)ppGpp in the regulation of various cellular processes and the expression of genes has been well studied, its involvement in the adaptation of cell wall peptidoglycan metabolism under nutrient deprivation remains unclear. We hypothesized that (p)ppGpp might be involved in this process since elevated levels of this nucleotide confer resistance to the narrow-spectrum β-lactam mecillinam. Moreover, (p)ppGpp has been shown to be essential for broad-spectrum β-lactam resistance mediated by redirecting the flux of peptidoglycan precursors towards the β-lactam-insensitive polymerase LdtD (YcbB). Here, we unexpectedly show that these (p)ppGpp-dependent β-lactam resistance mechanisms do not rely on the regulation of genes involved in peptidoglycan metabolism. Instead, (p)ppGpp-mediated downregulation of ribosomal RNA transcription was sufficient for resistance, even though β-lactams have no known impact on ribosomes. This paradox suggests that negative control of ribosomal RNA transcription is critical to prevent the downstream effects of β-lactam-mediated inactivation of peptidoglycan polymerases. Our findings provide novel insights into the complex mechanisms underlying bacterial adaptation to nutrient deprivation and antibiotic resistance.

Authors names: Henri Voedts, Constantin Anoyatis-Pelé, Jean-Emmanuel Hugonnet, Michel Arthur

Affiliations: Centre de Recherche des Cordeliers, Sorbonne Université, INSERM, Université Paris Cité, F-75006 Paris, France.

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From: Carolin Zeiher <carolin.zeiher@medizin.uni-leipzig.de>

Title: Peptidoglycan as a major bacterial compound accumulates with age in several human brain regions and distinct cell types

Text: In this project, we explore the localization of peptidoglycan and peptidoglycan-fragments (PG) in postmortem human brain samples. PG as a major bacterial compound is ubiquitous at epithelial barriers in animals and humans, notably in the gut microbiota. PG and its signaling receptors affect physiological brain maturing, social behavior and psychiatric disorders in animal models.
Since it is unknown if and in which cell types, PG is localized in the human brain, we analyzed postmortem brain samples of human donors without any known neuropathological disease by immunohistochemistry and immunofluorescence. In adult brain tissue of various age (between 35 and 85 years old) and sex, we detected PG signals in seven different brain regions, including the hippocampus, olfactory bulb, and frontal cortex. PG signals were located intra- and extracellularly. By co-staining with cell type specific antibodies, we found intracellular signals in neurons, astrocytes, oligodendrocytes and endothelial cells. Conversely, in human newborn (stillbirth to four weeks old) no PG signals except endothelial signals could be identified by our method.
As positive controls we used human brain tissue of a body donor diagnosed with sepsis and human liver tissue. Sepsis tissue showed a higher PG load in the brain suggesting PG load dependency from e.g. bacterial systemic infection. As the liver is the first organ reached by gastrointestinal blood through the portal vein, containing bacteria and bacterial degradation products, we expected a high PG load throughout the tissue. Our methods revealed widespread intracellular PG signals in hepatocytes and PG accumulation around blood vessels.
To shed light on the localization and composition of PG fragments reaching the brain, we currently use further methods like immunocapture, mass spectrometry and plan on performing electron microscopy on human postmortem brain samples.

Authors names: Carolin Zeiher (1), Heidrun Kuhrt (1), Aline Rifflet (2), Ivo Gomperts Boneca (2), Ingo Bechmann (1) and Jon D. Laman (3)

Affiliations: (1) Institute of Anatomy, University of Leipzig, Germany (2) Institut Pasteur, University Paris Cité, CNRS UMR6047, INSERM U1306, Biology and Genetics of Bacterial Cell Wall Unit, Paris, France (3) Dept. Pathology & Medical Biology, University Medical Center Groningen (UMCG), Groningen

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From: Alessandra Martorana <alessandra.martorana@unimi.it>

Title: Non-essential peptidoglycan remodeling factors required for cell survival under outer membrane stress

Text: The multi-layered envelope of Gram-negative bacteria is composed by the inner membrane (IM), the peptidoglycan cell wall and an asymmetric outer membrane (OM) containing Lipopolysaccharide in the outer leaflet. This complex structure behaves as a selective chemical barrier and allows cells to sustain large turgor pressures. In Escherichia coli additional robustness to the envelope is conferred by the covalent attachment of OM to PG mediated by Braun lipoprotein Lpp. Bacterial cells need to coordinate the growth of OM and PG layers to maintain cellular integrity during cell cycle and enable survival in challenging or stressful environments. In this context we are characterizing PG remodeling factors whose functions is absolutely required to avoid cell lysis when the OM asymmetry is lost because of disruption of LPS outer layer or defective LPS biogenesis. These factors include: i) the LD-transpeptidase LdtD and LdtE that introduce the non-canonical 3-3 cross-links in the PG layer, ii) the DpaA enzyme that detaches Lpp from the PG and iii) ActS a novel regulator of amidases, the enzymes that hydrolyze septal PG during cell separation. These factors appear to be part of a complex network where DpaA plays a central role in that not only directly controls covalent linkage between OM and PG but also indirectly modulates both ActS activity and 3-3 cross-link level in the cell. Our results support a model in which PG remodeling and OM biogenesis are coordinated to maintain cell viability under envelope stress conditions.

Authors names: Alessandra Maria Martorana[1], Marta Zaccaria[1], Jacob Biboy[2], Laura Alessandrini[1], Desara Hysa[1], Waldemar Vollmer[2,3] and Alessandra Polissi[1]

Affiliations: [1]Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli studi di Milano, Milano, Italy [2]Centre for Bacterial Cell Biology, Biosciences Institute, Newcastle University, Newcastle upon Tyne, UK [3]Institute for Molecular Biosciences, The University of Queensland, Brisbane, Australia

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From: Nadia Nikulin <ns646@cornell.edu>

Title: Molecular Dynamics of *Klebsiella pneumoniae* in Spheroplast Recovery

Text: Antibiotics are an essential component of modern medicine. With their discovery came an increase in human life expectancy; antimicrobial infections no longer posed as great of a threat. Over years of continued use and misuse of these miracle agents came the evolutionary pressure for bacteria to evolve and become resistant. Research has shown that antibiotic tolerance, the ability of a microorganism to survive exposure of an antibiotic in a peptidoglycan-deficient state, is a steppingstone to antibiotic resistance. Tolerant microbes can become resistant; thus it is of great importance to study antibiotic tolerance. Many individuals have been pursuing research on how tolerance occurs, but it is also of interest to learn how microorganisms recover from a tolerant state. Here, we explore the molecular dynamics of proteins involved in various cell wall and membrane building processes.

Authors names: Nadia Nikulin, Ziyu Xue, Tobias Doerr

Affiliations: Cornell University-- 1. Weill Institute for Cell and Molecular Biology 2. Department of Microbiology

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From: Quentin Gaday <quentin.gaday@pasteur.fr>

Title: Structural insights into cell septation in Corynebacteriales

Text: The bacterial cell wall is a complex structure that is essential for bacterial survival and virulence. In Corynebacteriales, the D,L endopeptidase RipA has emerged as a major peptidoglycan (PG) hydrolase for cell separation, and RipA defects have major implications for the virulence of human pathogens such as Mycobacterium tuberculosis and Corynebacterium diphtheriae. However, the precise mechanisms by which RipA mediates cell separation remain elusive. In our recent publication [1], we reported the phylogenetic, biochemical, and structural analysis of the *C. glutamicum* homologue of RipA, Cg1735. The structure of Cg1735 shows the enzyme in an auto-inhibited conformation mediated by the conserved N-terminal domain of RipA. Based on structural and bioinformatical evidence, we proposed a mechanism of activation mediated by the transmembrane protein SteB (Cg1604), which was recently identified as a corynebacterial cell division protein [2]. We have since obtained structural evidence that the SteB-RipA mechanism of action is conserved in M. tuberculosis. We are now aiming to understand the intracellular process that controls the SteB-mediated activation of RipA in *C. glutamicum*. We are investigating the role of SteA, another corynebacterial transmembrane protein which was shown to interact with SteB [2]. Our recent findings in a steA-B knock-out strain of *C. glutamicum* enabled us to gather evidence of how these proteins work together to promote cell separation. These findings are clinically relevant, as SteA and SteB are conserved in pathogenic Corynebacteriales and involved in *C. glutamicum* tolerance to antibiotics. Our work provides important insights into the fundamental biological processes and specificities that underlie corynebacterial cell division.

[1] Gaday et al. PNAS. 2022 ; [2] Lim et al. PLoS Genet. 2019

Authors names: Quentin Gaday (1), Giacomo Carloni (1), Daniela Megrian (1), Mariano Martinez (1), Bohdana Sokolova (1), Mathilde Ben Assaya (1), Pierre Legrand (2), Sebastien Brûlé (3), Ahmed Haouz (4), Rosario Duran (5), Anne Marie Wehenkel (1) and Pedro M. Alzari (1)

Affiliations: (1) Unité de Microbiologie Structurale, Institut Pasteur, CNRS, Université Paris Cité, 75015 Paris, France ; (2) Synchrotron SOLEIL, 91192 Gif-sur-Yvette, France ; (3) Plate-forme de biophysique moléculaire, C2RT-Institut Pasteur, CNRS UMR 3528, F-75015 Paris, France ; (4) Plate-forme de cristallographie, C2RT-Institut Pasteur, CNRS UMR 3528, F-75015 Paris, France ; (5) Analytical Biochemistry and Proteomics Unit, Institut Pasteur Montevideo, Uruguay

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From: Kate Willett <k.willett.1@warwick.ac.uk>

Title: Exploration of the structure-activity relationship of Y196 in *Staphylococcus aureus* PBP2

Text: *Staphylococcus aureus* is a commensal organism and opportunistic pathogen of both humans and livestock. Current treatments for infection target the transpeptidation stage of peptidoglycan biosynthesis, but the rise of antibiotic resistance has highlighted the urgent need for identification of novel antimicrobial targets. Peptidoglycan synthesis is also dependent on polymerisation of lipid II by the glycosyltransferase (GT) activity of SEDS proteins and Class A PBPs, presenting an as yet under-explored target for novel treatments. Previous work by Rebets et al.[1] identified a Y196D mutant of *S. aureus* PBP2 which provided resistance to moenomycin *in vivo*, reduced glycan chain length, and affected cell morphology. In this work, we aim to further investigate the structure activity relationship around this key residue. *In vitro* assays were used to explore the impact of various mutations on GT activity and moenomycin resistance. Our results suggest that Y196 forms a pi-stacking interaction with the newly synthesised glycan strand which is required for normal lipid II polymer formation and consequently cell wall peptidoglycan formation.

Reference:
Rebets, Y., Lupoli, T., Qiao, Y., Schirner, K., Villet, R., Hooper, D., Kahne, D., Walker, S., 2014. Moenomycin Resistance Mutations in *Staphylococcus aureus* Reduce Peptidoglycan Chain Length and Cause Aberrant Cell Division. ACS Chem. Biol. 9, 459–467.

Authors names: K. E. Willett, N. Thornhill, J. Deering, P. J. Stansfeld, D. I. Roper

Affiliations: University of Warwick, Midlands Integrative Biosciences Training Partnership (MIBTP), Biotechnology and Biological Sciences Research Council (BBSRC)

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From: Francesca Gillett <f.gillett@warwick.ac.uk>

Title: Lytic transglycosylases as future targets in peptidoglycan synthesis

Text: Peptidoglycan synthesis is a vital process in most bacteria, maintaining cell shape and withstanding turgor. Inhibition of its synthesis results in cell lysis, highlighting just how important this process is. Peptidoglycan is very dynamic and needs to be constantly remodelled due to the environment. As a result, there is a fine balance between peptidoglycan growth and breakdown. the lytic transglycosylases are a family of enzymes that break down peptidoglycan and they are involved in peptidoglycan remodelling. There is evidence in Pseudomonas aeruginosa that a lytic transglycosylase interacts with RodA-PBP2, the core machinery of the elongasome involved in cell growth. This interaction is thought to be very important and lately lytic transglycosylases are being investigated as potential future targets for antibiotics.
Lytic transglycosylases are targeted by a secondary metabolite of the bacterium Paraburkholderia acidophila called Bulgecin A. This natural product is synthesised in combination with a β-lactam antibiotic which increases the efficacy of β-lactam antibiotics in resistant bacteria, resulting in a 'Bulging' phenotype that leads up to cell lysis. Part of this project will involve characterising the binding partner of RodA-PBP2 through different binding experiments e.g., SPR. Future experiments will characterise which lytic transglycosylases are inhibited by Bulgecin A using a new novel assay for visualising lytic transglycosylase activity.

Authors names: Francesca Gillett, Dr Allister Crow, Professor David Roper

Affiliations: University of Warwick, Midlands Integrative Biosciences Training Partnership (MIBTP), Biotechnology and Biological Sciences Research Council (BBSRC), Micropathology Ltd

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From: Jed Fisher <jfisher1@nd.edu>

Title: *In vitro* studies of the protein-interaction network of cell-wall lytic transglycosylase RlpA of *Pseudomonas aeruginosa*.

Text: The protein networks of cell-wall-biosynthesis assemblies are largely unknown. A key class of enzymes in these assemblies is the lytic transglycosylases (LTs), of which eleven exist in P. aeruginosa. We have undertaken a pulldown strategy in conjunction with mass-spectrometry based proteomics to identify the putative binding partners for the eleven LTs of P. aeruginosa. A total of 71 putative binding partners were identified for the eleven LTs. A systematic assessment of the binding partners of the rare lipoprotein A (RlpA), one of the pseudomonal LTs, was made. This 37-kDa lipoprotein is involved in bacterial daughter-cell separation by an unknown process. RlpA participates in both the multi-protein and multi-enzyme divisome and elongasome assemblies. We reveal an extensive protein-interaction network for RlpA involving at least 19 proteins. Their kinetic parameters for interaction with RlpA were assessed by microscale thermophoresis, surface-plasmon resonance, and isothermal-titration calorimetry. Notable RlpA binding partners include PBP1b, PBP4, and SltB1. Elucidation of the protein-interaction networks for each of the LTs, and specifically for RlpA, opens opportunities for the study of their roles in the complex protein assemblies intimately involved with the cell wall as a structural edifice critical for bacterial survival.

Authors names: Avila-Cobian, L. F.; De Benedetti, S.; Kim, C.; Feltzer, R.; Champion, M. M.; Mobashery, S.; Fisher, J. F.

Affiliations: University of Notre Dame, USA

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From: Matthew Herdman <matthew.herdman@jic.ac.uk>

Title: Cell Cycle Dependent Orchestration of Surface Layer Biogenesis in *Caulobacter crescentus*

Text: Surface layers (S-layers) are proteinaceous, two-dimensional crystals that constitute the outermost components of many prokaryotic cell envelopes. In this study, we investigated principles of S-layer biogenesis in the model organism *Caulobacter crescentus*. Fluorescent microscopy revealed localised incorporation of new S-layer at the poles and mid-cell, consistent with elongation and division phases of the cell cycle. Next, light microscopy and electron cryotomography investigations of drug-treated bacteria revealed that bacterial actin MreB is crucial for localised S-layer insertion. We further uncovered that S-layer biogenesis follows new peptidoglycan synthesis and localises to regions of high cell wall turnover. Finally, correlated cryo-light and electron microscopy analysis of regions of S-layer insertion showed the presence of gaps in the underlying hexagonal S-layer lattice, contrasting with archaeal S-layers completed by defined defects. Our findings provide insight into how *C. crescentus* cells form an ordered S-layer on their surface, with implications on fundamental prokaryotic cell biology, providing evidence for coordination between the biogenesis of the cell envelope at multiple levels.

Authors names: Matthew Herdman, Andriko von Kügelgen, Ulrike Schulze, Alan Wainman, Tanmay A.M. Bharat
Affiliations: Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom / Structural Studies Division, MRC Laboratory of Molecular Biology, Cambridge CB2 0QH, United Kingdom

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From: Raffaele Ieva <raffaele.ieva@univ-tlse3.fr>

Title: Gets a foot in the door of the LPS translocon: YifL/LptM promotes oxidative maturation of LptD by substrate binding mimicry.

Text : Lipopolysaccharide (LPS) inserts in the external leaflet of the Gram-negative bacterial outer membrane (OM) forming a protective shield against noxious molecules. LPS insertion into the OM is gated by the LPS translocon, consisting of a β-barrel membrane protein, LptD, and a lipoprotein, LptE. The β-barrel assembly machinery (BAM) assembles LptD together with LptE to form a plug-and-barrel structure. In the enterobacterium Escherichia coli, formation of two native disulfide bonds in LptD controls LPS translocon activation. We report the discovery of LptM (formerly YifL), a lipoprotein conserved in many proteobacteria, that is assembled by the BAM complex together with LptD and LptE giving rise to the heterotrimer LptDEM. We demonstrate that LptM stabilizes a conformation of LptD, which can efficiently form native disulfide bonds. Inactivation of LptM causes the accumulation of non-natively oxidized LptD, making disulfide bond isomerization by DsbC become essential for viability. Our structural and biochemical analyses indicate that tri-acylated LptM binds to sites in both LptD and LptE that are proposed to coordinate gating of LPS into the OM. Together, our results suggest that, by mimicking LPS, LptM i) facilitates oxidative maturation of LptD and ii) primes the opening of the LPS translocon gate via which LPS inserts into the OM.

Authors names: Yiying Yang, Haoxiang Chen, Robin A. Corey, Violette Morales, Yves Quentin, Carine Froment, Anne Caumont-Sarcos, Odile Burlet-Schiltz, David Ranava, Phillip J. Stansfeld, Julien Marcoux, Raffaele Ieva

Affiliations: Laboratoire de Microbiologie et Génétique Moléculaires, Centre de Biologie Intégrative (CBI), Université de Toulouse, CNRS, UPS, Toulouse 31062, France

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From: Sam Benedict <STB578@student.bham.ac.uk>

Title: Friend and foe? Novel cell wall hydrolases in mycobacterial physiology

Text: Mycobacteria are a group of important human pathogens, responsible for a huge range of human illnesses. The unique tripartite cell wall of mycobacteria and related species is a defining trait of these unusual bacteria. This cell wall acts as both entryway and barrier to environmental insult such as chemical agents, as well as other biological entities, such as phage. A component of this cell wall is the polysaccharide arabinogalactan, which acts as a scaffold between the inner peptidoglycan sacculus and the exterior mycomembrane. We have discovered a novel group of enzymes encoded in all mycobacteria for maintenance of their cell wall through D-arabinan cleavage. We have characterised the products of the two endogenous families and these contrast with those generated by the phage-encoded family members. Surprisingly, many mycobacteriophage have weaponised this same enzyme family to aid in lysis. We assert that these enzymes act in the late phase of the lytic phage cycle, lysing cells and allowing release of nascent virions into the environment. Using a combination of structural and biochemical approaches we have investigated the mechanism by which these proteins induce lysis. We believe this work will be of key importance in understanding host range specificity of phage therapeutics in the clinic, as well as perhaps representing novel enzybiotics in the future.

Authors names: Samuel Benedict; Patrick Moynihan; Elisabeth Lowe; Jenn Ross; Omar Al-Jourani

Affiliations: University Of Birmingham; University of Newcastle

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From: Royer Marie <marie.royer@pasteur.fr>

Title: The mecillinam resistome in *Klebsiella pneumoniae*

Text: Antibiotic resistance is a major public health issue worldwide, with Klebsiella pneumoniae being one of the main species responsible. Indeed, it is a common cause of hospital-acquired infections, with carbapenem-resistant strains being particularly feared. Carbapenems are last resort drugs that belong to the ß-lactams, the most widely prescribed class of antibiotics in humans. Different penicillin-binding proteins (PBPs) can be targeted by ß-lactams, depending on the molecule. But despite their long-standing availability the detailed process of bacterial killing is still a matter of debate. To get insight into the mechanisms of killing by these antibiotics in *K. pneumoniae*, I first focused on resistance to a particular ß-lactam, mecillinam. This antibiotic only targets PBP2 and so can be used as a model to study part of the killing process.
I investigated the mecillinam resistome in *K. pneumoniae* by sequencing the genome of 101 mecilliam-resistant mutant strains (Illumina technology). Candidate causative mutations of resistance were detected in a broad range of functional categories, including amino-acid metabolism, central carbon metabolism, translation, and cell envelope. Interestingly, most of these mutations were predicted to either directly or indirectly increase the expression of the bacterial division protein FtsZ to bypass PBP2 inhibition. In line with this hypothesis, I also identified transient resistance due to unstable large genomic amplifications encompassing the *ftsQAZ* locus. In a second step, I phenotypically characterized six mutant strains (antibiotic resistance, fitness, live/dead staining), representative of the different classes. All these mutants were highly resistant (MIC > 256 mg/L) but there was no correlation between a functional category and a specific phenotype. And interestingly, despite their high level of resistance they all have a growth defect in the presence of mecillinam.
In *E. coli*, *ftsZ* expression is controlled by central regulatory systems such as RpoS or the stringent response. To check whether MecR mutants were also dependent on the stringent response, I constructed derivatives with a (p)ppGpp synthetase/hydrolase RelA defect. Based on the results of qRT-PCR for *ftsZ* and a (p)ppGpp reporter gene, as well as engineered strains and literature knowledge, I showed that the majority of mutants have an increased ftsZ expression and that their phenotype was not RelA-dependant. Thus, in *K. pneumoniae*, the mecillinam resistance is mostly mediated by *ftsZ* overexpression independently from RelA.

Authors names: Marie Royer*, Nicolas Cabanel*, Guilhem Royer*, Isabelle Rosinski-Chupin*, Claude Loverdo**, Philippe Glaser*

Affiliations: *Ecology and Evolution of Antibiotic Resistance, Pasteur Institute, Paris, France. Laboratoire Jean Perrin, Sorbonne University, Paris, France

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From: Folcher <victor.folcher@i2bc.paris-saclay.fr>

Title: Mechanistic basis of Enterocin C targeting the undecaprenyl pyrophosphate phosphatase BacA from *Enterococcus faecalis*

Text: *Enterococcus faecalis* is an important opportunistic pathogen often isolated from nosocomial infections and its natural resistance to some β-lactams makes it difficult to treat. One therapeutic strategy could rely on the potentiation of β-lactams by another drug interfering with a critical membrane step of peptidoglycan biosynthesis, such as the recycling of the carrier lipid undecaprenyl phosphate (C55-P). In this line, lowering the recycling rate in *Escherichia coli* was found to provoke a synergistic response with β-lactams leading to rapid cell lysis. In this study, we have shown that the two-peptide bacteriocin called Enterocin C (EntC) specifically targets the undecaprenyl phosphate recycling enzyme BacA from *E. faecalis*. Each peptide is inactive alone and acts synergistically at the nanomolar range to kill the target cell. We used a combination of biochemical and biophysical approaches to understand the molecular mechanism of EntC. Our results show a sequential binding of the peptides to the membrane protein BacA. This binding event leads to a significant BacA stabilization, the inhibition of BacA enzymatic activity together with membrane permeabilization to protons on BacA-containing liposomes. Our data suggest that EntC parasitizes BacA structure in a way to induce ion leakage. Structural modelling, molecular dynamics and mutagenesis are under way to figure out this mechanism. This work may open the way to bacteriocin engineering in order to specify and/or modify its spectrum of action in a global antibacterial strategy.

Authors names: Victor Folcher, Rodolphe Auger, Clara Louche, Pascale Serror, Thierry Touzé

Affiliations: Institute for Integrative Biology of the Cell (I2BC)

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From: Manuela Alvarado <ma2229@cornell.edu>

Title: Carboxypeptidases are central regulators of bacterial cell wall turnover homeostasis

Text: Peptidoglycan (PG) is the main component of the bacterial cell wall; it maintains cell shape while protecting the cell from internal osmotic pressure and external environmental challenges. PG synthesis is essential for bacterial growth and survival, and a series of PG modifications are required to allow expansion of the sacculus. Endopeptidases (EPs), for example cleave the crosslinks between adjacent PG strands to allow the incorporation of newly synthesized PG. EPs are essential for bacterial growth and must be carefully regulated to prevent sacculus degradation and cell death. However, EP regulation mechanisms are poorly understood. Here, we used *Tn*Seq to uncover novel EP regulation factors in Vibrio cholerae. This screen revealed the carboxypeptidase DacA1 as a novel factor for mitigation of EP toxicity. Surprisingly, dacA1 is essential for viability on LB medium, but EP overexpression mitigated this essentiality. To further understand the role of DacA1 in cell wall homeostasis, we performed a suppressor screen of Δ*dacA1* in LB medium. This screen revealed hypomorphic mutants in the PG synthesis pathway, as well as mutations that promote PG degradation. Our data thus suggest a novel role of DacA1 in maintaining the balance between PG synthesis and degradation.

Authors names : Manuela Alvarado Obando, Tobias Doerr

Affiliations: Department of Microbiology, Cornell University, Ithaca, NY; Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca, NY

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From: Adrián Izquierdo Martínez <aizquierdomartinez@itqb.unl.pt>

Title: DipM controls multiple autolysins and mediates a regulatory feedback loop promoting cell constriction in *Caulobacter crescentus*

Text: Proteins containing a catalytically inactive LytM-type endopeptidase domain are important regulators of cell wall-degrading enzymes in bacteria. Here, we study their representative DipM, a factor mediating proper cell division in *Caulobacter crescentus*. We show that DipM interacts with multiple autolysins, including the lytic transglycosylases SdpA and SdpB, the amidase AmiC and the putative carboxypeptidase CrbA, and stimulates the activities of SdpA and AmiC. Its crystal structure displays a conserved groove, which is predicted to represent the docking site for autolysins by modeling studies. Mutations in this groove indeed abolish the function of DipM in vivo and its interaction with AmiC and SdpA in vitro. Notably, DipM and its regulatory targets SdpA and SdpB stimulate each other's recruitment to the division site, establishing a self-reinforcing cycle that gradually increases autolytic activity as cytokinesis progresses. DipM thus acts at the intersection of different peptidoglycan-remodeling pathways and coordinates their activities to ensure proper cell constriction and daughter cell separation.

Authors names: Adrian Izquierdo-Martinez (a,b), Maria Billini (a,*), Vega Miguel-Ruano (c,*), Rogelio Hernández-Tamayo (d,g), Pia Richter (a), Jacob Biboy (e), María T. Batuecas (c), Timo Glatter (f), Waldemar Vollmer (e), Peter L. Graumann (d,g), Juan A. Hermoso (c), Martin Thanbichler (§,a,b,g)

Affiliations: [a] Department of Biology, University of Marburg, Marburg, Germany. [b] Max Planck Fellow Group Bacterial Cell Biology, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany. [c] Department of Crystallography and Structural Biology, Instituto de Química-Física "Rocasolano", Consejo Superior de Investigaciones Científicas, Madrid, Spain. [d] Department of Chemistry, University of Marburg, Marburg, Germany. [e] Centre for Bacterial Cell Biology, Biosciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom. [f] Mass Spectrometry and Proteomics Facility, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany. [g] Center for Synthetic Microbiology (SYNMIKRO), Marburg, Germany. * These authors contributed equally to this study.

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From: Upasana Basu <ub39@cornell.edu>

Title: The cell division protein ZapC mitigates stress induced by lytic transglycosylase insufficiency and promotes morphogenesis in *Vibrio cholerae*

Text: The peptidoglycan (PG) cell wall is a unique feature of most bacteria, and it maintains cell shape and provides strength to withstand high turgor pressure. The synthesis and degradation of PG needs to be finely regulated to maintain structural integrity, especially during processes like cell division and growth. PG degradation is mediated by diverse classes of lytic enzymes called autolysins. While cell wall synthesis is well-studied in many bacteria, we lack a clear understanding of the in vivo regulation of autolysin activity. The lytic transglycosylases (LTGs), a class of autolysins, have been biochemically well-characterized, but little is known about their physiological roles. The LTGs catalyze the non-hydrolytic cleavage of the glycosidic bond between the MurNAc and GlcNAc residues of the glycan strands in PG. In this study, we aim to understand the essentiality and apparent redundancy (encodes eight) of LTGs in *Vibrio cholerae*. To identify genes that are conditionally essential during LTG insufficiency, a *Tn*Seq screen was performed using the minimal LTG strain Δ6LTG, where six out of the eight LTGs are absent. Among other factors, we found that that deletion or depletion of two uncharacterized response regulators: vca_0578 and vca_0600, as well as the poorly characterized cell division protein ZapC, cause cell filamentation in a Δ6LTG background. Overexpressing *zapC* rescued the filamentation phenotype in these response regulator deletion mutants. The transcriptomics profile of these mutants further emphasized that these response regulators independently, and redundantly control ZapC levels, vca_0578 being the major contributor. We hypothesize that cell division is delayed during LTG insufficiency, pointing to a novel regulatory pathway for cell division in these cell wall mutants.

Authors names: Upasana Basu (1,2); Tobias Doerr (1,3)

Affiliations: 1. Weill Institute for Cell and Molecular Biology; 2. Cornell University College of Veterinary Medicine; 3. Department of Microbiology, Cornell University

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From: Xiaofang Li <x.li@umcg.nl>

Title: Functional profiling of CHAP domain-containing peptidoglycan hydrolases of *Staphylococcus aureus* USA300 uncovers potential targets for antistaphylococcal therapies

Text: *Staphylococcus aureus* is a Gram-positive bacterium responsible for many hospital- and community- acquired infections worldwide. S. aureus employs a thick cell wall for protection against physical and chemical insults. This cell wall plays important roles in maintaining barrier integrity and in bacterial growth and division. The main cell wall component is peptidoglycan. Accordingly, the bacteria produce so-called peptidoglycan hydrolases (PGHs) that cleave glycan strands to facilitate growth, cell wall remodeling, separation of divided cells and release of exported proteins into the extracellular milieu. A special class of PGHs contains so-called 'cysteine, histidine-dependent amidohydrolase/peptidase' (CHAP) domains.
In the present study, we profiled the roles of 11 CHAP PGHs encoded by the core genome of *S. aureus* USA300 LAC. Mutant strains lacking individual CHAP PGHs were analyzed for growth, cell morphology, autolysis, and invasion and replication inside human lung epithelial cells. The results showed that several investigated CHAP PGHs contribute to different extents to extracellular and intracellular growth and replication of *S. aureus*, septation of dividing cells, daughter cell separation and autolysis. In particular, the CHAP PGHs Sle1 and SAUSA_2253 control intracellular staphylococcal replication and the resistance to β-lactam antibiotics including oxacillin. This makes the *S. aureus* PGHs in general, and the Sle1 and SAUSA300_2253 proteins in particular, attractive targets for future prophylactic or therapeutic anti-staphylococcal interventions.
Next, we will investigate the cell wall composition and structure of the USA300 wild-type strain and derivative CHAP PGHs mutant strains. We will also investigate the host-pathogen interactions of CHAP PGHs mutant strains in lung epithelial cells in our further research.

Authors names: Xiaofang Li, Min Wang, Francis M Cavallo, Harita Yedavally, Elisa J.M. Raineri, Elias Vera Murguia, Jeroen Kuipers, Anna Salvati, Zhenhua Zhang, Jan Maarten van Dijl, and Girbe Buist

Affiliations: Department of Medical Microbiology - Molecular Bacteriology, University Medical Center Groningen, University of Groningen, the Netherlands.

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From: Brandon Jutras <bjutras@vt.edu>

Title: The unusual features of *Borrelia Burgdorferi* peptidoglycan and their impacts of the biology and pathogenesis of Lyme disease

Text: Both cell wall synthesis and peptidoglycan composition of the spirochete Borrelia burgdorferi—the causative agent of Lyme disease—are unlike any previously described. For instance, *B. burgdorferi* elongates to nearly 50 microns in length by producing multiple zones of peptidoglycan (PG) synthesis that are spatially and temporally regulated. In addition, not only does *B. burgdorferi* PG peptides contain Ornithine (Orn) instead of Lysine or Diaminopimelate, but we have also discovered unprecedented glycan stoichiometry. High-resolution LCMS methods, coupled with metabolic labeling studies and NMR, indicate that *B. burgdorferi* PG contains the trisaccharide GlcNAc-GlcNAc-MurNAc. This modification to PG glycans can be partially explained by acquisition and cell wall incorporation of the tick-vector sugar chitobiose (GlcNAc-GlcNAc). Atomic force microscopy studies reveal that GlcNAc-GlcNAc-MurNAc glycan organization is a novel means to 1) increase distance between stem peptides; 2) produce more flexible PG; and 3) collectively optimize motility to withstand the torque of endoflagella. Earlier analysis of *B. burgdorferi* PG also indicated additional unusual features, such as the presence of several isobaric structures. Our unpublished data show that *B. burgdorferi* produces muropeptides that are both alpha- and gamma-carbon linked between amino acids in the second (D-Glu) and third (Orn) positions. We discuss our findings in the context of spirochete biology, evolution, and pathogenesis.

Authors names: Mecaila McClune, Saadman Ahmad, Tanner G. DeHart, Rebecca, Trimble, Rich Helm, and Brandon L. Jutras

Affiliations: Virginia Tech

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From: Noortje Dannenberg <N.Dannenberg@biology.leidenuniv.nl>

Title: Mycobacteria form viable cell wall-deficient cells that are undetectable by conventional diagnostics

Text: The cell wall is considered essential for most bacteria and the enzymes involved in cell wall synthesis are therefore among the prime targets of effective antibiotics. Considering the importance of this structure, it is surprising to find that many bacteria can transiently shed their cell wall. In this cell wall-deficient state the bacteria are insensitive to cell wall-targeting antibiotics, more competent for DNA uptake and can revert to their walled state when the stressful conditions have ended. Importantly, recent observations suggest a link of such wall-deficient cells to chronic infections. Whether shedding the cell wall also occurs in mycobacteria, responsible for devastating diseases such as Tuberculosis and leprosy, has not been established unambiguously. In this study we provide compelling evidence that a wide range of mycobacterial species, including *Mycolicibacterium smegmatis*, *Mycobacterium marinum*, *Mycobacterium avium*, *Mycobacterium bovis* BCG, *Mycobacterium tuberculosis* and mycobacterial clinical and endophytic isolates, form cell wall-deficient cells in response to environmental stressors that is stimulated by the presence of cell wall-targeting agents. Confocal microscopy and cryo-transmission electron microscopy confirm that these cells contain DNA but lack their cell wall. We furthermore show that these cells are viable and can revert to a walled state. Importantly, conventional diagnostic media used for detection of mycobacteria do not sustain these cells, perhaps indicating that such cells have been largely overlooked in clinical settings.

Authors names: Noortje Dannenberg1, Victor J. Carrion Bravo1, 2, 3, Tom Weijers1, Herman P. Spaink1, Tom H. M. Ottenhoff4, Ariane Briegel1, 5 & Dennis Claessen1*

Affiliations: 1 Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands. 2 Departamento de Microbiología, Instituto de Hortofruticultura Subtropical y Mediterránea 'La Mayora', Universidad de Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Universidad de Málaga, Málaga, Spain 3 Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, the Netherlands 4 Infectious Diseases, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands 5 Netherlands Centre for Electron Nanoscopy (NeCEN), Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands *Correspondence: d.claessen@biology.leidenuniv.nl

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From: Ragnhild Sødal Gjennestad <ragnhild.sodal.gjennestad@nmbu.no>

Title: The contributions of low-affinity penicillin binding proteins to a branched structured cell wall in *Streptococcus pneumoniae*

Text: The primary peptidoglycan layer in *Streptococcus pneumoniae* is synthesised by the SEDS transglycosylates FtsW and RodA working alongside their dedicated transpeptidases PBP2x and PBP2b respectively. Recent evidence suggest that this primary peptidoglycan is matured and strengthened by bi-functional (have both transglycosylase and transpeptidase activity) class A PBPs (PBP1a, PBP1b and PBP2a). Penicillin works by blocking the transpeptidase activity of PBPs. This, however, is avoided in penicillin resistant pneumococci, which share three main characteristics: (i) expression of low-affinity PBP2x, PBP1a and PBP2b (low-affinity for penicillin), (ii) a branched structured cell wall and (iii) an altered version of MurM, an enzyme responsible for adding additional amino acids to the lipid II peptidoglycan precursor creating branched structured muropeptides. The presence of branched lipid II molecules is critical for resistance, since in many isolates, deletion of *murM* renders the pneumococcus sensitive to penicillin. To explore which low-affinity PBPs that is involved in the incorporation of branched muropeptides, we introduced low-affinity versions of PBP2x, PBP1a and PBP2b into the penicillin sensitive *S. pneumoniae* R6 and monitored how it affected the stem peptide composition of the peptidoglycan. Introduction of low-affinity PBP2b gave higher ratios of branched muropeptides in the cell wall, while low-affinity PBP2x and PBP1a did not. We hypothesised that the low-affinity version of PBP2b could have higher preference towards branched muropeptide substrates, or that the enzyme is less efficient and calls for other PBPs, which prefers branched lipid II, to help with the transpeptidation. We knocked out or depleted either *pbp* genes to investigate whether the absence of one of the PBPs influenced the level of branched muropeptides. In this talk we will present our results from these experiments as well as a possible new model for the regulation of the peptidoglycan synthesis machinery in *S. pneumoniae*.

Authors names: Ragnhild Sødal Gjennestad(1), M. V. Heggenhougen(1), J. Molstad(1), A. R. Winter(1) V. Eldholm(2), L. S. Håvarstein(1), M. Kjos(1), D. Straume(1)

Affiliations: (1) Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway; (2) Department of Bacteriology, Norwegian Institute of Public Health, Oslo, Norway

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From: Victor Pinedo <victor.pinedo@umu.se>

Title: Characterization of a novel penicillin-binding protein in *Vibrio cholerae*

Text: Penicillin-binding proteins (PBPs) are membrane-associated proteins that catalyze the synthesis of peptidoglycan (PG), the polymer that constitutes the bacterial cell wall. Thanks to a genome wide high-throughput screening of PG composition in *Vibrio cholerae*, we were able to identify several novel genes that could play a potential role in PG homeostasis. Among these, we found a gene with locus tag vc1321, which was previously annotated as a hypothetical protein but showed functional and sequence homology to the other high-molecular weight PBPs in *V. cholerae*. We discovered that this unique PBP, named PBP1V (after Penicillin-binding protein 1 in Vibrio), helps *V. cholerae* survive in low-salt conditions and overall contributes to outer membrane stability, as its deletion mutant shows a dysregulation on the lipopolysaccharide (LPS) layer. Furthermore, through proteomic studies we have also uncovered a potential role of this protein in virulence since the deletion mutant is altered in the ToxRS virulence system and related outer-membrane proteins. PBP1V is conserved in different Gamma proteobacteria species, suggesting its evolutionary and biological importance. Further characterization of PBP1V might uncover novel mechanisms of how PG homeostasis and PBPs can affect central systems in bacteria such as virulence or envelope stability, as well as potentially new therapeutic targets in infection.

Authors names: Víctor Pinedo, Barbara Ritzl-Rinkenberger, André Mateus, Felipe Cava

Affiliations: Department of Molecular Biology and Laboratory for Molecular Infection Medicine Sweden; Umeå University; Umeå; Sweden. Department of Chemistry and Laboratory for Molecular Infection Medicine Sweden; Umeå; Sweden.

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From: Julia Fairbairn <julia.fairbairn@warwick.ac.uk>

Title: Synthesis of peptidomimetic inhibitors for the MraY-protein E interaction site as potential anti-microbial therapeutics.

Text: MraY, an essential prokaryotic enzyme, catalyses the first lipid-linked step of peptidoglycan biosynthesis, provides a promising target for the development of novel antimicrobials. Protein E from bacteriophage phi-X174 induces host cell lysis in E. coli through a protein-protein interaction with MraY, offering a previously unexplored route for enzyme inhibition, leading to bacterial cell death.
Previous work in our group has shown that a peptide motif Arg-Trp-x-x-Trp (RWxxW) within protein E is responsible for the activity against MraY, and that this motif can be mimicked using an Arg-Trp-octyl ester (Ref. 1) or alpha-helical peptidomimetics (Ref. 2), which both show antimicrobial activity.
This study presents the design, synthesis and evaluation of a 2nd-generation of RWxxW peptidomimetics, consisting of a small library of substituted heterocycles based on a triazinedione scaffold. These compounds show antimicrobial activity against *Escherichia coli* C43 (MIC 1 – 16 ug/mL) as well as *Pseudomonas fluorescens* (MIC 2 – 256 ug/mL), and show inhibition of particulate E. coli MraY (20 – 50% inhibition at 200 uM concentration) using a fluorescence-based assay. These results further validate this strategy for targeting MraY, and may contribute towards the development of novel antimicrobial therapeutics.

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Authors names: Julia Fairbairn, Prof. Tim Bugg, Dr Andy Merritt

Affiliations: University of Warwick, LifeArc, funding provided by MRC.

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From: Yijie Zhang <yijie.zhang@i2bc.paris-saclay.fr>

Title: Identification of the mycoloylome in *C. glutamicum*

Text: Corynebacteriales is an order of bacteria that comprises several human pathogens, including Mycobacterium tuberculosis and Corynebacterium diphtheriae, as well as economically valuable species like *Corynebacterium glutamicum*. All these species share a common very atypical cell envelope composed of arabinogalactan-modified peptidoglycan and an outer membrane mainly composed of mycolic acid-derived glycolipids. In 2010, Huc et al. discovered the presence of protein O-mycoloylation in *C. glutamicum*, a post-translational modification catalyzed by the enzyme mycoloyltransferase C (MytC). MytC has been shown to transfer a mycolate chain from trehalose mono mycolate (TMM) to the serine of unusual porins, namely PorAH and PorBC. An additional unknown protein, ProtX, was also reported to be mycoloylated. (Issa et al. 2017 and Carel et al. 2017).
In order to gain a more in-depth understanding of protein mycoloylation, we undertook a proteomic global approach to identify the mycoloylome of *C. glutamicum*. For this, we performed specific metabolic labelling of mycoloylated proteins using a unique synthetic alkyne-tagged TMM (alk-TMM) analogue as a mycoloyl donor. Potential candidates were identified by mass spectrometry. The results were further refined using a MytC knockout mutant, which led us to identify 14 new high-confidence candidates in fine. Interestingly, all these candidates are predicted to be exported in the cell envelope and do contain an unstructured region (Alphafold prediction) with a serine-rich sequence.
We are currently engaged in the biochemical validation of protein mycoloylation candidates. This involves determining their molecular mass and localization and identifying the mycoloylated sites through mass spectrometry analysis. By employing these methodologies, we aim to uncover further insights into the occurrence and importance of mycoloylated proteins in *C. glutamicum*, and to explore the specific function of this unusual post-translational modification, compared to the coexisting N-acylation, in cell envelope protein trafficking. Additionally, this work opens the possibility of using our validated protocol to identify mycolated proteins in other genera and unravel their fundamental importance more generally in the order of Corynebacteriales

Authors names: Yijie Zhang[1], Cecile Labarre[1], Yann Bourdreux[2], Emilie Lesur2, Dominique Guianvarc'h[2], Christiane Dietrich[1], Laila Sago[1], David Cornu[1], and Nicolas Bayan[1]
Affiliations: 1 Institute for Integrative Biology of the Cell (I2BC), Université Paris-Sud, Université Paris-Saclay, CEA, CNRS, Gif-sur-Yvette Cedex, France. 2 Institut de Chimie Moléculaire et des Matériaux d'Orsay (ICMMO), UMR 8182, Université Paris-Sud, Université Paris-Saclay, CNRS, F-91405 Orsay, France.

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From: Beiyan Nan <bnan@tamu.edu>

Title: The coordination between penicillin-binding protein 1a (PBP1a) and the hydrolytic peptidase DacB determines the integrity of bacterial cell poles

Text: Peptidoglycan (PG) defines cell shape and protects bacteria against osmotic stress. The growth and integrity of PG require coordinated actions between synthases that insert new PG strands and hydrolases that generate openings to allow the insertion. However, the mechanisms of their coordination remain elusive. Here we show that moenomycin that inhibits a family of PG synthases known as Class-A penicillin-binding proteins (aPBPs), triggers cell lysis despite aPBPs being non-essential for cell integrity. We demonstrate that inhibited PBP1a2, an aPBP, accelerates the degradation of cell poles by DacB, a hydrolytic PG peptidase, in the bacterium *Myxococcus xanthus*. Moenomycin reduces the mobility of DacB molecules through PBP1a2, potentially promoting the binding between DacB and PG. Conversely, DacB also regulates the distribution and dynamics of aPBPs. These findings reveal the lethal action of moenomycin and suggest that disrupting the coordination between PG synthases and hydrolases could be more lethal than eliminating individual enzymes.

Authors names: Huan Zhang, Srutha Venkatesan, Emily Ng and Beiyan Nan

Affiliations: Texas A&M University, College Station, Texas 77843, USA

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From: Thomas Delerue <thomas.delerue@pasteur.fr>

Title: PBP2/MreC: an ideal target to identify new antibacterial agents

Text: The synthesis of PGN during cell elongation is a process tightly regulated by the rod-complex, a critical multi-protein complex. Among the core components of this complex are two bitopic proteins: the Penicillin-Binding Protein 2 (PBP2), that insert new PGN into the existing structure through transpeptidation, and the cell shape determining protein MreC, which acts as a scaffolding platform for other actors involved in PGN synthesis. The deletion of either protein triggers loss of cell shape, leading ultimately to cell death. Our group has previously demonstrated, using the pathogenic bacteria *Helicobacter pylori*, that inhibiting the interaction between PBP2 and MreC induces the same detrimental phenotype. This finding highlights the potential of the PBP2/MreC complex as a yet unexplored biological target for novel drug development. Given the conservation of this complex across both gram-negative and gram-positive bacteria, targeting the PBP2/MreC interface represents a promising strategy for creating antibiotics with a wide range of targets.
With an *in-silico* approach based on the crystal structure of the complex PBP2/MreC obtained from *H pylori*, we are currently designing small peptides inhibitors that mimic MreC and contain residues essential for the PBP2/MreC interaction. To expand the range of tested compounds and to increase the probability of discovering successful inhibitor candidates, we have been mining the surface properties of PBP2 and MreC, to identify druggable hotspots using the software InDeep. The druggable hotspots are being virtually screening on MolPort, a catalogue that contains 3.5 million compounds and is available at Institut Pasteur with AutoDock Vina.
The most promising candidate molecules will then be tested 1) *in vitro* on a Homogeneous Time Resolved Fluorescence (HTRF) assay using the purified recombinant proteins His-PBP2 and GST-MreC, and 2) in vivo in E coli on a newly developed Bioluminescence Resonance Energy Transfer (BRET) using PBP2 and MREC tagged with mNeonGreen and NanoLuciferase. We will extend our assays to existing libraries of small molecules starting with the PPi-chem from the Institut Pasteur.
The selected candidates will finally be tested in *H. pylori*. We will first assess the effect of the peptides and small molecules on the bacteria growth and morphology. Finally, we will validate the target using the variant of the BRET in *H pylori*, to directly measure the interaction between PBP2 and MreC in the presence of the drugs.

Authors names: Thomas Delerue, Emma Melotti, Karine Nozeret, Olivier Sperandio, Yves-Marie Coic, Laurence Mullard, Ivo G Boneca

Affiliations: Institut Pasteur Paris

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From: Alexander Anderson <aander14@uoguelph.ca> and Anthony Clarke <ajclarke@wlu.ca>

Title: Structure and ligand binding of peptidoglycan *O*-acetyltransferase B (PatB) from Gram-negative pathogens: a potential new antibacterial target

Text: The O-acetylation of peptidoglycan (PG) occurs in many bacteria, including most important human pathogens, such as *Staphylococcus aureus*, *Campylobacter jejuni*, *Helicobacter pylori,* and *Neisseria gonorrhoeae* [1]. This modification to the C6 position of *N*-acetylmuramoyl residues in PG inhibits the action of lysozymes of innate immune systems and it totally precludes the activity of the lytic transglycosylases, bacterial autolysins that are involved with the biosynthesis, modification and turnover of the PG sacculus. We have characterized two distinct systems for PG O-acetylation. In Gram-negative bacteria, an integral membrane protein, PG *O*-acetyltransferase (Pat) A, is proposed to translocate acetate from cytoplasmic pools of acetyl CoA through the cytoplasmic membrane to the periplasm for its transfer to PG by PatB [2]. With Gram positives, a single protein, *O*-acetyltransferase (OatA), represents a fusion of PatA and PatB to catalyze both the translocation and transfer of acetyl groups for PG O- acetylation [3].

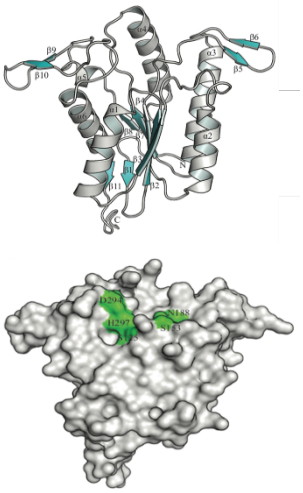

Fig. 1. Ribbon and space-filling models of *N. gonorrhoeae* PatB.

We present the X-ray crystal structure of PatB from both *N. gonorrhoeae* and *C. jejuni* which has the atypical α/β hydrolase fold expected for the SGHN/GDSL hydrolases. The catalytic triad of Ser-His-Asp residues is appropriately aligned on the surface in the center of the enzyme; no active site cleft or pocket exists that is typical of other PG-active carbozymes. Unique to all PatB homologs compared to all other SGNH/GDSL hydrolases is the existence of two pairs of short β-strands that we hypothesized to extend the substrate-binding surface of the enzyme. With continued failure to obtain crystals of PatB in complex with any ligand, we tested this hypothesis by generating 25 site-specific variants of *N. gonorrhoeae* PatB and determined their kinetic parameters as enzymes acting as both *O*-acetyl esterases and transferases, as well as their ability to be inhibited by purpurin, an effective inhibitor of the wild-type enzyme. We identified a pair of Gln residues important for binding acetyl donors, and additional Arg, Lys, Phe and Tyr residues that span across 11 putative subsites for the binding of glycans. These data, together with theoretical considerations, explain how PatB as a peripheral membrane protein interacts with its insoluble PG substrate, important information for the development of the enzyme as an antibacterial target.

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Authors names: Alexander Anderson[1*], Ashley Brott[1], Stefen Stangherlin[2], Anthony J. Clarke[1,2]

Affiliations: [1]University of Guelph, Guelph, Ontario, Canada. [2]Wilfrid Laurier University, Waterloo, Ontario, Canada.

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From: Olivier Danot <olivier.danot@pasteur.fr>

Title: A new regulator of peptidoglycan remodeling and cell shape in *Helicobacter pylori*

Text: To enable bacterial growth and morphological changes, peptidoglycan needs to be constantly remodeled. This process involves not only peptidoglycan synthases such as Class A penicillin binding proteins or SEDS proteins like RodA, but also numerous peptidoglycan hydrolases whose activity can target virtually any glycosidic or peptide bond within the cell wall. The potentially lethal activity of these hydrolases needs to be tightly controlled and coordinated to prevent peptidoglycan rupture. This control often involves an autoinhibition component but more and more examples show that the activity of peptidoglycan hydrolases can also be regulated and coordinated by dedicated periplasmic or membrane proteins such as NlpI in *Escherichia coli*.
In a search for candidates for this role in *Helicobacter pylori*, we came across a protein of unknown function (tentatively named Tfl for thioredoxin fold lipoprotein) that was predicted by a two-hybrid screen to interact with an important peptidoglycan hydrolase, HdpA. HdpA is a carboxy- and endopeptidase, whose disruption affects virulence, competence and the transition to the coccoid shape, a hallmark of H. pylori which was proposed to favor immune evasion. We have shown that Tfl has two antagonistic effects on HdpA: on one hand it strongly protects it from proteolysis *in vivo*, but on the other hand it inhibits its activity in a purified system, thus probably keeping HdpA in check until it reaches the location where its activity is needed. We have solved the three-dimensional structure of the Tfl-HdpA complex, which shows that Tfl interacts with an auto-inhibitory domain of HdpA suggesting a simple mechanism for the inhibition and the stabilization exerted by Tfl. In vivo, the deletion of the *tfl* gene recapitulates the morphological phenotypes of the deletion of *hdpA*, including a strong defect in the formation of coccoids. However, since the peptidoglycan composition of the two mutants is different, we propose that Tfl also regulates additional components of the peptidoglycan remodelling machinery and might play a role analogous to that of NlpI in *E. coli*.

Authors names: Olivier Danot[1], Ariel Mechaly[2], Ahmed Haouz[2], Ivo Gomperts Boneca[1]

Affiliations: [1] Institut Pasteur, Université Paris Cité, CNRS UMR6047, INSERM U1306, Unité Biologie et génétique de la paroi bactérienne, F-75015 Paris, France. [2] Institut Pasteur, Université Paris Cité, Plate-Forme de Cristallographie, C2RT, Institut Pasteur, CNRS UMR3528, Paris, France.

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From: Adrien Ducret <adrien.ducret@ibcp.fr>

Title: The placement of the division site of *S. pneumoniae* is dictated by the local composition of peptidoglycan

Text: The proper placement of the bacterial division site is essential for the generation of two viable and identical daughter cells. This process generally involves the identification of the middle of the cell followed by the recruitment of the division machinery. In the oval-shaped bacterium, *Streptococcus pneumoniae* (pneumococcus), the placement of the future division sites is directly linked to its mode of growth. In fact, pneumococcal cells elongate only from mid-cell. Peptidoglycan (PG)-synthesis builds the new cell-halves from the cell center while the old cell-halves are pushed apart. Consequently, the frontier between new and old cell wall material (i.e. cell equator) keeps moving away from mid-cell as the cell elongates. We previously identified that the protein MapZ specifically localizes at the moving cell equator through its extracellular domain, forming a ring that ultimately positions the division machinery. However, what drives the localization of MapZ remained elusive. Here, using a PG-labelling approach utilizing timed pulses of fluorescent D-amino acids, we demonstrated that the localization and the dynamic of MapZ at the cell equators is strongly affected in mutants deficient in some hydrolytic activities. Our results show that cell equators could be specifically enriched in a signature which results from the coordinated activity of these hydrolases and which is specifically recognized by the extracellular domain of MapZ. As a matter of fact, a chimeric version of MapZ in which the extracellular domain was replaced with the catalytic domain of a protein which binds to this signature, localized specifically at the cell equators. By looking at the specific sub-cellular localization of these hydrolases, we demonstrated they display different dynamics during the cell cycle. By consequence, the enrichment of this signature at the cell equators could result from the lag between their recruitment at mid-cell. This study provides evidence that MapZ-mediated selection of the division site in S. pneumoniae is dictated by a local composition of the PG, which derive from the precise timing of two sequential hydrolase activities.

Authors names: Adrien Ducret, Céline Freton & Christophe Grangeasse

Affiliations: Molecular Microbiology and Structural Biochemistry, UMR 5086, Université de Lyon, CNRS, Lyon, France.

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From: Simon Schäper <sschaeper@itqb.unl.pt>

Title: Processive movement of the essential peptidoglycan synthesis enzyme complex driving cell constriction in *Staphylococcus aureus*

Text: The relationships between FtsZ treadmilling and the movement and activity of the septum-specific peptidoglycan (PG) synthases vary across organisms. We aimed to elucidate the relationship between dynamics of FtsZ in the Z-ring and of the essential PG synthase complex FtsW-PBP1 that mediates septal PG incorporation in the gram-positive pathogen Staphylococcus aureus.
We used *S. aureus* strains containing mutations known to interfere with the enzymatic activities of FtsZ and PBP1 to study their effect on FtsZ treadmilling, cell constriction, and movement of the essential PG synthase complex. *S. aureus* FtsZ T111A mutants exhibited a reduced treadmilling speed while showing wildtype-like growth and cell constriction rates. Contrarily, the PBP1 active site mutation S314A caused cells to constrict at slower rates, whereas FtsZ treadmilling and cell growth rates were not affected by this mutation.
We also characterized the movement dynamics of nine conserved cell division proteins using single-molecule imaging. The PG synthases FtsW and PBP1, as well as their putative activator protein DivIB, directionally moved around the division site with the same velocity. FtsW and DivIB velocity was slowed down or completely stopped in cells with perturbed PG synthesis, but not in cells with impaired FtsZ treadmilling.
Our findings indicate that in S. aureus the processive movement of the FtsW-PBP1 complex is coupled to its catalytic activities and does not scale with FtsZ treadmilling. Moreover, active FtsW-PBP1 complexes and the regulatory subcomplex DivIB-DivIC-FtsL likely stably interact to drive septum synthesis in this organism.

Authors names: Simon Schäper(1), António M. Brito(1,2), Bruno M. Saraiva(1,2), Georgia R. Squyres(3), Matthew J. Holmes(3), Ethan C. Garner(3), Ricardo Henriques(2), Mariana G. Pinho(1)

Affiliations: (1) Instituto de Tecnologia Química e Biológica António Xavier, Universidade NOVA de Lisboa, Oeiras, Portugal. (2) Instituto Gulbenkian de Ciência, Oeiras, Portugal. (3) Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA, USA.

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From: Ofélia Godinho <ofeliagodinho95@gmail.com>

Title: The cell wall of Planctomycetota is still a mystery to unveil: non-essentiality of the canonical peptidoglycan biosynthesis genes

Text: Members of the phylum Planctomycetota have been under scrutiny for different aspects of their biology, but none has been as long lasting as the structure of their cell wall. Initial studies in the 1980's reported lack of peptidoglycan in their cell wall and proposed the presence of a proteinaceous cell wall instead. Later in 2015, reports suggested the presence of classical peptidoglycan and of genes of its known biosynthetic pathway in their genomes. However, more recent studies have found some contradicting evidence: 1) for some orders within the phylum their genomes lack almost if not all of the genes of the pathway; 2) even in those orders that do possess such genes, some of them are not essential, and their knockout does not produce strong phenotypes if they produce any. Here we expand that knowledge with the exhaustive study of the peptidoglycan biosynthesis in *Planctopirus limnophila*, where multiple knockout mutants for different stages of the peptidoglycan biosynthesis pathway have been constructed and phenotypically characterized. With this work we extend the list of demonstrated non-essential genes for the synthesis of peptidoglycan in P. limnophila and take a step further in the elucidation of the planctomycetotal pathway for the synthesis of peptidoglycan.

Authors names: Ofélia Godinho[1,2,$], Elena Rivas-Marin[3,$], David Moyano Palazuelo[3], Sandra Quinteira[1,4,5], Olga M. Lage[1,2], Damien P. Devos[3]
$ Both author contribute equally.

Affiliations: (1) Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Porto, Portugal; (2) CIMAR/CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Porto, Portugal; (3) Centro Andaluz de Biología del Desarrollo, CSIC, Universidad Pablo de Olavide, Seville, Spain.; (4) BIOPOLIS/CIBIO-InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, Vairão, Portugal; (5) TOXRUN-Toxicology Research Unit, University Institute of Health Sciences, CESPU, CRL., Gandra PRD, Portugal

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From: Maria Disen Barbuti <maria.disen.barbuti@nmbu.no>

Title: Uncovering a novel mechanism influencing lipoteichoic acid biosynthesis in *Staphylococcus aureus*

Text: *Staphylococcus aureus* possesses a cellular envelope composed of a thick layer of peptidoglycan and teichoic acids situated outside of a single lipid membrane. Teichoic acids are attached to the peptidoglycan layer (wall teichoic acids, WTAs) or the cytoplasmic membrane (lipoteichoic acids, LTAs) and extend through the cell wall. In *S. aureus*, these anionic polymers are important virulence factors and modulators of antibiotic susceptibility, in addition to maintaining cell integrity and facilitating proper cell division. The paralogous membrane proteins CozEa and CozEb have previously been reported to influence staphylococcal cell division in S. aureus SH1000. In this work, the functions of the conserved CozE membrane proteins were further investigated in methicillin-sensitive- and methicillin-resistant *S. aureus* (MSSA and MRSA). Construction and characterization of single and double *cozE* deletion and knockdown mutants, confirmed that *cozEa* and *cozEb* constitute a synthetic lethal gene pair across different MSSA and MRSA strains. Starting from CRISPR-interference based genetic screens, we found that proteins involved in synthesis and flipping of the glycolipid anchor of LTA, modulate the essentiality of CozE proteins. Notably, the essentiality of CozEa and CozEb was alleviated in cells lacking the flippase activity (ΔltaA), while the opposite was found in cells devoid of glycolipid synthesis (ΔugtP). Furthermore, anti-LTA immunoblot assays revealed that CozEb plays a unique role in controlling LTA polymer length and stability. Together, the results demonstrate that the CozE proteins have a direct or indirect role in controlling LTA biosynthesis in S. aureus.

Authors names: Maria Disen Barbuti[1], Ine Myrbråten[1], Maria Victoria Heggenhougen[1], Marita Torrissen Mårli[1], Adrien Ducret[2], Christophe Grangeasse[2], Daniel Straume[1], and Morten Kjos[1]

Affiliations: [1]Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway; [2]Molecular Microbiology and Structural Biochemistry, CNRS, Université de Lyon, Lyon, France.

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From: Morten Kjos <morten.kjos@nmbu.no>

Title: SmdA is a key cell morphology determinant in *Staphylococcus aureus*

Text: Cell division and cell wall synthesis in staphylococci need to be precisely coordinated and controlled, in order to allow the cell to multiply while maintaining their nearly spherical shape. From a combination of large-scale knockdown-screens, we identified a hitherhto uncharacterized membrane protein, named SmdA (for staphylococcal morphology determinant A), with major influence on cell morphology in *Staphylococcus aureus*. Using CRISPR interference knockdown and overexpression combined with different microscopy techniques, we demonstrate that proper levels of SmdA, is necessary for cell division, including septum formation and cell splitting. Pulldown- and bacterial two-hybrid interaction experiments showed that SmdA interacts with several known cell division- and cell wall synthesis proteins, including penicillin binding proteins (PBPs). Synthetic lethal interactions with PBP3 and PBP4 were identified and notably, SmdA also affects susceptibility to cell wall targeting antibiotics, particularly in methicillin-resistant S. aureus (MRSA). SmdA is indispensable for growth at high temperatures and by characterizing suppressor interactions we show that SmdA is a key player in a complex network of factors required to carry out proper cell division.

Authors names: Maria Disen Barbuti, Ine S. Myrbråten, Zhian Salehian, Marita T. Mårli, Tiril Mathiesen Knudsen, Mathilde Hustad, Daniel Straume and Morten Kjos

Affiliations: Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Norway.

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From: Roy van Beekveld <r.a.m.vanbeekveld@uu.nl>

Title: Understanding the Molecular Mechanism of Polymyxin by Solid-State NMR

Text: Gram-negative bacteria are intrinsically resistant to many antibiotics due to their near-impermeable outer membrane (OM). A large part of the OM is composed of a unique lipid, lipopolysaccharide (LPS), that forms a formidable target to fend off Gram-negative infections. A well-known antibiotic class that does so is the group of polymyxins. Nevertheless, what polymyxins do to the OM has been elusive for decades, likely due to the fact that OMs have proven difficult to be studied in their native environment at high-resolution thus far. Here, we present the first steps in our work towards solving the complex formed by polymyxins with the bacterial OM using solid-state NMR spectroscopy in near-native conditions. Using this approach, we can readily study complexes of LPS and drugs that target this lipid at atomic level. Moreover, it allows for the study of LPS variants that are linked to polymyxin resistance and offers atomic details that can help overcome resistance caused LPS modifications. Ultimately, this work provides the first high-resolution insights in the mode of action of polymyxins. Furthermore, it offers novel strategies to study OM targeting antibiotics on an atomic level, offering opportunities to overcome the Gram-negative permeability barrier.

Authors names: Roy A.M. van Beekveld, Lisa Renee van Haften, Maik G.N. Derks, Eefjan Breukink, Markus Weingarth

Affiliations: Bijvoet Centre for Biomolecular Research, Department of Chemistry, Faculty of Science, Utrecht University, Utrecht, The Netherlands

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From: Patrick Viollier <parick.viollier@unige.ch>

Title: Molecular dissection of a diderm envelope using large membrane-impermeable antibiotics

Text: Despite its diderm envelope structure, the alpha-proteobacterium *Caulobacter crescentus* is very sensitive towards large cell-wall targeting antibiotics that are normally excluded by the outer membrane (OM) in other Gram-negative bacteria. By contrast, *C. crescentus* is highly resistant towards beta-lactam and first-generation quinolone antibiotics owing to a chromosomally-encoded metallo-beta-lactamase, an RND-type efflux pump and/or a natural polymorphism the gyrase A gene. Our negative-selection screen for sensitive transposon mutants revealed strains that exhibit hypersensitivity to vancomycin and bacitracin, yet elevated resistance to moenomycin. On the basis of these results, we launched suppressor studies with these sensitive mutants as entry mutant to understand why *C. crescentus* is sensitive towards these large cell-wall targeting antibiotics. We learned that these traits are governed by a regulatory circuit involving a new type of OM sensor, two-component signaling pathways, cell wall biosynthesis enzymes (PBPs), and inducible TonB-dependent transporter and antibiotic efflux pump.

Authors names: Jordan Costafrolaz , Patrick Viollier

Affiliations: University of Geneva, Switzerland

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From: Girbe Buist<g.buist@umcg.nl>

Title: Predicted immunogenic domains of surface-associated virulence factors of *Staphylococcus aureus* are recognized by IgGs from cows with mastitis

Text: Bacterial surface-exposed protein epitopes are ideal antigens to elicit host immune responses. Consequently, they are promising candidates for the development of protein-based vaccines. The human and livestock pathogen Staphylococcus aureus displays a broad spectrum of surface-exposed proteins that are not strictly conserved among different staphylococcal lineages. Some surface proteins of S. aureus are known to trigger adaptative immune responses by the host. Accordingly, the present study aimed at the in-silico dissection of conserved domains in virulence-related surface proteins of S. aureus with the long-term objective to develop future vaccines against bovine mastitis. A specific focus was therefore placed on S. aureus isolates from cows with mastitis. Following an immunoproteomics approach, highly conserved potentially antigenic regions and Bcell epitopes were predicted in surface proteins of the inspected S. aureus isolates, which were shown to belong to 16 different sequence types. In particular, 23 strictly conserved surface proteins were identified in S. aureus isolates implicated in bovine mastitis, with predicted numbers of exposed immunogenic epitopes ranging from 1 to 15. Subsequent verification studies using sera from 12 cows with mastitis revealed IgG responses to the cell wall hydrolases Aly, LytM and Sle1. In particular, the Sle1 protein emerged as a potential candidate for developing a sub-unit vaccine formulation against mastitis. Altogether, our present observations form the basis for further validation studies to explore the immunogenicity of the predicted S. aureus surface protein epitopes and their potential use for the development of effective candidate vaccines that could protect cattle against mastitis caused by S. aureus.

Authors names: E. Vera Murguia, N. Couto, Y. Mora Hernandez, A. Revtova, F. Akhter Chowdhury, M. Wang, A.H. Visser, L. Liu, P. Hernández Jauregui, J. W. A. Rossen, J. M. van Dijl , G. Buist

Affiliations: Department of Medical Microbiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

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From: Alessandro Pagliuso <alessandro.pagliuso@inrae.fr>

Title: A bacterial pathogen sheds its cell wall during the transition into VBNC state

Text: Microorganisms can survive in extreme environments. Some bacterial species have evolved the ability to produce highly resistant structures, called spores, to enter dormancy until environmental conditions are met to resume vegetative growth. In non-sporulating bacteria, the viable but non-culturable" (VBNC) state is an alternative between the active growth and sporulation. In this state, bacteria are viable and metabolically active, but resistant to cultivation in their routine growth media. Although hundreds of bacterial species have been reported to transition into the VBNC state, the underlying molecular mechanisms remain elusive. Here we report that, during several weeks of nutrient starvation, *Listeria monocytogenes* bacilli progressively transform into coccoidal forms, after shedding their cell wall in a moulting-like process. By developing an antibody able to specifically detect these wallless VBNC bacteria, we further show that this property is shared by other Listeria species, indicating that cell wall shedding is an adaptive process common to *Listeria* genus. Our work unveil that cell wall deficient bacteria (also known as L-forms) can naturally arise without the need of osmotic stabilizers and may represent a strategy to persist in oligotrophic conditions.

Authors names: Filipe Carvalho (1), Alexis Carreaux (1), Marie-Pierre Chapot-Chartier (1), Anna Sartori-Rupp (2), Eliane Milohanic (1), Hélène Bierne (1) and Alessandro Pagliuso (1)

Affiliations : (1) Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en-Josas, France; (2) Institut Pasteur, Paris

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From: Megan Keller <mrk269@cornell.edu>

Title: The Central Carbon Metabolism of *Vibrio cholerae* and its Unexplored Role in Antibiotic Susceptibility

Text: Like many gram-negative pathogens, *V. cholerae* utilizes a wide range of energy sources and pathways. This enables these gram-negative pathogens the ability to infiltrate the host and thrive in niche environments. Gram-negative bacteria are also common evaders of antibiotic efficacy, posing an increasing threat to public health. While the energy state of a bacterium has been linked to how effective an antibiotic is, *V. cholera*'s central metabolism remains unexplored in its connection to drug susceptibility, particularly towards the cell wall targeting β-lactams. Using a cell wall stress sensing system and a transposon mutagenesis genetic screen, we found that when central carbon metabolism is disrupted at the stage of early glycolysis (phosphoglucose isomerase, encoded by the *pgi* gene), the bacterial cell wall is subsequently damaged and V. cholerae becomes more susceptible to cell wall-active antibiotics, such as the β-lactams. Additionally, we observed severe morphological defects and sensitivity to low osmolarity conditions, further supporting the idea that the *pgi* mutant has a damaged cell wall. Suppressor mutations within the phosphotransferase system (PTS) restored all defects associated with the *pgi* mutation, suggesting that excess sugar phosphates are deleterious in a *pgi* mutant. We propose that these sugar phosphates act as a competitive inhibitor of the cell wall precursor enzyme, GlmM, displacing its natural substate glucosamine-6-phosphate. With GlmM's activity decreased, the bacterial cell wall struggles to maintain efficient cell wall synthesis and thus is more susceptible to β-lactams. The external addition of the cell wall precursor GlcNAc surprisingly rescued the observed defects. GlcNAc is converted to glucosamine-6-phosphate, which likely relieves competitive inhibition of GlmM. This work demonstrates the need for further research into bacterial metabolism's ability to enhance antibiotic susceptibility, and the creation of new antibiotic targets.

Authors names: Megan Keller, Molly Han, Tobias Doerr

Affiliations: Cornell University

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From: Robert Smith <robert.smith@pasteur.fr>

Title: Identification of the first hydrolase from the cell walled Archaea *Methanobrevibacter smithii* resolves the structure of archaeal pseudopeptidoglycan

Text: While Archaea and their envelopes are traditionally considered atypical, multiple archaea, possess a pseudopeptidoglycan (pPG) which is similar to bacterial peptidoglycan (PG) in its macromolecular architecture. Yet several key structural differences have been observed including replacement of MurNAc with TalNAc and the use of only L amino acids. Concordantly, these archaea encode the *mur* genes, *mreB*, and *ftsZ*. However currently there is a large gap in our knowledge concerning how pPG is crosslinked and hydrolyzed. Here I present the discovery and characterization of the first archaeal pseudopeptidoglycan hydrolase. Using *Methanobrevibacter smithii*, a prominent member of the human gut microbiota. Candidate pPG hydrolases were identified using a bespoke bioinfomatic pipeline. Candidates were then expressed in E. coli and assessed for pPG hydrolytic activity using zymograms. We obtained an enzyme encoding both glycosyl hydrolase and peptidase domains, henceforth named talozyme. Talozyme digestion of pPG and talopeptide analysis by RPLC-MS showed that both enzymatic domains are active and surprisingly revealed the presence of a previously undescribed 3-beta-methyl-alanine group replacing the lactyl group from PG. Phylogenetic analysis and mapping show that talozyme is widely present throughout the Methanobacteriales and is not found in bacteria. This conservation suggests that talozyme is the responsible for septum cleavage in Methanobacteriales and has been acquired in archaeal evolution as part of their reinvention of the PG wheel. In addition to understanding its biological role, talozyme is an essential tool in pPG structural analysis replacing the role of mutanolysin and opening up the possibility to study pPG from the diversity of walled archaea.

Authors names: Robert Smith[1], Nika Pende[1], Aline Rifflet[2], Richard Wheeler[2], Najwa Taib[1], Pierre Garcia[1], Iñaki Guijarro[3] Ivo G Boneca[2], Simonetta Gribaldo[1]

Affiliations: 1 Institut Pasteur, Evolutionary biology of the microbial cell, Department of Microbiology, Paris, France; 2 Institut Pasteur, Biology and genetics of the bacterial cell wall, Department of Microbiology, Paris, France 3 Institut Pasteur, Biological NMR and HDX technology platform Department of Microbiology, Paris, France

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From: Antoni Planas <antoni.planas@iqs.url.edu>

Title: Peptidoglycan de-N-acetylases as antibacterial targets. Specificity and structural insights for GlcNAc and MurNAc deacetylation.

Text: Pathogenic bacteria and fungi modify their own cell wall polysaccharides as a strategy to evade the host immune responses at initial stages of infection. Plant pathogenic fungi partially deacetylate their cell wall chitin to be resistant to plant chitinases or deacetylate the released chitooligosaccharides to escape recognition by chitin receptors and evade the plant immune responses. Human pathogenic bacteria utilize deacetylation (2-N-deacetylation of GlcNAc and/or MurNAc residues) and acetylation (6-O-acetylation of MurNAc) of their cell wall peptidoglycan (PG) to become resistant to host lysozymes and evade detection by the innate immune system. Both chitin and PG deacetylases share high sequence and structural similarities and belong to Family 4 Carbohydrate esterases (CE4 enzymes) acting by metal-assisted general acid-base catalysis [1].
Our objective is to understand the different specificities (GlcNAc vs. MurNAc PG deacetylases) and evaluate them as novel therapeutic targets for antimicrobials. PG GlcNAc deacetylases participate in the dynamic control of autolysis and are virulent factors in pathogenic bacteria (lysozyme resistance) [2]. They are active on intact PG and also on chitooligo-saccharides (COS). Less studied are PG MurNAc deacetylases involved in the formation of the spore cortex PG during sporulation. They act on PG devoid of the stem peptide (requiring the previous action of an amidase) to lead to the δ-lactam modification of spore cortex PG, and are inactive on COS. Although PG GlcNAc and MurNAc deacetylases have mutually exclusive specificities, the determinants of the different specificities are unknown since no 3D structures of any PG deacetylase in complex with substrates have been solved, and no clear sequence signature has been assigned to both types of activities. A novel PG MurNAc deacetylase, PdaC from *B.subtilis*, was identified, showing activity on intact PG but also active on COS (an activity thought to be restricted to GlcNAc deacetylases), not involved in sporulation and with an unknown function. We have solved the 3D structure of BsPdaC (free enzyme) and characterized its mode of action on COS [3]. Here we will present recent results aimed at unraveling the structural determinants of substrate specificity within the diversity of PG deacetylases:
- Bioinformatic analysis of annotated whole-genome PG deacetylases based on Conserved Unique Peptide Patterns (CUPPS [4]) for clustering and functional annotation, which resulted in a family subclassification for different specificities.
- Structural and mutational studies on BsPdaC aimed at identifying the protein-ligand interactions that define the dual MurNAc and GlcNAc specificities.
Based on these results, a panel of PG deacetylases covering predicted differential specificities are being recombinantly expressed and characterized to deepen the structure-specificity-function relationships of this family of enzymes and evaluate them as potential therapeutic targets against infectious diseases.

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Authors names: Mònica Texido, Aitor Vega, Xevi Biarnés and Antoni Planas*

Affiliations: Laboratory of Biochemistry, Institut Químic de Sarrià, University Ramon Llull, Barcelona, Spain

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From: Coralie Fumeaux <coralie.fumeaux@unil.ch>

Title: Role(s) of a new operon in cell wall homeostasis and antibiotic resistance in *Pseudomonas aeruginosa*

Text: Peptidoglycan is an essential cross-linked polymer that surrounds most bacterial cells to prevent osmotic rupture of the cytoplasmic membrane. Its synthesis relies on shape, elongation, division and sporulation proteins (SEDS) and penicillin-binding proteins (PBPs). PBPs are the targets of a widely used class of antibiotics, the beta-lactams. Many Gram-negative bacteria, including the opportunistic pathogen Pseudomonas aeruginosa, are resistant to beta-lactams due to a chromosomally-encoded beta-lactamase called AmpC. In *P. aeruginosa*, expression of the *ampC* gene is tightly regulated and its induction is linked to cell wall stress. We reasoned that a reporter gene fusion to the *ampC* promoter would allow us to identify mutants defective in maintaining cell wall homeostasis and thereby uncover new factors involved in the process. A library of transposon mutagenized P. aeruginosa was therefore screened for mutants with elevated *ampC* promoter activity. As an indication that the screen was working as expected, mutants with transposons disrupting the *dacB* gene were isolated. Defects in DacB (PBP4) have previously been implicated in *ampC* induction and clinical resistance to beta-lactam antibiotics. The screen also uncovered mutants in an uncharacterized operon composed of three genes. We present genetic evidence that these genes contribute to resistance to not only beta-lactams, but also aminoglycosides and polymyxins and possibly play a role in peptidoglycan synthesis.

Authors names: Joël Gyger and Coralie Fumeaux
Affiliations: Institute of Microbiology, Lausanne University Hospital (CHUV), Switzerland

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From: Maarten Lubbers <m.lubbers@biology.leidenuniv.nl>

Title: Using wall-deficient bacteria for the rational design of cell morphology

Text: Bacteria come in a wide variety of shapes, ranging from rod-shaped unicellular cells to complex multicellular structures. These shapes have evolved to benefit the organism in its natural environment. However, industry often takes such organisms from their natural environment aimed at producing useful molecules that favor mankind. Their natural morphology is often far from optimal for use in an industrial setting. Therefore, various morphology engineering approaches have successfully been used but typically only yield incremental improvements. One limitation of these approaches is the oftentimes essential nature of fundamental genes involved in shaping cells. We here propose to use a radical approach to redesign cell morphology using cell wall-deficient L-forms as the starting point. These cells can proliferate without the otherwise essential cell wall synthesis and division genes, enabling a bottom-up approach to cell morphology design. These developments would not only vastly improve our understanding of the factors controlling cell morphology but also spearhead optimization of industrial production purposes in recalcitrant microbes.

Authors names: Maarten Lubbers, Dennis Claessen

Affiliations: Institute of Biology, Leiden University, The Netherlands

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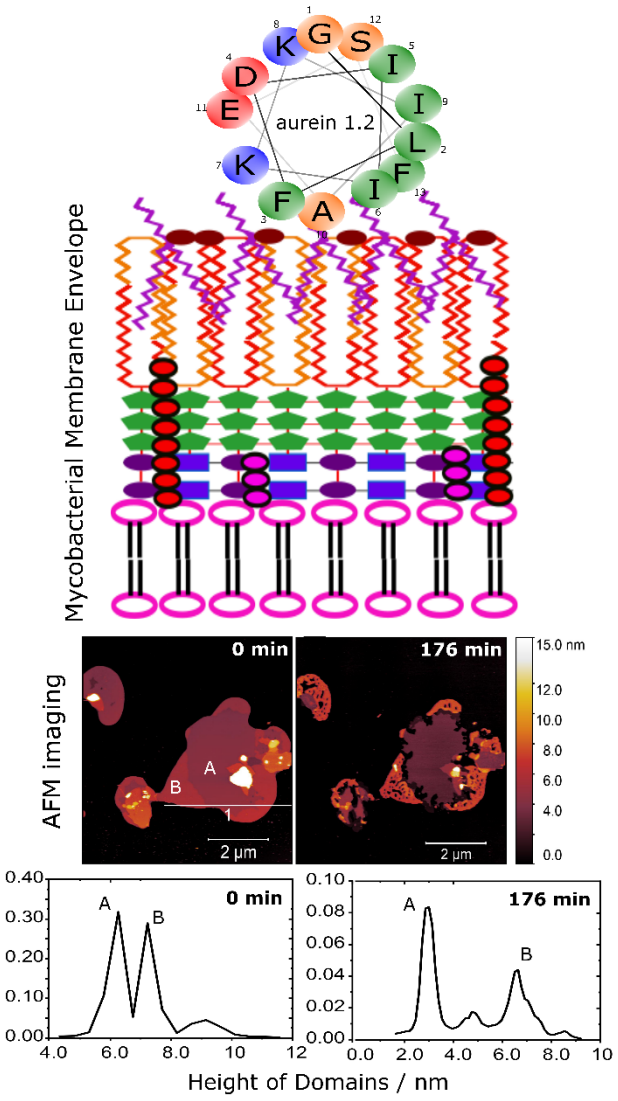
From: Anjana Peethambaran Menon <ANJANA.NAMBALAT@GMAIL.COM>

Title: Lipidome-Specific Development of Combination Therapy to combat Infection Stage-Dependent Antibiotic Resistance

Text: Anti-tubercular therapy is a long-term process during which the chances of antibiotic resistance are high due to its inaccurate distribution. The bioavailability of the antibiotic is limited due to the pharmacokinetic parameters and cell membrane barriers [1, 2]. Rational designing of the antibiotics is essential to validate the required entry into the target cell to eliminate chances of resistance [3, 4]. A thorough membrane lipid distribution among different infection stages could help in developing combinational therapies against resistant variants. In the first part of the work, a thorough lipidomics of the mycobacterial outer and inner membranes were conducted which marks the deciding factor of lipids specific in resisting drug entry. Notably, increase of fatty acids and lipoarabinomannans and decrease of glucose mono-mycolate was observed during the late infection stage. Australian frog peptides aurein1.2 and maculatin1.1 have membrane-active properties which could be exploited to enhance the drug uptake in Mycobacterium. This work later uses a combination of biophysical techniques to study the peptide interaction patterns on these mycobacterial cell envelope layers. Microscale thermophoresis studies showed that aurein1.2 (~1µM) has a greater affinity towards the mycobacterial membrane than maculatin1.1 (~5µM). Therefore, atomic force microscopy (as shown in the graphical abstract) and surface plasmon resonance studies were performed on individual membrane lipid bilayers, revealing that aurein1.2 insertion leads to lipid removal in the mycobacterial inner membrane lipids. In contrast, maculatin1.1 insertion leads to the blebbing of the bilayer. Dye-leakage assay performed using confocal microscopy promises aurein1.2 to effectively permeabilize outer and inner mycobacterial membranes, while maculatin1.1 permeabilizes selectively. However, flow cytometry studies indicated greater permeabilization of



propidium iodide into the whole mycobacterial cell when treated with maculatin1.1, indicating the formation of a larger surface area of blebbing compared to the area of lipid removal by aurein1.2. These peptides when used in combination with the anti-tubercular drug amikacin, these molecules spurred up the amikacin uptake to enhance intracellular antibiotic concentrations. Leveraging the membrane-drug interaction profiles unique to mycobacteria, specific chemotypes consisting of outer membrane-disruptive agents and anti-tubercular drugs may provide strategies for new combinational TB therapies [5].

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Authors names: Anjana Peethambaran Menon[1,2,3], Tzong-Hsien Lee[2,3], Marie-Isabel Aguilar[2,3], Shobhna Kapoor[1,2,4]

Affiliations: [1]Department of Chemistry, Indian Institute of Technology Bombay, Mumbai 400076, India; [2]IITB-Monash Academy, Indian Institute of Technology Bombay, Mumbai 400076, India; [3]Department of Biochemistry & Molecular Biology, Monash University, Clayton, Vic 3800, Australia; [4]Graduate School of Integrated Sciences for Life, Hiroshima University, Hiroshima, 739-8528, Japan

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From: Marjolein Crooijmans <m.e.crooijmans@biology.leidenuniv.nl>

Title: Insights into the Proliferation of Wall-deficient *Escherichia coli*

Text: The bacterial cell wall is a crucial structure that protects cells from external stressors and environmental changes. However, some bacteria can shed their cell walls and continue to grow in osmoprotective conditions after exposure to chemical or enzymatic agents that target the cell wall. This ability has important implications for their survival in industrial and medical settings, as well as for the development of antibiotic resistance. Despite this, the mechanisms underlying the proliferation of Gram-negative wall-less bacteria are not well understood.

To further investigate this extraordinary growth mechanism, we engineered an *E. coli* L-form strain that can proliferate with or without a cell wall. We found that their growth is not dependent on an intact outer membrane, but likely due to blebbing of the inner membrane caused by the accumulation of lipopolysaccharide precursors.

While studying this phenomenon, we discovered that the L-form can grow during treatment with antibiotics that target peptidoglycan precursors in the periplasmic space. However, targeting upstream precursors of peptidoglycan synthesis in the cytoplasmic space resulted in lethality. These findings provide new insights into the mechanism of cell growth and the tight regulation between early peptidoglycan and lipid A biosynthesis.

Authors names: Marjolein Crooijmans, Dennis Claessen

Affiliations: Institute of Biology, Leiden University, the Netherlands

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From: Aline Rifflet <arifflet@pasteur.fr>

Title: Accumulation of bacterial cell wall moieties in the aging brain and neurophysiopathology: evidence and perspectives.

Text: Bacterial peptidoglycan (PGN) and its derivative fragments (muropeptides) elicit a variety of host responses and play a major role in mediating gut-brain communication. Recent research highlights the role of PGN during homeostasis in appetite and body temperature control (1). However, PGN has also been shown to be a driver of chronic brain inflammation (2). Our results, suggest that this macromolecule tends to accumulate in different brain regions with age. Specific monoclonal antibodies were used to track and observe this neuro-aggregation. Unfortunately, it is not clearly understood how and what kind of bacterial cell wall features, the paratopes are able to recognize in the brains of elderly donors.
In this work, biochemicals and complementary approaches were used to identify the epitope for each testing antibody. We characterized four different antibodies described as recognizing PGN (2E9, MAB995, 2E7 and Ch'tiMAB).
First, antibodies were evaluated against purified PGN and muropeptides solutions in dot blots to discriminate the size, the nature and cross-link rate of the potential target peptidoglycan patterns. We used a variety of mammalian and bacterial PGN hydrolases to generate different PGN structures. The results indicate that polymeric peptidoglycans are preferentially recognized by the antibodies although Ch'tiMAB was also able to detect soluble muropeptides. In addition, immunofluorescence staining was performed directly on purified sacculi of bacteria and intestinal tissues of mice for microscopy analysis.
High resolution mass spectrometry was also used to go even further in structural elucidation of epitopes of interest. Using antibody coated magnetic beads, we selectively purified and concentrated the epitopes from complex matrices as homogenates of brain, spleen and liver. The results confirmed that the antibodies exhibit a preferential binding affinity for soluble polymeric peptidoglycan instead of smaller muropeptides.

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Authors names: RIFFLET Aline [1], ZEIHER Carolin (PhD)[2], KUHRT Heidrun[2], BECHMANN Ingo2, LAMAN Jon D.[3] and GOMPERTS BONECA Ivo[1]

Affiliations: [1] Unité BGPB, Université Paris Cité, UMR CNRS 2001, INSERM U1306, Département de Microbiologie, Institut Pasteur, Paris, France; [2] Institute of Anatomy, Leipzig University; [3] Department of Biomedical Sciences of Cells and Systems, Section of Molecular Neurobiology, University Medical Center Groningen

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From: Berliza Marie Soriano <berliza.soriano@ucsf.edu>

Title: Builders and Breakers: Enzymes Involved in Peptidoglycan Homeostasis

Text: The cell wall (CW) is a macromolecule that encases bacteria providing structural integrity, an essential feature for cell viability. A major component of the CW is a polymeric layer called the peptidoglycan (PG), an organizing scaffold for hundreds of proteins. The assembly of PG is a tightly coordinated process that involves the action of opposing enzymatic activities ie. polymerases and hydrolases that are predicted to associate in a multi-subunit protein complex. The overarching goal of this study is to unravel, at an atomic level, the nature of the polymerase-hydrolase complex structure by implementing a holistic structural pipeline. We are focusing on the polymerase, penicillin-binding protein (PBP), and the soluble lytic transglycosylase (SLT) complex from an important human pathogen P. aeruginosa. PBP was confirmed as a binding partner to SLT by co-expression and purification using a His-tag system followed up by size exclusion chromatography. After collecting a data set of a single particle electron microscopy from a negative stain we were able to create a low-resolution 3D reconstruction of the PBP-SLT complex. This 3D reconstruction brings us one step closer to elucidating the inner workings of the PBP/SLT complex machinery and how they govern peptidoglycan synthesis.

Authors names: Berliza Marie Soriano [1], Allison H. Williams [2]

Affiliations: 1. Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA, 94158, USA. 2. Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA, 94158, USA; Chan Zuckerberg Biohub, San Francisco, CA, USA.

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From: Beatriz de Pina Mariz <b.mariz@campus.fct.unl.pt>

Title: Bacterial strategies to produce branched peptidoglycan

Text: *Streptococcus pneumoniae* is considered one of the top six priority bacterial pathogens by the world health organization (WHO). Infections by pneumococcal bacteria are frequently treated with β-lactam antibiotics, such as penicillin, which inactivate penicillin-binding proteins (PBPs) and impair peptidoglycan (PGN) biosynthesis.
Penicillin resistance has emerged in *S. pneumoniae* due to the acquisition of mosaic PBPs with low affinity for β-lactams. Besides these PBPs, MurM and MurN enzymes, which produce branched PGN, are also required for the expression of pneumococcal penicillin resistance. These proteins catalyse non-ribosomal peptide synthesis reactions using aminoacyl tRNAs, with MurM linking the first amino acid (L-serine or L-alanine) of the bridge that connects different PGN stem peptides, and MurN linking the second amino acid(L-alanine).
Several bacteria from the Firmicutes phylum have been identified as having branched PGN, synthesized by MurMN-like enzymes. Given the relevance that cell wall biosynthesis has for bacterial survival and the role of PGN branching enzymes in antibiotic resistance, we decided to determine which bacterial species produce MurMN-like enzymes and have the potential to eliminate toxic mis-acylated tRNAs.
A phylogenetic analysis has identified that bacterial organisms from 37 phyla carry sequences in their genome that encode MurMN-like enzymes. As expected, the phylum with the greatest representation is the Firmicutes (53.8%). We have also found MurMN-like sequences in Actinobacteria (23.2%) and Chloroflexi (17.6%). Other less represented phyla include the Armatimonadetes (0.6%), Tenericutes (0.4%), Deinococcus-Thermus (0.4%) and the Cyanobacteria/ Melainabacteria group (0.4%). We are currently analyzing the ability of plasmid encoded, heterologous, MurMN-like proteins to branch pneumococcal PGN and to support penicillin resistance in an *S. pneumoniae* murMN mutant.

Authors names: Beatriz Mariz (1), Sérgio R. Filipe (1), Carla Gonçalves (2)

Affiliations: (1) Laboratory of Bacterial Cell Surfaces and Pathogenesis, (2) Laboratory of Yest Genomics, UCIBIO-Applied Molecular Biosciences Unit, Department of Life Sciences, NOVA School of Science and Technology, Universidade Nova de Lisboa, Monte da Caparica, Portugal.

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From: Clare Thomas <CXT255@student.bham.ac.uk>

Title: LpqF is a low molecular weight penicillin binding protein that contributes to lateral wall homeostasis in mycobacteria.

Text: Species of mycobacteria, which kill over 1.5 million people annually, have a complex cell wall contributing to their resistance to many front-line antibiotics. The inner-most layer of the cell wall is made of peptidoglycan which is comprised of a repeating disaccharide of N-acetylglucosamine and N-acetylmuramic acid. These glycan chains are cross-linked by short peptides, creating a mesh-like sacculus. To investigate genes responsible for cell envelope biosynthesis, we conducted a transposon-mediated colony morphology screen in *Mycobacterium marinum*. One of the hits was mapped to a previously uncharacterised gene predicted to encode a penicillin-binding-protein (PBP) called LpqF. Here we show that a strain of *Mycobacterium marinum* lacking *lpqF* produces longer and wider cells. We have also solved the X-ray crystal structure of M. tuberculosis LpqF showing that it is comprised of a classical penicillin-binding-protein domain and an NTF2 domain which hints at an unusual mode of auto-inhibition. Given its structural similarity to penicillinases, we sought to clarify the biochemical function of LpqF. Our assays show that LpqF is not a beta-lactamase and that the protein binds peptidoglycan directly through its NTF2 domain, likely through a distinct mechanism from other peptidoglycan-binding NTF2 domains. Together, our data show that LpqF plays an important, but previously unknown role in mycobacterial peptidoglycan biogenesis.

Authors names: Clare Thomas, Rudi Sullivan, Greg Goudge, Abigail Layton, Jiemin Chen, Ian Cadby, Gurdyal Besra, Apoorva Bhatt, Manuel Banzhaf, Andrew Lovering, & Patrick Moynihan.

Affiliations: University of Birmingham and University of Bristol.

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From: Dr Laia Pasquina Lemonche <l.pasquinalemonche@sheffield.ac.uk>

Title: Using Atomic Force Microscopy and advanced image analysis to study Peptidoglycan orientation in *B. subtilis*

Text: The current antibiotics do not work to combat common bacterial infections because the cells are developing resistance mechanisms. One of the most common antibiotic targets is the bacterial cell wall. We need to use biophysical approaches to understand the synthesis and organisation of the cell wall to find better solutions against AMR bacterial strains.

After years of research using atomic force microscopy (AFM), the conclusion is that the cell wall, composed mainly of peptidoglycan is a highly porous heterogeneous hydrogel with four different architectures [1]. In this project, we deciphered the molecular architecture (on the order of 1 nm) of *Bacillus subtilis* cell wall. Then, we applied the same methods to study genetically modified strains where their two main synthesis mechanisms were controlled with inducer conditions. *B. subtilis* is a rod and it has two types of synthesis machineries with different trajectories: the *mreB* protein moves in helical movements along the short axis of the cell and the pbp2 protein moves randomly distributed along the cell [2], when these enzymes are not in balance the cells either loose or exaggerates the rod shape. The mutant strains where the activity of *mreB* or *pbp2* proteins can be overexpressed or underexpressed were imaged with AFM with unprecedented resolution. The peptidoglycan fibres forming their internal architecture were detected and analysed with a custom-made automated image analysis routine [3]. The aim of this work is to understand the relationship between these protein trajectories, the cell shape and the peptidoglycan organisation at nanometric level.

Authors names: Laia Pasquina Lemonche (Presenter, 1,2), Oliver Meacock(3), Abimbola Feyisara-Olulana (1), Simon Foster (2), Ethan Garner (4) and Jamie Hobbs (1)

Affiliations: (1) Department of Physics and Astronomy, University of Sheffield, UK, (2) School of Biosciences, University of Sheffield, UK, (3) EPFL, Laussane, Switzerland, (4) Department of Molecular and Cellular Biology, Harvard, USA

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From: Xiaobo Zhong <x.zhong@biology.leidenuniv.nl>

Title: The stomatin-like protein StlP confines apical growth in filamentous actinobacteria under hyperosmotic stress

Text: Filamentous actinobacteria are ubiquitous in almost all soil environments, where they are frequently exposed to environmental insults. Recent work indicates that some actinobacteria have a natural ability to shed their cell wall under the influence of hyperosmotic stress, while others, such as the model organism *S. coelicolor*, appear unaffected. To enable growth, Streptomyces expands the cell wall by inserting new cell wall material at hyphal tips, which is guided by the apical localized cytoskeletal protein DivIVA. We here identify the stomatin-like protein StlP as a crucial factor for Streptomyces growth under hyperosmotic stress conditions. StlP localizes at hyphal tips, where it is important to spatially confine growth. In the absence of StlP, filaments start to branch frequently coinciding with a delocalized pattern of cell wall synthesis and an altered membrane fluidity at hyphal tips. Surprisingly, filaments of the *S. coelicolor* Δ*stlP* mutant extrude cell-wall-deficient cells, while the constitutive expression of StlP in actinobacteria that naturally form such cells blocks their extrusion. Consistent with other stomatin proteins, we show that StlP oligomerizes and interacts with several proteins involved in tip growth. Altogether, these data indicate that StlP plays a central role in coordinating tip growth by organizing cell wall synthesis in localized microdomains and imply that stomatin-like proteins provide a competitive advantage to actinobacteria that are frequently exposed to hyperosmotic stress.

Authors names: Xiaobo Zhong, Sarah Baur, Shraddha Shitut, Erik Vijgenboom, Marc Bramkap, Dennis Claessen

Affiliations: Institute of Biology, Leiden University, the Netherlands

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From: Maryam Sadat Alehashem <maryamalehashem5@gmail.com>

Title: Investigating the Role of Lytic Transglycosylases in Peptidoglycan Degradation

Text: Lytic transglycosylases (LTs) are redundant enzymes that break down the glycan strand of peptidoglycan, allowing for remodeling of the bacterial cell wall. LTs form multiprotein complexes that contribute to peptidoglycan degradation. In this study, we analyze the role of LTs in peptidoglycan degradation and examine their function in protein-protein interactions during bacterial cell wall degradation. LtgA and LtgD are lytic transglycosylases from the human pathogen Neisseria meningitidis that are involved in cell division and separation, and we have shown that they form a 149 kilodaltons peptidoglycan-degrading protein complex. We further demonstrate utilizing negative stain and cryo-electron microscopy, that there are extensive interactions between LtgD and LtgA and that their overall shape is reminiscent of Lambda complex where a dimer of LtgD is bound to a monomer of LtgA. Our study reveals LtgD and LtgA work together to enhance the efficiency of peptidoglycan degradation which is important for cell wall metabolism.

Authors names: Maryam S. Alehashem and Allison Williams

Affiliations: University of California-San Francisco, San Francisco, California, United States.

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From: Phuong Y Mai <phuong-y.mai@pasteur.fr>

Title: Untargeted metabolomics analysis of *Helicobacter pylori*

Text: *Helicobacter pylori* is a bacterial pathogen in the microbiota of more than half of the population and causes several diseases, including chronic active gastritis, gastric lymphoma, peptic ulcer, and gastric carcinoma. *H. pylori*, identified as the primary cause of gastric cancer and the second most common cause of cancer-related deaths worldwide, is a high-priority pathogen for antimicrobial development by the World Health Organization [1]. However, identifying new compounds' modes of action (MoAs) represents a major bottleneck in the drug discovery pipeline.
Our project aims to use a metabolomics approach based on LC-MS/MS to study *H. pylori* after treatment with a library of chemical compounds, including most antibiotics that target the cell wall. Since the antibiotics with similar MoAs induce similar metabolic responses, the metabolomics data will be used to predict the MoAs of potential antibiotics [2] and to develop an artificial intelligence pipeline to cluster gene networks with the MoAs of antibiotics.

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Authors names : Phuong Y MAI, Sandrine Aros-Calt, Ivo G. Boneca

Affiliations : Institut Pasteur, Université de Paris, CNRS UMR6047, INSERM U1306, Unité de Biologie et Génétique de la paroi bactérienne, Paris, France

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From: Cassandra Lenoir <cassandra.lenoir@ibcp.fr>

Title: MapZ phosphorylation and cell division of *Streptococcus pneumoniae*

Text: Bacterial cell division starts with the recruitment of a multiproteic dynamic complex called the divisome. The divisome assembly is organized and coordinated by FtsZ, a tubulin-like protein that treadmills at the cell center. The later should therefore be accurately selected to allow successful division and generation of two viable daughter cells. In Streptococcus pneumoniae, the positioning of FtsZ is controlled by MapZ, a membrane protein with a cytoplasmic domain phosphorylated by the serine-threonine protein kinase StkP. MapZ interacts with FtsZ independently of its phosphorylation state. However, MapZ phosphorylation is critical for proper cell morphogenesis and division. Therefore, the role of MapZ phosphorylation remains to be elucidated. In this aim, we constructed two MapZ probes mimicking a permanently phosphorylated or dephosphorylated form of MapZ and tracked their localization during the cell cycle. Strikingly, only the dephosphorylated probe displayed a divisome-like localization, suggesting that MapZ interacts with a cell division protein. Co-immunoprecipitation coupled to mass-spectrometry identified that MapZ interacts with the divisome protein EzrA. This interaction was confirmed both in vivo and in vitro. Three-color imaging allowed to track the localization of MapZ, EzrA and StkP in single cells and showed that dephosphorylated MapZ co-localize with EzrA but not StkP in a specific window of the cell cycle. Last, we demonstrated that the dynamics of EzrA is dependent on the phosphorylation state of MapZ using TIRF imaging. Collectively, these data show that the phosphorylation of MapZ is crucial for cell division by modulating the functioning of the divisome.

Authors names: Cassandra Lenoir, Céline Freton, Hugo Millat, Jean-Pierre Lavergne, Adrien Ducret and Christophe Grangeasse

Affiliations: CNRS - Université de Lyon

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From: Hugo Millat <hugo.millat@ibcp.fr>

Title: RopA, a novel regulatory cell division protein in the human pathogen *Streptococcus pneumoniae*

Text: Bacterial cell division is achieved by a multiprotein complex called the divisome. Within this complex protein network, Class A PBPs (aPBPs) play a key role in the biosynthesis of peptidoglycan (PG), a major component of the bacterial cell wall. *Streptococcus pneumoniae* produces three aPBPs, which are dedicated to PG repair and remodeling. Their activity should therefore be finely regulated in time and space to confer the ovoid cell shape of the pneumococcus. To date, some aPBPs regulators have been identified in pneumococcus but their mode of action remains elusive. In this work, we elucidated the function of an unknown protein, now identified as RopA (Regulator of PBPs class A) which turned out to be a core component of the *S. pneumoniae* divisome. We observed that the deletion of *ropA* induced morphological defects characterized by thinner cells and, strikingly, the formation of mini rounded cells. RopA localizes at the division site and interacts with several cell division proteins, including the three aPBPs. Remarkably, *ropA* displays a synthetic lethal interaction with some of its partner expression. Collectively, these data demonstrate that RopA is a novel morphogenic protein that links the divisome to aPBPs to control their function during cell division of *S. pneumoniae*.

Authors names: Hugo Millat, Cassandra Lenoir, Adrien Ducret and Christophe Grangeasse

Affiliations: CNRS - Université de Lyon

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From: Xin Xu <xuxin01@nus.edu.sg>

Title: Mechanistic insights into the regulation of cell wall hydrolysis by FtsEX and EnvC at the bacterial division site

Text: The peptidoglycan (PG) cell wall produced by the bacterial division machinery is initially shared between the daughters and must be split to promote cell separation and complete division. In gram-negative bacteria, enzymes that cleave PG called amidases play major roles in the separation process. To prevent spurious cell wall cleavage that can lead to cell lysis, amidases like AmiB are autoinhibited by a regulatory helix. Autoinhibition is relieved at the division site by the activator EnvC, which is in turn regulated by the ATP-binding cassette (ABC) transporter-like complex called FtsEX. EnvC is also known to be autoinhibited by a regulatory helix, but how its activity is modulated by FtsEX and the mechanism by which it activates the amidases have remained unclear. Here, we investigated this regulation by determining the structure of Pseudomonas aeruginosa FtsEX alone with or without bound ATP, in complex with EnvC, and in a FtsEX-EnvC-AmiB supercomplex. In combination with biochemical studies, the structures reveal that ATP binding is likely to activate FtsEX-EnvC and promote its association with AmiB. Furthermore, the AmiB activation mechanism is shown to involve a regulatory helix swap. In the activated state of the complex, the inhibitory helix of EnvC is released, freeing it to associate with the regulatory helix of AmiB, which liberates its active site for PG cleavage. These regulatory helices are found in many EnvC proteins and amidases throughout gram-negative bacteria, suggesting that the activation mechanism is broadly conserved and may be a good target for lysis-inducing antibiotics that misregulate the complex.

Authors names: Xin Xu[1, #], Jianwei Li[1, #], Wan-Zhen Chua[2], Martin Alcorlo[3], Jian Shi4, Juan A. Hermoso[3, *], Thomas Bernhardt[5, 6, *], Lok-To Sham[2, *], and Min Luo[1, 4, *]

Affiliations : [1] Department of Biological Sciences, Faculty of Science, National University of Singapore [2] Infectious Diseases Translational Research Programme and Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore [3] Department of Crystallography and Structural Biology, Instituto de Química-Física “Rocasolano”, Consejo Superior de Investigaciones Científicas, Madrid, Spain [4] Center for Bioimaging Sciences, Department of Biological Sciences, National University of Singapore 5 Department of Microbiology, Blavatnik Institute, Harvard Medical School, Boston, MA 02115. [6] Howard Hughes Medical Institute, Boston, United States. [#] These authors contributed equally. [*]Corresponding author.

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From: Nienke Buddelmeijer <nienke.buddelmeijer@pasteur.fr>

Title: Role of lipoprotein modification enzyme Lgt on cell shape and pathogenicity in proteobacteria

Text: Bacterial lipoproteins play an important role in bacterial physiology and virulence. They are part of protein complexes involved in cell envelope biogenesis and they function as signaling molecules of the innate immune system. The first step in the lipoprotein modification pathway is catalyzed by integral membrane enzyme Lgt that is essential for cell viability in proteobacteria such as Escherichia coli and Helicobacter pylori. We recently showed that Lgt is essential in E. coli in the absence of major lipoprotein Lpp that covalently cross-links the outer membrane to the peptidoglycan. This suggests that other outer membrane lipoproteins have an important role in viability. We further demonstrated that low levels of Lgt sustains cell growth but leads to an aberrant cell morphology due to inefficient outer membrane localization these lipoproteins. Upon extensive growth under Lgt depletion conditions so-called revertants appeared, growth of which depended on low levels of Lgt and an as yet unidentified factor. We hypothesize that alterations in metabolism compensate for a growth defect in absence of Lgt.

Authors names: Tristan RUFFIOT, Simon LEGOOD*, Ivo G BONECA and Nienke BUDDELMEIJER

Affiliations: Institut Pasteur, U. Paris Cité, Unité Biologie et génétique de la paroi bactérienne, CNRS UMR6047, INSERM U1306, F-75015 Paris, FRANCE
*current address U. Birmingham, UK

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From: Abimbola Feyisara Olulana <a.adedeji@sheffield.ac.uk>

Title: Mapping Nanoscale Phenotype in MRSA Peptidoglycan using High-Resolution Atomic Force Microscopy

Text: In *Staphylococcus aureus*, peptidoglycan (PG) is a 3D mesh-like macromolecule that surrounds the cell, playing an essential role in cell survival and stability by maintaining the shape of the cell during division and protecting the cell against its internal turgor pressure. Its biosynthesis proteins known as penicillin-binding proteins (PBPs) are the targets of ß-lactams antibiotics. The chemical composition of PG is well understood but little is known about its 3-D nanoscale architecture. In our previous works, we have shown the detailed molecular architecture of methicillin-sensitive *Staphylococcus aureus* (MSSA) peptidoglycan and the nanostructural changes on the PG when treated with antibiotics1,2. In this study, we have been focusing on the peptidoglycan associated with the methicillin-resistant *S. aureus* (MRSA) and exploring the following questions; 1) what are the imprints of resistance on the MRSA PG architecture? 2) Is there differences in PG architecture when MRSA cell is treated with methicillin? 3) Are these differences related to the level of resistance—high or low? 4) Can we genetically switch ON and OFF these nanoscale architectural phenotypes without using antibiotics? And 5) What happens to the PG architecture when one of the essential PBPs is turned OFF—say for example transpeptidase activity of PBP1 or PBP2? Addressing these questions could give an understanding of how MRSA evades antibiotic-induced cell death.
To this end, we use high-resolution atomic force microscopy (AFM) to map and capture the nanoscopic phenotype induced by antibiotic-treatment and genetic manipulation on the MRSA peptidoglycan. I look forward to presenting our discoveries and results at the symposium.

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Authors names: Abimbola Feyisara Olulana[1,3], Katarzyna Wacnik[2,3], Bohdan Bilyk[2,3], Laia Pasquina-Lemonche[1,3], Mariana Tinajero-Trejo[2,3], Simon J. Foster[2,3], Jamie K. Hobbs[1,3]

Affiliations: 1. Department of Physics and Astronomy, University of Sheffield, Sheffield, UK. 2. School of Biosciences, University of Sheffield, Sheffield, UK. 3. The Florey Institute for Host-Pathogen Interactions, University of Sheffield, Sheffield, UK.

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From: Jianwei Li <ljw26@nus.edu.sg>

Title: Structure insights into the regulation of the Cell Division by the FtsEX system

Text: The FtsEX complex plays a crucial role in regulating peptidoglycan (PG) degradation for cell division in bacteria. In certain genera, the FtsEX system directly activates PG-hydrolases, but the underlying mechanism is not well understood. Our investigation into the non-canonical regulator FtsEX in a pathogenic bacterial species revealed high basal ATPase activity. By using cryo-EM structures, we have uncovered details about the signal transduction mechanism that leads to the activation of NlpC-like protein. Our findings suggest that NlpC-like protein is recognized through a "Match and Fit" mechanism, resulting in an asymmetric rearrangement of the extracellular domains of FtsX and a unique binding mode of NlpC-like protein. our study sheds light on the molecular mechanisms of FtsEX and NlpC-like protein regulation in the context of a critical human pathogen and could inform the development of drugs targeting PG remodeling.

Authors names: Jianwei Li[1], Xin Xu[1], Jian Shi[2], Juan A. Hermoso[3], Lok-To Sham[4], and Min Luo[1,2]

Affiliations: 1 Department of Biological Sciences, Faculty of Science, National University of Singapore. 2 Center for Bioimaging Sciences, Department of Biological Sciences, National University of Singapore. 3 Department of Crystallography and Structural Biology, Instituto de Química-Física “Rocasolano”, Consejo Superior de Investigaciones Científicas, Madrid, Spain. 4 Infectious Diseases Translational Research Programme and Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore

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From: Kelly Rosch <kmr257@cornell.edu>

Title: *Vibrio cholerae* endopeptidase ShyA mediates adaptation to osmotic stress via its LysM domain

Text: The bacterial cell wall is a covalently closed meshwork that protects cells from osmotic lysis and maintains cell shape. The cell wall is made of peptidoglycan (PG), which consists of polysaccharide strands that are connected via short peptide crosslinks. As bacteria elongate and divide, PG is constantly remodeled through the activity of both cell wall synthases and hydrolases. PG hydrolysis is inherently dangerous to the cell, as unregulated PG cleavage activity results in cell lysis. Therefore, synthesis and hydrolysis are presumably tightly coupled to prevent structural integrity failure; but how this balance is maintained remains poorly understood. One such PG hydrolase, the endopeptidase ShyA, is regulated through conformational switching between an open (active) conformation and a closed (inactive) conformation. Despite exhibiting potent endopeptidase activity, ShyA does not cause cell lysis when overexpressed. These observations led us to hypothesize that ShyA is produced in the closed (inactive) conformation, and is activated by local PG stretching. To investigate this, I targeted potential PG-binding residues in the LysM carbohydrate-binding domain using site-directed mutagenesis and assessed cell viability and morphology on salt-free medium, where we expect enhanced PG stretching due to increased turgor pressure. Indeed, the LysM domain of ShyA is required for adaptation to osmotic stress, consistent with a model where PG binding is required for optimal ShyA function in fluctuating environments.

Authors names: Kelly Rosch, Tobias Doerr

Affiliations: Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca, NY

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From: Tiffany Tsui <tttsui@indiana.edu>

Title: Negative regulation of MurA and MurZ activity by IreB(*Spn*) underlies GpsB and StkP essentiality in *Streptococcus pneumoniae* D39

Text: GpsB links peptidoglycan synthases to other proteins that determine the shape of the respiratory pathogen *Streptococcus pneumoniae* (pneumococcus; *Spn*) and other low-GC Gram-positive bacteria. GpsB is also required for phosphorylation of proteins by the essential StkP(*Spn*) Ser/Thr protein kinase. Here we report three classes of frequently arising chromosomal duplications (≈21-176 genes) containing *murZ* (MurZ-family homolog of MurA) or *murA* that suppress Δ*gpsB* or Δ*stkP*. These duplications arose from three different repeated sequences and demonstrate the facility of pneumococcus to modulate gene dosage of numerous genes. Overproduction of MurZ or MurA alone or overproduction of MurZ caused by Δ*khpAB* mutations suppressed Δ*gpsB* or Δ*stkP* phenotypes to varying extents. Δ*gpsB* and Δ*stkP* were also suppressed by MurZ amino-acid changes distant from the active site, including one in commonly studied laboratory strains, and by inactivation of the homolog of IreB(ReoM). IreB(*Spn*) is phosphorylated by StkP at a single Thr residue in ≈90% of WT cells. Co-IP and B2H assays showed that IreB(*Spn*) interacts directly with MurZ and MurA, and forms complexes with StkP and GpsB. In contrast to the *Listeria* homolog, IreB(*Spn*) does not regulate MurZ and MurA amounts, and Δ*clpP* or Δ*clpC* does not suppress Δ*gpsB* or Δ*stkP*. Phosphomimetic changes of the phosphorylated Thr in IreB(*Spn*) prevented interaction with MurZ, were viable, and suppressed Δ*gpsB*, whereas phosphoablative changes affected cell shape, allowed MurZ interactions, were viable in a *gpsB*$^{+}$ strain, but inviable in a Δ*gpsB* mutant. Unlike in other Gram-positive bacteria, MurZ is predominant to MurA in pneumococcal cells. These results support a model in which regulation of MurZ and MurA activity, likely by IreB(*Spn*), is the only essential requirement for StkP-mediated protein phosphorylation in exponentially growing D39 pneumococcal cells.

Authors names: Ho-Ching Tiffany Tsui[1†], Merrin Joseph[1†], Jiaqi J. Zheng[1], Amilcar J. Perez[1], Irfan Manzoor[1], Britta E. Rued[1], John D. Richardson[1], Berenice Furlan[3], Pavel Branny[2], Linda Doubravová[2], Orietta Massidda[3], and Malcolm E. Winkler[1].

Affiliations: [1]Department of Biology, Indiana University Bloomington, Bloomington, IN, USA; [2]Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic; [3]Department of Cellular, Computational, and Integrative Biology, University of Trento, Italy. [†]Contributed equally.

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From: Wilhelm Linus <linus.wilhelm@ibcp.fr>

Title: Role of CozEa in *Streptococcus pneumoniae*: from peptidoglycan synthesis to membrane homeostasis

Text: The cell envelope of the bacterial pathogen *Streptococcus pneumoniae* consists of a single membrane surrounded by a thick layer of an intricate glycan network called the peptidoglycan (PG). Both the PG and the membrane are decorated by anionic polymers, which are either covalently linked to the PG (wall teichoic acid, WTA) or attached to a glycolipid embedded in the cell membrane (lipoteichoic acid, LTA). Thus, the assembly of a native cell wall requires a tight coordination between PG and TA synthesis. However, the link between these two processes remains elusive. In this context we re-evaluated the function of cozEa, an essential gene encoding a protein initially described as directing the activity of a PG synthase. Indeed, the absence of *cozEa* was shown to affect cell growth and to lead to severe morphological defects. However, and surprisingly, we found that the composition of the PG produced in absence of cozEa was similar to that of WT cells. In parallel, we observed that the deletion of cozEa led to the presence of suppressor mutations. Using whole-genome sequencing, we found that all the suppressor mutations mapped to a single gene encoding a protein involved in the biosynthesis of glycolipids, including the lipid anchor of the LTA. Our experiments however showed that glycolipid biosynthesis was not affected by CozEa depletion. Remarkably, we found that altering the membrane fluidity of a CozEa depletion mutant partially restored a wild type growth and morphology. In addition, Western blot monitoring of LTA assembly revealed differential patterns upon CozEa depletion. Collectively, our results show that CozEa is likely required for membrane homeostasis and teichoic acid biosynthesis.

Authors names: Linus Wilhelm[1], Maria Disen-Barbuti[2], Gro-Anita Stamsas[2], Marie-Pierre Chapot-Chartier[3], Daniel Straume[2], Morten Kjos[2], Adrien Ducret[1], Christophe Grangeasse[1]

Affiliations: [1] Molecular Microbiology and Structural Biochemistry, UMR 5086, Université de Lyon, CNRS, Lyon, France. [2] Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway [3] Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en- Josas, France.

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From: Yousef Alanazi <Y.N.N.Alanazi2@newcastle.ac.uk>

Title: Modulation of Bacterial Cell Surface Charge and CAMPs Resistance: The Role of FmtA, Flp, and PbpX in Teichoic Acid D-Alanylation in *S. aureus* and *B. subtilis*

Text: The primary innate immune system defence against invasive microbial infections is provided by cationic antimicrobial peptides (CAMPs) (Zasloff, 2002). By modifying negatively charged teichoic acids (TAs) with D-alanine(D-Ala), Gram-positive bacteria are able to withstand CAMPs (Neumann et al., 2016). In *Staphylococcus aureus* and *Bacillus subtilis*, *dlt* operon is responsible for activating and transferring the D-alanine from the cytosol to the outer membrane and then attaching it to the Lipoteichoic acid (LTA) (Neumann et al., 2016) (Percy, M. G., & Gründling, A. (2014). FmtA in *S. aureus* was identified as a factor that affects methicillin resistance in *S. aureus* strains (Bertsche et al., 2013). FmtA was shown to interact with teichoic acids and to localize to the cell division septum (Bertsche et al., 2013). FmtA was later shown to hydrolyze the ester bond between D-Ala and the backbone of teichoic acids, which are polyglycerol-phosphate or polyribitol-phosphate polymers found in the *S. aureus* cell envelope. FmtA contains two conserved motifs found in serine active-site penicillin-binding proteins (PBPs) and β-lactamases (Bertsche et al., 2013). Flp in *S. aureus* and PbpX in *B. subtilis* is an analogous proteins to the FmtA in *S. aureus*, *pbpX* that contains contain similar motifs, but its function was unclear. Our recent results points to PbpX mediating the transfer of D-ala from LTA to WTA, the missing step in the D-alanylation pathway. In addition, our analysis indicated that *flp* of *S. aureus* does the same function, whereas fmtA has the opposite function, removing D-ala from teichoic acids.

We first evaluated the effect of deleting FmtA, Flp, or PbpX on bacteria cell surface charge, which is presumed to be influenced by the LTA and Wall Teichoic Acids (WTA) D-alanylation. In *S. aureus ΔfmtA* shifts the cell surface charge slightly to the positive during exponential growth and that increases significantly during the stationary phase. In contrast, *Δflp* shifts the cell surface charge to the negative during both exponential and stationary phases. Analysis of the *ΔpbpX* mutant in *B. subtilis* indicated a similar increase in negative surface charge. We then evaluated the sensitivity of these strains to the cationic antimicrobial peptide CAMA. The *ΔfmtA* was slightly less sensitive compared to the wild type. However, the *Δflp* and *ΔpbpX* strains become more sensitive compared to the wild type. Finally, we assessed the amount of D-alanine associated with WTA and LTA in all strains and compared them to the wild type and we found that in *ΔfmtA*, the D-alanine associated with WTA and LTA is slightly higher than the wild type. Interestingly, the D-alanine that is attached to theWTA was significantly lower than that determined for equivalent samples of the wild type, whereas for LTA the D-ala abundance was slightly higher than the wild type. In a previous model published by Werner Fischer and colleagues, they showed that LTA is first D-alanylated and then the D-ala was transferred to the WTA (Fischer et al., 2021). Our results indicate that Flp in *S. aureus* and PbpX of *B. subtilis* provide this function. Our findings indicate that bacteria cell surface charge and resistance to CAMPs is mediated by modification of both the WTA and LTA, although it seems that modification of the LTA is more biologically significant.

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Authors names: Yousef Alanazi, Richard Daniel

Affiliations: Centre for Bacterial Cell Biology, Newcastle University Biosciences Institute.

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From: Mariana Tinajero Trejo <m.tinajero-trejo@sheffield.ac.uk>

Title: The role of *Staphylococcus aureus* cell division proteins DivIB, DivIC and FtsL

Text: Cell division is a fundamental, multi-stage process that requires the formation of dynamic macromolecular, protein interactions. Many cell division proteins are still of unknown function but conserved across species, alluding to a common mechanism of division. A case in point is the transmembrane trimeric complex involving DivIB, DivIC and FtsL in Gram-positives and their orthologues in Gram-negatives. By constructing individual conditional lethal mutants of *divIB*, *divIC* and *ftsL* in *S. aureus*, we have begun to determine their essentiality and unveil independent, hierarchal roles in cell division. A better understanding of the cell division mechanisms in *S. aureus* and other pathogenic microorganisms can provide possibilities for the development of new, more effective treatments for bacterial infections.

Authors names: Mariana Tinajero-Trejo[1,2], Oliver Carnell[1,2], Laia Pasquina-Lemonche[2,3], Lucia Lafage[1,2], Jamie K Hobbs[2,3] and Simon J Foster[1,2*]

Affiliations: 1 School of Biosciences, University of Sheffield, Sheffield, UK. [2] The Florey Institute for Host-Pathogen Interactions, University of Sheffield, Sheffield, UK [3] Department of Physics and Astronomy, University of Sheffield, Sheffield, UK

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From: Suraj Kumar Meher <surajkumar@ccmb.res.in>

Title: Understanding the mechanism of cell wall expansion in *Escherichia coli*

Text: Most bacteria are surrounded by a mesh-like macromolecule called peptidoglycan (PG) to protect them from osmotic lysis and harsh external environmental conditions. Several glycan polymers with alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues linked through β(1→4) glycosidic bonds form the backbone of this macromolecule. Each NAM residue is covalently linked to a stem peptide chain which in turn forms cross-links with adjacent peptides of neighbouring glycan strands to generate a mesh-like sacculus encasing the bacterial cytosolic membrane. As PG is a continuous mesh, it needs to be cleaved for its expansion during growth of a cell. In E. coli it is known that hydrolysis of cross-links between the peptide chains by D,D-endopeptidases is essential to make space for the insertion of nascent PG strands. Here, we find that cleavage of the stem peptide itself generates space in the PG sacculus to contribute to its expansion. We find that overexpression of a PG hydrolytic enzyme, RipA from *Mycobacterium tuberculosis* rescues the cell lysis of *E. coli* mutants lacking the essential cross-link specific endopeptidases. Using both *in vivo* and *in vitro* experiments we show that the activity of a D,L-endopeptidase, RipA that cuts within the peptide chain of intact *E. coli* PG sacculi is the basis for the rescue of the mutants lacking the essential D,D-endopeptidases. In addition, CwlO, a D,L endopeptidase from *B. subtilis*, also compensated the absence of cross-link specific endopeptidases in *E. coli*. Overall, our results suggest that opening the PG mesh by cleavage within the stem peptides (and not strictly the cleavage of cross-links), is sufficient to generate space for the new glycan strand insertion for successful expansion of PG in *E. coli*.

Authors names: Suraj Kumar Meher, Manjula Reddy*

Affiliations: CSIR-Centre for Cellular and Molecular Biology, Hyderabad, 500007 India, Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, 201002 India

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From: Sebastien Rigo <sebastien.rigo@roche.com>

Title: Essentiality of lipooligosacharride transport in *A.baumannii*

Text: Carbapenem resistant *A.baumannii* is ranked as critical by World Health Organization with urgent need for novel antibiotics. LipoOligoSacharride (LOS) synthesis and transport are attractive target pathway but their essentiality in *A.baumannii* is not clear. Previous work identified differences in the ability to generate LOS deficient mutants in two *A.baumannii* ATCC strains, 17978 and 19606. This raises the question of how clinical isolates would behave under the inhibition of LOS pathway.
Disruption of the phospholipid homeostasis machinery PldA (phospholipase A1) in combination with mutation(s) in *lpxA*, *lpxC* or *lpxD* result in resistance to the last line-antibiotic colistin in *A.baumannii*.
Therefore, the regulation of PldA is key to survive in vitro under LOS loss. The deletion of the phospholipid degradation enzyme PldA allows us to delete genes involved in the LOS transport and LOS synthesis. As target validation is important for antibiotic research and development, in this work we explored the fitness, virulence and antibiotic susceptibility of *A.baumannii* ATCC 17978 Δ*pldA* in the absence of *lptF* or *lpxC*

Authors names: Sebastien Rigo, William Riboulet, Pauline Misson, Sarah Gartenmann, Severine Louvel, Andrej Trauner, Valentine Rebischung, Christoph Bieniossek, Claudia Zampaloni and Andreas Haldimann

Affiliations: Roche Pharma Research and Early Development, Immunology, Infectious Diseases and Ophthalmology Disease Therapeutic Area, Roche Innovation Center, Basel, F.Hoffmann-La Roche Ltd, Grenzacherstrasse 124, 4070 Basel, Switzerland

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From: Alaa Aljohani <a.a.aljohani2@newcastle.ac.uk>

Title: Peptidoglycan Dynamics in *Bacillus subtilis*

Text: The cell envelope is an essential component of bacterial cells, responsible for maintaining their structural integrity and protecting them from rupturing due to internal osmotic pressure. The main structural element of the cell envelope is the peptidoglycan, which is a matrix composed of glycan polymers cross-linked by peptide side chains. In Gram-positive bacteria, the cell envelope is dynamic, with old outer material degrading while new peptidoglycan is synthesized near the cell membrane. To prevent the degradation of newly synthesized peptidoglycan or the random breakdown of the peptidoglycan matrix, there needs to be coordinated regulation of the secreted peptidoglycan hydrolases, which are enzymes responsible for degrading the peptidoglycan. Autolytic enzymes are collectively though to provide this function, and their substrate specificity has been assigned by similarity to characterized enzymes. However, their specific roles are unclear and functional redundancy seem to be present. Using Bacillus subtilis as a model system and a combination of genetic and cell biological methods we have obtained an insight into the functional redundancy of the autolysins. In addition, from this and other data we propose a mechanism by which the turnover of peptidoglycan through post-synthesis "maturation" of the peptidoglycan as a way to prevent futile synthesis and degradation.

Authors names: Alaa Aljohani

Affiliations: Newcastle University

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From: Céline Freton <celine.freton@ibcp.fr>

Title: Molecular Basis of the final step of cell division in *Streptococcus pneumoniae*

Text: The assembly of the peptidoglycan mesh, the main component of bacterial cell wall, is an essential process to maintain the cell shape and integrity. It requires several synthases such as PBP and SEDS proteins but also several hydrolases that are essential for its remodeling. In *Streptococcus pneumoniae*, the N-acetylglucosaminidase LytB is essential at the late step of cell division to allow the final separation of the two daughter cells. The deletion of lytB leads thus to long chains of cells linked by the tip of the new cell pole. Recently, LytB activity was found to be regulated by StkP, the receptor serine/threonine-kinase governing pneumococcal cell division. However, both the catalytic mechanism of LytB and the molecular mechanism underlying StkP-mediated regulation remain largely unknown.
In this study, we report an integrative structure-function analysis providing a comprehensive understanding of the mode of action of LytB and its regulation by StkP. The crystallographic structures of the catalytic domain of LytB in complex with synthetic substrates supported by pneumococcal cell imaging reveal the catalytic mechanism of LytB. On the other hand, the same methodological approach allows to pinpoint the differentiate roles of the huge and unique choline-binding module of LytB demonstrating that a specific domain binds wall-teichoic acid to allow cell separation whereas the other domains are involved in the regulation by StkP. Collectively, these data allow to draw a model depicting the final step of cell separation during pneumococcal cytokinesis.

Authors names: Siseth Martínez-Caballero (1), Céline Freton (2), Rafael Molina (1,), Sergio G. Bartual (1), Virginie Gueguen-Chaignon (3), Chryslène Mercy (2), Federico Gago (4), Kiran V. Mahasenan (5), Inés G. Muñoz (6), Mijoon Lee (5), Dusan Hesek (5), Shahriar Mobashery (5), Juan A. Hermoso (1) and Christophe Grangeasse (2)

Affiliations: (1). Department of Crystallography and Structural Biology, Instituto de Química-Física “Rocasolano”, Consejo Superior de Investigaciones Científicas, Madrid, Spain. (2). Molecular Microbiology and Structural Biochemistry, UMR 5086, Université de Lyon, CNRS, Lyon, France. (3). SFR Biosciences, Université de Lyon, CNRS UAR3444, ENS de Lyon, Inserm US8, Lyon, France. (4). Department of Biomedical Sciences & Instituto de Química Médica-CSIC Associated Unit, School of Medicine and Health Sciences, University of Alcalá, E-28805 Alcalá de Henares, Spain (5). Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556, United States. (6). Structural Biology Programme, Spanish National Cancer Research Center (CNIO), 28029 Madrid, Spain.

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From: Andreia Duarte <andreia.duarte@itqb.unl.pt>

Title: Exploring the roles of *Staphylococcus aureus* PknB in cell division, morphology and antibiotic resistance

Text: *Staphylococcus aureus* is a life-threatening bacterium since methicillin-resistant *S. aureus* (MRSA) strains are resistant to virtually all beta-lactam antibiotics. This extensive resistance is due to the acquisition of a low affinity penicillin-binding protein - PBP2A.
However, several other proteins are also required for *S. aureus* beta-lactam resistance. One of this is the eukaryote-like serine/threonine kinase (eSTK) PknB, an auxiliary factor that contributes to beta-lactam resistance. Together with its cognate phosphatase, Stp, PknB is involved in the regulation of crucial cellular processes such as purine biosynthesis, central metabolism, cell wall synthesis and autolysis, cell division, virulence, antibiotic resistance as well as antibiotic persistence.
Interestingly, the well-studied MRSA *S. aureus* strains COL and JE2 have different alleles of *pknB*. While JE2 encodes a full-length PknB, COL encodes a truncated protein due to a G-to-T transversion at nucleotide 1165 which introduces a stop codon in *pknB*.
To provide new insights into the cellular role of PknB we have studied its localization and we have constructed JE2 and COL mutants lacking *pknB* or encoding truncated or full-length PknB. These mutants were studied in terms of antibiotic resistance, cell wall composition, cell cycle progression.

Author names: Duarte AM, Brito AD and Pinho MG.

Affiliations: Laboratory of Bacterial Cell Biology, Instituto de Tecnologia Química e Biológica, Universidade NOVA de Lisboa, Oeiras, Portugal

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From: Daniel Karadzas <daniel.karadzas@warwick.ac.uk>

Title: Investigating the interaction of RodA with lipid II in a range of pathogenic bacteria

Text: Novel antibiotics are desperately needed as multi-drug resistant bacteria become increasingly prevalent. The peptidoglycan glycosyltransferase, RodA, is recognized as a target with vast potential for novel antibiotics. The interaction of RodA with its substrate is necessary to form the glycan chains of peptidoglycan. Although the mechanism of *E.coli* RodA has been recently elucidated, little is known about the mechanism of RodA in other species. Here, the interaction of RodA and lipid II is investigated in a range of pathogenic bacteria using coarse-grain molecular dynamics simulations of RodA models. It is shown that the proposed binding sites of E. coli RodA with lipid II are conserved across all species. This extends our understanding of the mechanism of RodA beyond *E.coli*. This knowledge could be exploited for the structure-guided development of novel antibiotics which block lipid II interaction.

Authors names:

Affiliations:

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From: Prabhjot Kaur <prabhjot.Kaur@mail.huji.ac.il>

Title: Cell Wall Re-modelling Enzymes mediate Toxic Protein Delivery to Recipient Bacteria

Text: Bacteria employ an array of mechanisms to compete amongst each other for food and resources, yet mechanisms of bacterial competition still remain largely enigmatic. We have previously discovered that the soil bacterium *Bacillus subtilis* (Bs) competitively inhibits the growth, and procures nutrition from, the closely related bacterium *Bacillus megaterium* (Bm), providing an attractive model to study bacterial competition. The growth inhibition by Bs was found to be mediated by a tRNase called WapA, delivered from Bs to Bm in a contact-dependent mechanism. To understand WapA delivery, biosynthesis, regulation, and prey recognition mechanisms, we designed a genetic screen searching for Bs mutants that are deficient in WapA delivery and subsequently allowing Bm growth. To this end, we employed the single gene knockout library of Bs, comprising ~4000 mutants, as the WapA donor strain. This high throughput screen uncovered 45 candidate mutants, which were defective in killing the prey. The list of candidates comprised genes involved in signaling, transcription, transport, cell wall remodeling, prophage genes, flagellar genes, and genes of unknown functions. One of the most significant candidates was the *cwlO* gene, encoding a crucial D,L cell wall endopeptidase, along with genes encoding its cofactors, FtsE and SweD. These proteins are known to be involved in peptidoglycan remodeling to facilitate cell elongation (ref). We observed that WapA is profoundly displayed on the cell wall of the mutant cells, but remarkably, it becomes trapped within the thick mutated cell wall failing to be properly released and transferred to the recipient Bm. We propose that cell wall modulation by the CwlO pathway, enables proper presentation and functionality of multiple cell wall associated proteins.

Authors names: Prabhjot Kaur[1], Ilan Rosenshine[1], Sigal Ben Yehuda[1]

Affiliations: 1Department of Microbiology and Molecular Genetics, IMRIC, Faculty of Medicine, The Hebrew University of Jerusalem, Israel

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From: Patricia Reed <preed@itqb.unl.pt>

Title: A CRISPRi-based genetic resource to study essential *Staphylococcus aureus* genes

Text: We have optimized a CRISPR interference system to facilitate gene knockdown in the gram-positive bacterial pathogen *Staphylococcus aureus*. For this, we used a CRISPRi system derived from *Streptococcus pyogenes* which requires the co-expression of the *dcas9* gene encoding a catalytically inactive Cas9 protein and a customizable single guide RNA (sgRNA). In the system described in this work, *dcas9* is expressed from a single copy in the chromosome of methicillin resistant S. aureus (MRSA) strains COL or JE2, under the control of a tightly regulated promoter inducible by anhydrotetracycline. The sgRNAs are expressed from a replicative plasmid under the control of a constitutively active promoter. This system enables high-efficiency, inducible, knockdown of both essential and nonessential genes and was used for the construction of the Lisbon CRISPRi mutant library (LCML) of 261 strains, in the background of JE2, containing sgRNAs targeting 211 essential genes/operons. This library allows the study of the function of essential S. aureus genes and is complementary to the Nebraska Transposon Mutant Library which consists of nearly 2000 strains, each containing a transposon insertion within a non-essential gene. Together the two libraries should facilitate the study of S. aureus pathogenesis and biology.

Authors names: Patricia Reed, Moritz Sorg, Dominik Alwardt, Lúcia Serra, Helena Veiga, Simon Schäper, Mariana G. Pinho

Affiliations: Bacterial Cell Biology, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Oeiras, Portugal

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From: Becca Bailey <becca.bailey@warwick.ac.uk>

Title: MreB: bacterial actin homologue interactions with the membrane in molecular dynamics simulations

Text: Antimicrobial resistance is a constantly growing global health concern. A better understanding of essential bacterial processes will facilitate structure-based design of new antibiotics. MreB is a bacterial actin homologue which defines the shape of rod-shaped bacteria by coordinating peptidoglycan synthesis. MreB forms filaments that interact with the cytosolic leaflet of the cell membrane as well as membrane proteins and other cytosolic proteins. In this study, interactions of MreB with the membrane are investigated by molecular dynamics simulations, and reveal cardiolipin recruitment and membrane bending towards the peptidoglycan caused by MreB filaments. Simulations with MreB homologues revealed conservation of bending across species, and MreB mutants showed redundancy of residues that cause bending. A cardiolipin concentration dependent effect on bending is also observed, suggesting an importance of cardiolipin in this system.

Authors names: Becca Bailey, Phillip Stansfeld

Affiliations: University of Warwick

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From: Berenice Furlan <berenice.furlan@unitn.it>

Title: Peptidoglycan analysis of *Streptococcus pneumoniae* serotype 19A isolates belonging to the same clonal complex with different susceptibility to β-lactams in the presence or absence of cefotaxime

Text: *Streptococcus pneumoniae*, the pneumococcus, is a clinically relevant human respiratory pathogen responsible for more than a million deaths per year worldwide. β-lactam antibiotics have been employed to treat infections caused by *S. pneumoniae* for decades. However, β-lactam resistant *S. pneumoniae* strains, frequently expressing multiple antibiotic resistance phenotypes, have increased dramatically since the 1980s and pose serious problems in the treatment of infections. The Hungary^{19A}-6 clone, a multiple antibiotic-resistant strain with unusually high-levels of β-lactam resistance was prevalent in Hungary during the 1990s. This clone spread also in the Czech Republic and Slovakia but not significantly to other areas. In this work, we analyzed the peptidoglycan (PG) composition of two serotype 19A strains from Hungary, strains Hu15 and Hu17 as well as of two Hu15 derivatives in which the mosaic *pbp2x* and *pbp2x* together with *pbp1a* were introduced. Both Hu15 and Hu17 isolates are members of the same clonal complex, ST226, a single-locus variant of the representative strain HUN663 of the Hungary^{19A}-6 clone. Hu17 exhibits high-level penicillin resistance, whereas Hu15 is penicillin sensitive. This unique situation allowed us to study the development of β-lactam resistance and to understand the impact of the main resistance determinants on the PG composition and, ultimately, on the resistant phenotype. The PG of four *S. pneumoniae* strains was extracted from culture grown in the presence or the absence of cefotaxime and its composition was analyzed by high-performance liquid chromatography (HPLC) and mass spectrometry (MS). Growth and viability under the same conditions were also determined. Compared to the laboratory strain R6, the Hu strains had a PG profile showing an enrichment in branched *vs* linear peptides, consistent with the presence of a mosaic *murM* allele that is responsible for the synthesis of branched muropeptides and complex dimers, mostly bound through Ala-Ala crosslinks. These indirect cross-links are necessary for β-lactams resistance but not sufficient for β-lactams resistance in the absence of the mosaic PBP determinants. Moreover, as expected, the fraction of pentapeptides increased in all strains upon cefotaxime treatment in comparison to the condition of growth in absence of antibiotic. Finally, a bacteriostatic effect was observed upon treatment of the Hu strains with 1× MIC of cefotaxime with different behaviour depending on the specific strain. The results support that the Hu strains change their PG composition upon cefotaxime treatment, including decreasing their cross-linked muropeptides and increasing fraction of pentapeptides, supporting the notion that cefotaxime selectively binds PBP2x and PBP3. Our results also show that β-lactam resistance in the pneumococcus is a complex event that involve modifications in factors other than PBPs and they highlight that a specific genetic background is required to express full resistance.

Authors names: Dalia Denapaite[1], Berenice Furlan[1], Michael Bernard Whalen[2], Jacob Biboy[3], Joe Gray[3], Waldemar Vollmer[3], Orietta Massidda[1]

Affiliations: [1]Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, Trento, Italy; [2]Institute of Biophysics, National Research Counsil (CNR), Trento, Italy, [3]Centre for Bacterial Cell Biology, Biosciences Institute, Newcastle University, Newcastle upon Tyne, United Kingdom.

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From: Choo Pei Yi <pychoo@ntu.edu.sg>

Title: Shortened peptidoglycan cross-bridge in the absence of FemB causes division defects in *Enterococcus faecalis*

Text: Surface proteins of Gram-positive bacteria are anchored to the peptidoglycan cross-bridge by sortases. The length of cell wall cross-bridge varies across different bacteria species and contributes to the architecture of the cell envelope. For example, the cross-bridge of ovococcal shaped bacteria is predominantly made up of mono or dipeptides, while the peptidoglycan cross-bridge of coccoid bacteria such as *Staphylococcus aureus* is made up of five glycines. In *Entercoccus faecalis*, the cell wall cross-bridge is made of two L-Alanine synthesized by FemA and FemB. To investigate the effects of a shortened cross-bridge in *E. faecalis* in cell envelope biogenesis, we knocked out FemB, a protein responsible for the addition of the second and final L-Alanine peptide onto the *E. faecalis* cross-bridge. As predicted we observed that FemB mutants exhibit altered cell morphology, where cells appear larger and more spherical. Interestingly, *femB* mutants also exhibit a growth defect. The cell division pattern of FemB mutants deviates from the wild type parallel division pattern, displaying irregular septation, dysregulated peptidoglycan organization, and loss of symmetry between daughter cells. To gain insight into the mechanism of the growth defect and altered peptidoglycan patterning, we generated *femB* suppressor mutants that overcame the growth defect. Whole genome sequencing of suppressor mutants and subsequent analysis revealed mutations in genes involved in cell wall stress and cell wall synthesis, suggesting a role in compensating for the division defects in femB mutants due to shortened peptidoglycan cross-bridge.

Authors names: Pei Yi Choo, Stephane Mesnage, Kimberly A. Kline

Affiliations: 1. Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University, Singapore, 2. School of Biosciences, University of Sheffield, Sheffield, S10 2TN, UK 3. Department of Microbiology and Molecular Medicine, University of Geneva, Geneva, Switzerland

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From: Ana Paiva <ana.oliveira-paiva@i2bc.paris-saclay.fr>

Title: L,D-transpeptidases and peptidoglycan cross-linking in *Clostridioides difficile*

Text: *Clostridioides difficile* is an anaerobic gram-positive bacterium that is able to form spores. C. difficile infection (CDI) is the major cause of antibiotic-associated diarrhea in hospitalized patients, and can lead to severe diseases, such as toxic megacolon and even death. CDI occurs with the presence of the spores within the gut, and upon disruption of the microbiota due to antibiotic treatment, the spores are able to germinate and colonize the gastrointestinal tract. β-lactams antibiotics are the most commonly prescribed class of antibiotics, and have been recognized as major cause for the development of CDI. However the underlying mechanisms of resistance are currently unknown.
It has been previously shown that *C. difficile* contains an unusual content of peptidoglycan 3-3 crosslinks, approximately 75%, and the proportion increases in the presence of ampicillin. Crosslinks 3→3 are generated by the activity of L,D-transpeptidases (Ldt) that are structurally distinct of the penicillin-binding proteins (PBP). In C. difficile, three Ldt paralogues with the conserved ykuD catalytic domain, designated Ldt1, Ldt2 an Ldt3 were identified and the implication of Ldt1 and Ldt2 in the 3→3 link formation was demonstrated.
In this study we aimed to understand the role of the Ldt on *C. difficile* peptidoglycan assembly and in antibiotic resistance. No major differences in peptidoglycan composition were observed between different *C. difficile* ribotypes. We built a triple mutant of the genes encoding the putative Ldt's. Surprisingly, 3→3 link formation was not abolished in vegetative cells as in spores, that also contain a small fraction of 3→3 links. Work is in progress to tentatively identify the remaining Ldt(s) with an undescribed catalytic domain. To explore the function of the Ldt's we analyzed *C. difficile* peptidoglycan in different medium and antibiotic growth conditions. We hypothesized that *ldt1* has a role during *C. difficile* infection and *ldt3* is involved in sporulation.

Authors names : Ana Oliveira Paiva (1), Pascal Courtin(2), Glenn Charpentier(1), Marie-Pierre Chapot-Chartier(2), Johann Peltier(1)
Affiliations : 1-Université Paris-Saclay, CEA, CNRS, I2BC, France; 2-Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, France.

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From: Josue Flores Kim <Josue.FloresKim3@umassmed.edu>

Title: WhyD tailors surface polymers to prevent premature bacteriolysis and direct cell elongation in *Streptococcus pneumoniae*

Text: Penicillin and related antibiotics disrupt cell wall synthesis in bacteria causing the downstream misactivation of cell wall hydrolases called autolysins to induce cell lysis. Despite the clinical importance of this phenomenon, little is known about the factors that control autolysins and how penicillins subvert this regulation to kill cells. In the pathogen *Streptococcus pneumoniae* (Sp), LytA is the major autolysin responsible for penicillin-induced bacteriolysis. We recently discovered that penicillin treatment of Sp causes a dramatic shift in surface polymer biogenesis in which cell wall-anchored teichoic acids (WTAs) increase in abundance at the expense of lipid-linked teichoic acids (LTAs). Because LytA binds to both species of teichoic acids, this change recruits the enzyme to its substrate where it cleaves the cell wall and elicits lysis. In this report, we identify WhyD (SPD_0880) as a new factor that controls the level of WTAs in Sp cells to prevent LytA misactivation and lysis during exponential growth. We show that WhyD is a WTA hydrolase that restricts the WTA content of the wall to areas adjacent to active peptidoglycan (PG) synthesis. Our results support a model in which the WTA tailoring activity of WhyD during exponential growth directs PG remodeling activity required for proper cell elongation in addition to preventing autolysis by LytA.

Authors names: Josué Flores-Kim, Genevieve S Dobihal, Thomas G Bernhardt, and David Z Rudner

Affiliations: UMass Chan Medical School, HHMI, and Harvard Medical School

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From: Joseph Boll <joeb2981@yahoo.com>

Title: Molecular interactions between peptidoglycan integrity maintenance and outer membrane lipid asymmetry in *Acinetobacter baumannii*

Text: The Gram-negative cell envelope is essential because it provides mechanical strength to counter the turgor and acts as a barrier to restrict the entry of toxins and antibiotics. The outer membrane (OM) and peptidoglycan are tightly linked during growth, but the molecular factors and pathways that coordinate assembly are poorly understood. Identifying cooperative factors that promote assembly between cell envelope layers may provide insights into how we can effectively target antimicrobials against Gram-negative pathogens. *Acinetobacter baumannii* is a nosocomial pathogen that has a high propensity to overcome antimicrobial treatment. LdtJ is a periplasmic LD-transpeptidase that promotes fitness during growth in *A. baumannii*. However, it is unknown how LdtJ enzymatic activity contributes to growth. Previous work in Escherichia coli showed that periplasmic tetrapeptide accumulation is toxic. Muropeptide analysis of Δ*ldtJ* showed increased tetrapeptide pools, which are LdtJ substrates, and this accumulation may also be toxic in *A. baumannii*. Transposon sequencing analysis suggested that deletion of mla genes in ΔldtJ background restored the growth defect. Maintenance of lipid asymmetry (Mla) retrograde phospholipid transport system removes mis localized surface-exposed phospholipids to maintain OM asymmetry. When Mla is disrupted, accumulation of surface-exposed phospholipids induces OM vesicle formation. Here, we tested the hypothesis that periplasmic tetrapeptide toxicity can be relieved by mla disruption, where increased OM vesicle formation may release tetrapeptides into the environment to reduce cellular toxicity. Thus, mla serves as a compensatory mutation to restore Δ*ldtJ* fitness defect in growth. This study will provide insights into how OM asymmetry and peptidoglycan integrity maintenance pathways are coordinated to maintain cell envelope homeostasis.

Authors names: Sinjini Nandy, Misha I. Kazi, Joseph M. Boll

Affiliations: University of Texas at Arlington

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From: Genevieve Dobihal <gsd2119@cumc.columbia.edu>

Title: Signaling networks controlling envelope remodeling and antibiotic resistance in *Klebsiella pneumoniae*

Text: Two-component regulatory systems (TCS) allow bacteria adapt to fluctuating conditions by inducing transcriptional changes. Importantly, TCS activity can also promote antibiotic resistance. In the Gram-negative pathogen *Klebsiella pneumoniae* (Kp), the CrrAB TCS induces resistance to polymyxins, antibiotics that disrupt the bacterial cell envelope, via the upregulation of non-essential factors that modify the envelope. Notably, my data shows that CrrAB also regulates essential envelope biogenesis pathways. The cell envelope of Gram-negative bacteria is a multi-layered structure, and the coordinated synthesis and remodeling of all layers is vital for cell growth. How these processes are coordinated to produce an organized envelope structure is unknown. My data indicate that CrrAB is a novel regulatory node linking drug resistance-inducing modifications to the outermost layers of the envelope with the synthesis and remodeling of the innermost layers to maintain envelope homeostasis. Additionally, the envelope is the interface between pathogen-host interactions, but how antibiotic-induced envelope remodeling pathways impact bacterial pathogenesis is unclear. My data indicates that CrrAB is critical for the virulence of Kp, but the mechanisms are undefined. This proposal will provide insights into how CrrAB coordinates envelope biogenesis pathways and reveal novel mechanisms by which TCS activity can drive antibiotic resistance and virulence.

Authors names: Dobihal, Genevieve S., Lewis, Kristen, McConville, Thomas H., Uhlemann, Anne-Catrin

Affiliations: Columbia University Medical Center

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From: Lucia Lafage <l.lafage@sheffield.ac.uk>

Title: Surface display of Staphylococcal cell wall components.

Text: The cell wall envelope *of Staphylococcus aureus* is made of multiple components that are collectively essential for the maintenance of cellular viability, growth, division and the interaction with the environment. The major structural polymer is peptidoglycan , that forms a single macromolecule around the cell and is responsible for cellular integrity. We have recently shown it to be an expanded hydrogel with an elegant architecture at the nanoscale, with an open mesh at its mature external face, a fine mesh at the inside and characteristic rings at the outside of the newly exposed septum after division. Here we have mapped other important envelope components on this molecular framework. Wall teichoic acids (WTA) were mapped using a novel probe, based on a phage receptor binding protein. WTA was found to have a surface, peripheral location, but missing from the septal ring architecture. Conversely, the WTA biosynthetic component TarO showed a septal location. Analysis of the surface display of the virulence determinant Surface Protein A (SpA) was also absent at the septal rings, even though it is known to be septally secreted. We hypothesise that these anomalies reveal a complex septal architecture with physiochemically distinct layers that permit both cell division and the localisation of surface components, contributing to the fidelity and function of the cell envelope, both in cellular homeostasis and interaction with the environment.

Authors names: Lafage, Lucia (1,2), Sutton, J.A.F. (1,2), Salamaga, B. (1,2), Hamer, T.L. (1,2), Foster, S.J. (1,2)

Affiliations: 1. School of Biosciences, University of Sheffield, Sheffield, United Kingdom 2. The Florey Institute for Host-Pathogen Interactions, University of Sheffield, Sheffield, United Kingdom.

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From: Lewis Jackson <lewis.jackson@warwick.ac.uk>

Title: Biochemical and structural characterisation of the Chlamydial MurC-Ddl fusion protein involved in peptidoglycan biosynthesis

Text: Most bacteria contain a peptidoglycan cell wall that encapsulates the bacterial cytoplasmic membrane, maintaining cell shape and integrity. Peptidoglycan biosynthesis begins in the cytoplasm and involves a series of Mur enzymes which catalyse the formation of the important intermediate UDP-MurNAc-pentapeptide. MurC is one of the enzymes involved and in Chlamydial species it is uniquely synthesised as a fusion with Ddl. The structure and enzymology of the Chlamydial MurC-Ddl fusion protein remains poorly defined. Additionally, it is still unclear what advantage this fusion provides since these enzymes do not act sequentially. Therefore, it is intended to determine the first crystal structure of MurC-Ddl and to characterise its enzyme kinetics. This fusion may hint that the enzymes involved in the cytoplasmic steps of peptidoglycan biosynthesis are organised into a larger complex. The clinical targeting of Mur ligases, such as MurC, remains under-developed and therefore these enzymes could represent good targets for the development of novel antibiotics. Hence, the final aim is to pursue novel anti-Chlamydial agents via high-throughput screening of compound libraries to identify inhibitors of MurC-Ddl.

Authors names: Lewis Jackson, Professor Christopher Dowson, Dr Adrian Lloyd

Affiliations: University of Warwick

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From: Patricia Rothe <rothep@rki.de>

Title: Growth conditions and genetic constellations controlling *in vivo* ReoM phosphorylation

Text: Gram positive bacteria are characterized by a thick cell wall mainly build up from peptidoglycan (PG) strands. The cell wall provides stability to withstand the turgor pressure and the inhibition of its biosynthesis, for example through antibiotics, can lead to cell death. A newly discovered regulation pathway (1,2) reveals that PG biosynthesis can be controlled through proteolytic de-/stabilization of MurA, the enzyme that performs the first step of PG biosynthesis. This pathway centers around the cytoplasmatic adapter protein ReoM that binds to MurA and directs it to the ClpCP protease for degradation. Whether MurA is degraded or not depends on the phosphorylation of ReoM at its essential Thr-7 residue by the PASTA-domain containing protein serine/threonine kinase PrkA.
To date, many aspects of this regulatory mechanism have not been clarified. Therefore, we here have studied the phosphorylation of ReoM through PrkA in vivo and have identified genetic constellations and growth conditions under which ReoM is phosphorylated or not. This uncovered that the late cell division protein GpsB is required for ReoM phosphorylation, as shown recently for *Bacillus subtilis* and *Enterococcus faecalis* (3,4). Our results strengthen the proposed PG biosynthesis regulation pathway through ClpCP-dependent control of MurA stability and suggest that cell division and peptidoglycan biosynthesis are closely intertwined. With further studies, we would like to clarify this link to cell division in more detail and try to identify conditions that inhibit ReoM phosphorylation and thus force the cell to shut down peptidoglycan biosynthesis.

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Authors names: Patricia Rothe (1), Sabrina Wamp (1), Lisa Rosemeyer (1), Jörg Döllinger (2) and Sven Halbedel (1,3)

Affiliations: (1) FG11 - Division of Enteropathogenic bacteria and Legionella, Robert Koch Institute, Burgstraße 37, 38855 Wernigerode, Germany, (2) ZBS 6 Proteomics and Spectroscopy, Robert Koch Institute, Seestraße 10, 13353 Berlin, Germany, (3) Institute for Medical Microbiology and Hospital Hygiene, Otto von Guericke University Magdeburg, 39120 Magdeburg, Germany.

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From: Jonny Burnett <jonny.burnett@warwick.ac.uk>

Title: Mechanisms and kinetics of novel insertion mutations in Ec FtsI

Text: First appearing in India, but now found global, novel insertion mutations YRIK/YRIN/TIPY in the beta2b-beta2c-beta2d region of *E. coli* divisisome protein PBP3 (FtsI) were identified as an important clinical determinant for the development of high levels of resistance, overcoming the previously effective aztreonam/avibactam combination therapy. Here we report the kinetic characterisation of these mutants in vitro and in vivo along with a novel crystal structure of the Ec PBP3 YRIK mutation.

Authors names: Jonathan Burnett, Chris Dowson

Affiliations: University of Warwick

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From: Bohumil Kubeša <b.kubesa@seznam.cz>

Title: Phosphorylation-independent regulation of Class A PBP2a by MacP in *S. pneumoniae* D39

Text: Survival of bacterial cells depends on their cell wall consisting of peptidoglycan (PG), which is essential for growth, division, and septum formation. Class A penicillin-binding proteins (aPBPs) are important for the proper maturation of essential PG, although the mechanisms by which they coordinate PG maturation with PG synthesis are not clearly understood. In the Gram-positive pathogen *Streptococcus pneumoniae*, MacP (SPD_0876) was identified as a membrane-anchored regulator of aPBP2a. MacP, forms a complex with aPBP2a, and is required for the *in vivo* activity of this PG synthase. MacP is a substrate of the Ser/Thr PASTA kinase StkP, and it was reported that phosphoablative and phosphomimetic substitutions in Thr32 of GFP-MacP prevents aPBP2a activation without affecting MacP-aPBP2a interaction. Here we provide *in vivo* and *in vitro* evidence that MacP is phosphorylated at Thr32 and Thr56. However, phosphoablative or phosphomimetic substitutions in T32 and T56 in MacP, as well as deletion of a cytoplasmic region containing T32 and T56, have no major effect on aPBP2a activity and MacP-aPBP2a interaction. Our results thus suggest that MacP regulation of aPbp2a activity is independent of the phosphorylation state of the cytoplasmic residues T32 and T56 under the growth conditions tested. In contrast, we show that the transmembrane region of MacP is essential for its function as a aPbp2a activator. We identified *macP* and *pbp2a* mutations in the transmembrane domain that are synthetically lethal to Δ*pbp1a*. Using B2H and Co-IP, we also show that MacP interacts with several cell division proteins that are part of the division and elongation machinery. Together, these results indicate that phosphorylation is not a primary signal for the complex regulation of aPBP2a activity by MacP in *S. pneumoniae*.

Authors names: Bohumil Kubeša[1,2‡], Merrin Joseph[3‡], Orietta Massidda[4], Ho-Ching T. Tsui[3], Pavel Branny[2], Malcolm E. Winkler[3] and Linda Doubravová[2]

Affiliations: [1]Charles University, Faculty of Sciences, Prague, Czech Republic; [2]Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague, Czech Republic; [3]Department of Biology, Indiana University Bloomington, Bloomington, Indiana, USA; [4]Department of Cellular, Computational and Integrative Biology, University of Trento, Via Sommarive 9, 38 123 Trento, Italy

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From: Thierry Touzé <thierry.touze@universite-paris-saclay.fr>

Title: Mechanistic basis of Enterocin C targeting the undecaprenyl pyrophosphate phosphatase BacA from *Enterococcus faecalis*

Text: *Enterococcus faecalis* is an important opportunistic pathogen often isolated from nosocomial infections and its natural resistance to some β-lactams makes it difficult to treat. One therapeutic strategy could rely on the potentiation of β-lactams by another drug interfering with a critical membrane step of peptidoglycan biosynthesis, such as the recycling of the carrier lipid undecaprenyl phosphate (C55-P). In this line, lowering the recycling rate in *Escherichia coli* was found to provoke a synergistic response with β-lactams leading to rapid cell lysis. In this study, we have shown that the two-peptide bacteriocin called Enterocin C (EntC) specifically targets the undecaprenyl phosphate recycling enzyme BacA from E. faecalis. Each peptide is inactive alone and acts synergistically at the nanomolar range to kill the target cell. We used a combination of biochemical and biophysical approaches to understand the molecular mechanism of EntC. Our results show a sequential binding of the peptides to the membrane protein BacA. This binding event leads to a significant BacA stabilisation, the inhibition of BacA enzymatic activity together with membrane permeabilisation to protons on BacA-containing liposomes. Our data suggest that EntC parasitizes BacA structure in a way to induce ion leakage. Structural modelling, molecular dynamics and mutagenesis are under way to figure out this mechanism. This work may open the way to bacteriocin engineering in order to specify and/or modify its spectrum of action in a global antibacterial strategy.

Authors names: Victor Folcher[1], Rodolphe Auger[1], Clara Louche[2], Pascale Serror[2], Thierry Touzé[1]

Affiliations: [1]Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC), FR-91198, Gif-sur-Yvette, France, [2]Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, F-78350, Jouy-en-Josas, France.

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From: Sarp Bamyaci <sarp.bamyaci@ki.se>

Title: The role of the serine/threonine protein kinase StkP in *Streptococcus pneumoniae* capsule formation

Text: *Streptococcus pneumoniae* (also called the pneumococcus), causes infections such as otitis media and pneumonia as well as life-threatening invasive infections such as sepsis and meningitis. It mainly affects individuals under 2 and over 65 years of age. The current main strategies against pneumococcal infections are antibiotic treatment and vaccines targeting the bacterial capsule. The pneumococcal capsule is one of the most well-studied virulence determinants of S. pneumoniae and there are approximately 100 different capsular serotypes described to date. However, serotypes not included in the vaccines are now becoming the most common cause of invasive pneumococcal infections. StkP, the pneumococcal eukaryotic-like serine/threonine protein kinase, has been shown to be essential for bacterial cell division and to interact with the cell wall synthesis machinery in addition to suggested roles in competence and virulence. The overall objective of this project is to extend the knowledge of the role of StkP in the bacterial life cycle with the long-term goal of identifying new drug targets.

In this study we hypothesize that StkP not only interacts with and regulates proteins involved in cell wall synthesis but also with capsular synthesis enzymes and we have preliminary data supporting a role of StkP in this process. Mutant bacteria lacking StkP (ΔstkP) and mutant bacteria with a non-functional StkP (kinase dead, stkP-K42M) were constructed both in encapsulated and unencapsulated S. pneumoniae strains. Quantitative phosphoproteomics, western blot-based quantification of capsule production, immunofluorescence, and electron microscopy as well as growth curve assays assessing fitness were performed using the obtained mutant strains as well as wild type (wt) strains. In the phosphoproteomics analysis both known and new substrates of StkP were detected including proteins involved in capsule biosynthesis and regulation. In addition, further experiments showed that wild type levels of capsule production required a functional StkP protein. We are now deciphering the role of StkP in the regulation of capsule biosynthesis, moreover if the kinase activity of StkP is involved in this regulation. Results from our study point to a role of StkP in capsule synthesis and regulation. A deeper understanding of the regulation of cell wall and capsule syntheses are crucial for the identification of new bacterial drug targets.

Authors names: Sarp Bamyaci [a], Vanessa Becker [b], Anuj Pathak [c], Lok-To Sham [d], Tanja Schneider [b], Staffan Normark [a], Birgitta Henriques-Normark [a,c], Anna Müller [b] and Karin Blomqvist [a,c]
Affiliations: [a] Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Sweden; [b] Institute for Pharmaceutical Microbiology, University Hospital Bonn, University of Bonn, Bonn, Germany; [c] Division of Clinical Microbiology, Karolinska University Hospital, Sweden; [d] Department of Microbiology and Immunology, National University of Singapore, Singapore

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From: Martin McAndrew <martin.mcandrew@warwick.ac.uk>

Title: Structure of an antimicrobial peptide resistance and sensing complex

Text: Antimicrobial resistance (AMR) is a growing challenge facing global healthcare systems and is at risk of undermining the many advances in modern medicine. Many Gram-positive bacteria use a multiprotein complex composed of an ABC transporter, histidine kinase and a DNA response regulator to sense, and provide resistance to, antibiotics such as vancomycin, nisin and bacitracin. In these systems, resistance is conferred by the activity of the ABC transporter which is thought to prise apart complexes formed between each antibiotic and their lipid targets. The ABC transporter also communicates its activity directly to the kinase which can further upregulate the expression of the transporter via activation of the response regulator. While structures of the ABC transporter are known, molecular details of the resistance mechanism and the process by which it communicates with the histidine kinase are incompletely understood. Here, we have used a combination of structural techniques and simulations to explore how these transporters facilitate signalling and resistance in the presence of antimicrobial agents.

Authors names: Martin McAndrew, Jonathan Cook, Susanne Gebhard, Phill Stansfeld, Allister Crow

Affiliations: University of Warwick, Johannes Gutenberg University of Mainz

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From: Parminder Singh Mavi <parminder_singh.mavi@biol.lu.se>

Title: PsmA is a newly identified regulator of cell growth in *Streptomyces*

Text: The phylum actinobacteria is populated majorly by gram-positive bacteria. Despite their diversity of shape and morphologies, they share a distinct property of polarized growth, wherein the new cell wall (a rigid polymer of N-acetyl glucosamine and N-acetyl muramic acid called peptidoglycan) is added to the existing cell wall in confined zones at the poles of the cell (hence named polar growth). The complex of proteins enabling this is termed polarisome. DivIVA is the essential coiled-coil protein responsible for organizing this polar growth across all Actinobacteria. DivIVA localises at the tip of the growing hyphae and co-ordinates polar growth in *Streptomyces*. In the spore-forming genus *Streptomyces*, the polarized growth is highly pronounced, where the lifecycle is divided between vegetative growth, where a unigenomic spore gives rise to a large branched, multigenomic mycelium without major compartmentalization of the chromosomal copies, that grows parallel and into the substrate. Ahead of formation of unigenomic spores, specialized hyphae form areal mycelium by growing away from the surface and creating compartments around individual chromosomal copies Aberrant expression of DivIVA affects both cell shape and viability. Scy and FilP are two related additional coiled-coil proteins involved in directing proper cell wall synthesis. The mechanism underlying polar growth and branching is poorly understood, and so are the partners in the process. Two structural proteins, the long coiled-coil Scy and the intermediate filament-forming FilP, are the best studied direct interaction partners of DivIVA so far in *Streptomyces*. Deletion of either causes modification in the hyphal growth pattern. Here, we have identified and characterized for the first time a Streptomyces-wide conserved pseudo-histidine kinase we've named PsmA, that interacts directly with DivIVA via its sensory domain and plays an important part in polar growth. We show that deletion of the gene *psmA* gives rise to pronounced defects in polar growth, leading to emergence of a distinct morphology, where the hyphal tips show unstable growth marked by repeated splitting. Combination of mutation of psmA with *scy* or *filP* leads to severe defect in polar growth and causes rapid division of the polarisome and severely effected cell growth and shape. Structurally, PsmA has a unique composition, with a hybrid histidine kinase like arrangement of domains, but with a long unstructured region connecting the N-terminal kinase domain to the C-terminal receiver domain. Interestingly, the catalytic histidine residue in the HisK domain in the N-terminus is missing. Purified N-terminal HisK portion of the protein gives no catalytic activity, Suggesting PsmA to be a pseudokinase with an important role in polar growth in Streptomyces.

Authors names: Parminder Singh Mavi, Klas Flärdh

Affiliations: Molecular Cell Biology Unit, Lund University, Sweden

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From: Pauline Macheboeuf <pauline.macheboeuf@ibs.fr>

Title: Architecture and reprogramming of the colibactin-producing complex for novel antibiotics development

Text: The continuous development of novel antibiotics is crucial in the actual context of bacterial resistance to classical antibacterial molecules. We are focusing our research on natural molecules production from the Polyketide/Non-Ribosomal Peptide (NRPS/PKS) family of enzymes that are often used by microorganisms to synthesize antibiotics or antifungal molecules. In particular we are interested in the pks operon present in several Enterobacteriaceae that is responsible for the production of colibactin, a multifaceted compound showing a mild inhibition against S. aureus and B. subtilis. We propose to elucidate the three-dimensional structure of the colibactin-producing megacomplex composed of 19 proteins at molecular and supra-molecular levels in order to better understand its function. Based on our structural results, we will reprogram this assembly line in order to synthesize new compounds and test their antibacterial activities against a bacterial strains collection. A better understanding of this fascinating natural machinery will pave the way for the discovery of new antibacterial drugs.

Authors names: Sarah Bonhomme, Carlos Contreras Martel, Andréa Dessen, Pauline Macheboeuf

Affiliations: Institut de Biologie Structurale, Grenoble, France

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From: Magdalena Kaus-Drobek <mdrobek@imdik.pan.pl>

Title: A novel chimeric M23 peptidoglycan hydrolase with anti-Listeria activity with unique role of SH3b binding domain.

Text: Listeriosis is a foodborne disease caused by *Listeria monocytoegnes*. It is relatively rare disease, but it is very serious due to high death rate (20–30%). The group of people particularly prone to listeriosis are pregnant women, new-borns, elderly, and those with severe immunodeficiency, like HIV infected patients or people after transplantations. The vast majority of cases of listeriosis are associated with ingesting *L. monocytogenes* from contaminated ready-to-eat food (RTE food), thus the biological control during the process of food production is crucial. Due to overuse of antibiotics, there is a need to find alternative treatment for bacterial infection. Among the candidates are peptidoglycan hydrolases (PGHs) that are able to cleave cell wall peptidoglycan (PG) leading to bacterial death. The newly discovered peptidoglycan hydrolase has been cloned, expressed, and purified. The sequence conservation and structure determination confirmed its M23 fold. The bactericidal activity against Listeria monocytogenes was confirmed in turbidity reduction and spot dilution assays. This enzyme was able to cleave peptidoglycan isolated from Listeria monocytoegnes and Bacillus subtilis, the Gram-positive species with meso-DAP type of PG. Addition of SH3b binding domain to M23 catalytic domain broaden the tolerance to ionic and pH conditions. Moreover, its activity was elevated and the specificity has been broaden. This chimeric protein was able to eradicate 108 CFU/ ml *Listeria monocytogenes* within 1 hr at RT. High activity and stability of chimera makes this PGH a good candidate to become an effective anti-Listeria agent.

Authors names: Magdalena Kaus-Drobek & Izabela Sabała

Affiliations: Mossakowski Medical Research Insitute, Polish Academy of Sciences

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From: Justyna Czarnecka <jczarnecka@imdik.pan.pl>

Title: *Listeria monocytogenes* amidase as a specific antimicrobial to prevent listeriosis

Text: Foodborne listeriosis is one of the most serious and severe foodborne diseases, caused by Listeria monocytogenes, bacteria which is widely distributed in nature. It can be found in soil, water, vegetation, and the faeces of some animals and can contaminate foods, especially long shelf-life products under refrigeration and foods that are consumed without further treatment, e.g., cooking. Ubiquitous nature of L. monocytogenes, high resistance to common preservative methods, such as the use of salt, smoke or acidic condition in the food, and its ability to survive and grow at refrigeration temperatures (around 5°C), makes food safety control even more challenging. We are investigating new peptidoglycan hydrolases encoded by *L. monocytogenes* genome and test their bacteriolytic activities as a potential antimicrobial. Recently studied enzyme sequence and structure indicate homology to N-acetylmuramoyl-L-alanine amidases (MurNAc-LAA), autolysins, which hydrolase the amide bond between N-acetylmuramoyl and L-amino acids in cell wall peptidoglycan. We have shown specific elimination of *Listeria monocytogenes* cells in optimized conditions (pH, conductivity, temperature), also for those strains, which were isolated from contaminated food. The efficiency and specificity of amidase was tested on Listeria biofilms, and its toxicity was investigated using Danio rerio model. Non-chemical and non-antibiotic nature or new antimicrobials allow to develop safe food preservatives and surface disinfectants in the future, which would not generate further increase of resistance.

Authors names: Justyna Czarnecka, Michał Zaród, Małgorzata Korzeniowska, Izabela Sabała, Elżbieta Jagielska

Affiliations: Mossakowski Medical Research Insitute, Polish Academy of Sciences

Participants contacts

Speakers

Given name	Surname	Affiliation	e-mail
Allister	Crow	U. Wawrwick	Allister.Crow@warwick.ac.uk
Anke	Becker	Univ Marburg	anke.becker@synmikro.uni-marburg.de
Bavesh	Kana	Univ Witwatersrand	Bavesh.Kana@wits.ac.za
Beate	Henrichfreise	Univ Bonn	bhenrich@uni-bonn.de
David	Sychantha	McMaster Univ	sychantd@mcmaster.ca
Dennis	Claessen	Leiden Univ	d.claessen@biology.leidenuniv.nl
Dominique	Missiakas	Univ Chicago	dmissiak@bsd.uchicago.edu
Ethel	Bayer-Santos	Univ São Paulo	ebayersantos@usp.br
Felipe	Cava	Univ Umëa	felipe.cava@umu.se
Irina	Shlosman	Harvard U, USA	shlosman@g.harvard.edu
Jean-Emmanuel	Hugonnet	Sorbonne Univ	jean-emmanuel.hugonnet@crc.jussieu.fr
Jeanne	Salje	Cambridge Univ	jss53@cam.ac.uk
Laure	El Chamy	Univ Saint Joseph	laure.chamy@usj.edu.lb
Luiz Pedro	Carvalho	The Francis Crick Institute	luiz.carvalho@crick.ac.uk
Malcolm	Winkler	Univ Indiana	winklerm@indiana.edu
Martin	Loessner	ETH Zurich	martin.loessner@ethz.ch
Natalia Luisa	Hiller	Carnegie Mellon Univ	lhiller@andrew.cmu.edu
Pamela	Brown	U. Missouri,	brownpb@missouri.edu
Qiao	Yuan	Nanyang Technological Univ	yuan.qiao@ntu.edu.sg
Seamus	Holden	U. Wawrwick	seamus.holden@warwick.ac.uk
Simon	Foster	U. Sheffield	s.foster@sheffield.ac.uk
Simonetta	Gribaldo	Institut Pasteur	simonetta.gribaldo@pasteur.fr
Sven	Halbedel	Robert Koch Institute	halbedels@rki.de
Tobias	Doerr	Cornell Univ	tdoerr@cornell.edu
Xavier	De Bolle	Univ Namur	xavier.debolle@unamur.be

Organisation

David	Roper	Univ Warwick	David.Roper@warwick.ac.uk
Elitza	Tocheva	Uuniv Bristish Columbia	elitza.tocheva@ubc.ca
Ivo	Gomperts Boneca	Institut Pasteur	bonecai@pasteur.fr
Natividad	Ruiz	Ohio State Univ	ruiz.82@osu.edu
Orietta	Massida	Univ Trento	orietta.massidda@unitn.it
Sérgio	Filipe	Univ Nova Lisboa	sfilipe@fct.unl.pt

Given name	Surname	Affiliation	Poster n°	e-mail
Abimbola	Feyisara olulana	University of Sheffield	94	a.adedeji@sheffield.ac.uk
Adria	Sogues	Vrije Universiteit Brussel	18	asoguesc@vub.be
Adrián	Izquierdo martínez	Instituto de Tecnologia Química e Biológica António Xavier	53	aizquierdomartinez@itqb.unl.pt
Adrien	Ducret	Université de Lyon	66	adrien.ducret@ibcp.fr
Alaa	Aljohani	Newcastle University	103	a.a.aljohani2@newcastle.ac.uk
Alessandra	Martorana	Università degli studi di Milano	41	alessandra.martorana@unimi.it
Alessandra	Polissi	Università degli studi di Milano	41	alessandra.polissi@unimi.it
Alessandro	Pagliuso	Université Paris-Saclay	74	alessandro.pagliuso@inrae.fr
Alexander	Anderson	University of Guelph	64	aander14@uoguelph.ca
Alicja	Razew	Institut de Biologie Structurale	22	alicja.razew@ibs.fr
Aline	Rifflet	Institut Pasteur	82	arifflet@pasteur.fr
Alix	Dachsbeck	de Duve Institute, Université catholique de Louvain	7	alix.dachsbeck@uclouvain.be
Ana	Paiva	Université Paris-Saclay	112	ana.oliveira-paiva@i2bc.paris-saclay.fr
Andrea	Dessen	Brazilian Biosciences National Laboratory	ST-5	andrea.dessen@ibs.fr
Andreia	Duarte	Instituto de Tecnologia Química e Biológica António Xavier	105	andreia.duarte@itqb.unl.pt
Andrew	McMahon	University of Warwick		andrew.mcmahon@warwick.ac.uk
Anjana	Peethambaran Menon	Indian Institute of Technology Bombay	80	ANJANA.NAMBALAT@GMAIL.COM
Anne Marie	Wehenkel	Institut Pasteur	34	anne-marie.wehenkel@pasteur.fr
Anthony	Clarke	Wilfrid Laurier University	64	ajclarke@wlu.ca

Antoni	Planas	University Ramon Llull	77	antoni.planas@iqs.url.edu
Barbara	Walenkiewicz	Indiana University	2	bwalenk@iu.edu
Beatriz	De Pina Mariz	Universidade Nova de Lisboa	84	b.mariz@campus.fct.unl.pt
Becca	Bailey	University of Warwick	109	becca.bailey@warwick.ac.uk
Beile	Gao	South China Sea Institute of Oceanology	32	gaob@scsio.ac.cn
Beiyan	Nan	Texas A&M University	62	bnan@tamu.edu
Berenice	Furlan	University of Trento	110	berenice.furlan@unitn.it
Berliza Marie	Soriano	University of California, San Francisco	83	berliza.soriano@ucsf.edu
Bohumil	Kubeša	Charles University	120	b.kubesa@seznam.cz
Brandon	Jutras	Virginia Polytechnic Institute and State University	56	bjutras@vt.edu
Brooks	Rady	University of Sheffield	36	b.j.rady@gmail.com
Camilla	Henriksen	University of Copenhagen	ST-4 and 10	camjen@sund.ku.dk
Camilo	Perez	Biozentrum of the University of Basel	15	camilo.perez@unibas.ch
Carolin	Zeiher	University of Leipzig	40	carolin.zeiher@medizin.uni-leipzig.de
Cassandra	Lenoir	Université de Lyon	90	cassandra.lenoir@ibcp.fr
Catherine	Tooke	University Walk	30	ct12425@bristol.ac.uk
Cécile	Morlot	Institut de Biologie Structurale	ST-1	cecile.morlot@ibs.fr
Céline	Freton	Université de Lyon	104	celine.freton@ibcp.fr
Chenyu	Li	Nanyang Technological University	27	lich0052@e.ntu.edu.sg
Choo Pei	Yi	Nanyang Technological University	111	pychoo@ntu.edu.sg
Christophe	Grangeasse	Université de Lyon		christophe.grangeasse@ibcp.fr
Clare	Thomas	University of Birmingham	85	CXT255@student.bham.ac.uk
Constantin	Anoyatis-pelé	Sorbonne Université	23	canopele@gmail.com

Coralie	Fumeaux	Lausanne University Hospital	78	coralie.fumeaux@unil.ch
Damien	Devos	Centro Andaluz de Biología del Desarrollo	11	damienpdevos@gmail.com
Daniel	Karadzas	University of Warwick	106	daniel.karadzas@warwick.ac.uk
David	Roberts	University of Warwick		David.M.Roberts@warwick.ac.uk
Dirk-Jan	Scheffers	University of Groningen		d.j.scheffers@rug.nl
Elena	Rivas-marin	Universidad Pablo de Olavide	68	erivmar@upo.es
Elisa	Pierre	de Duve Institute, Université catholique de Louvain	3	elisa.pierre@uclouvain.be
Elżbieta	Jagielska	Mossakowski Medical Research Institute, Polish Academy of Sciences	29	ejagielska@imdik.pan.pl
Francesca	Gillett	University of Warwick	45	f.gillett@warwick.ac.uk
Genevieve	Dobihal	Columbia University	115	gsd2119@cumc.columbia.edu
Girbe	Buist	University Medical Center Groningen	73	g.buist@umcg.nl
Gyanu	Lamichhane	Johns Hopkins University	1	gyanu@jhu.edu
Helen	Smith	University of Oxford		helen.smith@chem.ox.ac.uk
Helena	Veiga	Instituto de Tecnologia Química e Biológica António Xavier	ST-6	hveiga@itqb.unl.pt
Henri	Voedts	Sorbonne Université	39	henri.voedts@gmail.com
Hugo	Millat	Université de Lyon	91	hugo.millat@ibcp.fr
Izabela	Sabala	Mossakowski Medical Research Institute, Polish Academy of Sciences	20	isabala@imdik.pan.pl
Jean-Pierre	Simorre	Institut de Biologie Structurale	17	jean-pierre.simorre@ibs.fr
Jed	Fisher	University of Notre Dame	46	jfisher1@nd.edu

Jessica	Burnier	University of Lausanne	31	jessica.burnier@unil.ch
Jessica L	Davis	University of Sheffield	8	jldavis2@sheffield.ac.uk
Jianwei	Li	National University of Singapore	95	ljw26@nus.edu.sg
Jonny	Burnett	University of Warwick	119	jonny.burnett@warwick.ac.uk
Joseph	Boll	University of Texas at Arlington	114	joeb2981@yahoo.com
Joshua	Sutton	University of Sheffield	33	joshua.sutton@sheffield.ac.uk
Josue	Flores kim	UMass Chan Medical School	113	Josue.FloresKim3@umassmed.edu
Juan	Hermoso	Instituto de Química-Física "Rocasolano"	ST-2	xjuan@iqfr.csic.es
Julia	Fairbairn	University of Warwick	60	julia.fairbairn@warwick.ac.uk
Justyna	Czarnecka	Mossakowski Medical Research Institute, Polish Academy of Sciences	127	jczarnecka@imdik.pan.pl
Kate	Willett	University of Warwick	44	k.willett.1@warwick.ac.uk
Kelly	Rosch	Cornell University	96	kmr257@cornell.edu
Kelvin	Kho	Institut Pasteur	ST-4	kkho@pasteur.fr
Kevin	Whitley	Newcastle University	13	kevin.whitley@newcastle.ac.uk
Laia	Pasquina lemonche	University of Sheffield	86	l.pasquinalemonche@sheffield.ac.uk
Leonor Teixeira	Bismarck quintas marques	Instituto de Tecnologia Química e Biológica António Xavier	21	leonor.bismarck@gmail.com
Lewis	Jackson	University of Warwick	117	lewis.jackson@warwick.ac.uk
Lok-To	Sham	National University of Singapore	ST-6	miclts@nus.edu.sg
Lucia	Lafage	University of Sheffield	116	l.lafage@sheffield.ac.uk
Maarten	Lubbers	Leiden University	79	m.lubbers@biology.leidenuniv.nl
Magdalena	Kaus-drobek	Mossakowski Medical Research	126	mdrobek@imdik.pan.pl

		Institute, Polish Academy of Sciences		
Manuela	Alvarado	Cornell University	52	ma2229@cornell.edu
Marcel Gustavo	Alaman zarate	University of Sheffield	9	mgalamanzarate1@sheffield.ac.uk
Maria Disen	Barbuti	Norwegian University of Life Sciences	69	maria.disen.barbuti@nmbu.no
Mariana	Tinajero trejo	University of Sheffield	100	m.tinajero-trejo@sheffield.ac.uk
Marie	Royer	Institut Pasteur	50	marie.royer@pasteur.fr
Marie-Pierre	Chapot-chartier	Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute	6	marie-pierre.chapot-chartier@inrae.fr
Marjolein	Crooijmans	Leiden University	81	m.e.crooijmans@biology.leidenuniv.nl
Martin	McAndrew	University of Warwick	123	martin.mcandrew@warwick.ac.uk
Maryam Sadat	Alehashem	University of California-San Francisco	88	maryamalehashem5@gmail.com
Matthew	Herdman	University of Oxford	47	matthew.herdman@jic.ac.uk
Megan	Keller	Cornell University	75	mrk269@cornell.edu
Melina	Arts	University of Bonn	26	melina.arts@uni-bonn.de
Michel	Arthur	Sorbonne Université		michel.arthur@crc.jussieu.fr
Mijoon	Lee	Notre Dame Univrsity		mlee12@nd.edu
Morten	Kjos	Norwegian University of Life Sciences	70	morten.kjos@nmbu.no
Nadia	Nikulin	Cornell University	42	ns646@cornell.edu
Nicholas	Briggs	University of Warwick	16	n.briggs@warwick.ac.uk
Nienke	Buddelmeijer	Institut Pasteur	93	nienke.buddelmeijer@pasteur.fr
Nika	Pende	University of Vienna	ST-1	nika.pende@univie.ac.at
Noortje	Dannenberg	Leiden University	57	N.Dannenberg@biology.leidenuniv.nl
Ofélia	Godinho	Universidade do Porto	68	ofeliagodinho95@gmail.com
Olivier	Danot	Institut Pasteur	65	olivier.danot@pasteur.fr

Pablo	Sobrado	Virginia Polytechnic Institute and State University		psobrado@vt.edu
Parminder Singh	Mavi	Lund University	124	parminder_singh.mavi@biol.lu.se
Patricia	Reed	Instituto de Tecnologia Química e Biológica António Xavier	108	preed@itqb.unl.pt
Patricia	Rothe	Robert Koch-Institute	118	rothep@rki.de
Patrick	Viollier	University of Geneva	72	patrick.viollier@unige.ch
Pauline	Macheboeuf	Institut de Biologie Structurale	125	pauline.macheboeuf@ibs.fr
Pedro	Alzari	Institut Pasteur	35	pedro.alzari@pasteur.fr
Phuong Y	Mai	Institut Pasteur	89	phuong-y.mai@pasteur.fr
Prabhjot	Kaur	Hebrew University of Jerusalem	107	prabhjot.Kaur@mail.huji.ac.il
Quentin	Herail	Sorbonne Université	19	herailquentin1@gmail.com
Quentin	Gaday	Institut Pasteur	43	quentin.gaday@pasteur.fr
Quynh Mai	Nguyen	Institut de Biologie Structurale	ST-2	quynh-mai.nguyen@univ-grenoble-alpes.fr
Raffaele	Ieva	Université de Toulouse	48	raffaele.ieva@univ-tlse3.fr
Ragnhild	Sødal gjennestad	Norwegian University of Life Sciences	58	ragnhild.sodal.gjennestad@nmbu.no
Raj	Bahadur	University of Sheffield	36	r.bahadur@sheffield.ac.uk
Randy	Morgenstein	Oklahoma State University	4	randy.morgenstein@okstate.edu
Renata	C. Matos	Institut de Génomique Fonctionnelle de Lyon	ST-3	renata.matos@ens-lyon.fr
Richard	Daniel	Newcastle University	99	richard.daniel@ncl.ac.uk
Richard	Wheeler	Institut Pasteur	ST-3	rwheeler@pasteur.fr
Robert	Smith	Institut Pasteur	76	robert.smith@pasteur.fr
Roy	Van Beekveld	Utrecht University	71	r.a.m.vanbeekveld@uu.nl
Sam	Benedict	University Of Birmingham	49	STB578@student.bham.ac.uk
Sarp	Bamyaci	Karolinska Institutet	122	sarp.bamyaci@ki.se
Sebastien	Rigo	Roche	102	sebastien.rigo@roche.com

Simon	Schäper	Instituto de Tecnologia Química e Biológica António Xavier	67	sschaeper@itqb.unl.pt
Stephane	Mesnage	University of Sheffield	5	s.mesnage@sheffield.ac.uk
Suraj Kumar	Meher	CSIR-Centre for Cellular and Molecular Biology	101	surajkumar@ccmb.res.in
Thierry	Touzé	Université Paris-Saclay	121	thierry.touze@universite-paris-saclay.fr
Thimoro	Cheng	Institut Pasteur	37	thimoro.cheng@pasteur.fr
Thomas	Delerue	Institut Pasteur	63	thomas.delerue@pasteur.fr
Tiffany	Tsui	Indiana University	97	tttsui@indiana.edu
Tyler	Sisley	Harvard University	ST-5	tsisley@g.harvard.edu
Upasana	Basu	Cornell University	54	ub39@cornell.edu
Vanessa	Becker	University of Bonn	25	s6vabeck@uni-bonn.de
Victor	Folcher	Université Paris-Saclay	51	victor.folcher@i2bc.paris-saclay.fr
Victor	Pinedo	Umeå University	59	victor.pinedo@umu.se
Viktor	Hundtofte Mebu	University of Copenhagen	14	vhm@sund.ku.dk
Wilhelm	Linus	Université de Lyon	98	linus.wilhelm@ibcp.fr
Wolfram	Zueckert	University of Kansas School of Medicine	38	wzueckert@kumc.edu
Xiao	Jie	Johns Hopkins School of Medicine	28	xiao@jhmi.edu
Xiaobo	Zhong	Leiden University	87	x.zhong@biology.leidenuniv.nl
Xiaofang	Li	University Medical Center Groningen	55	x.li@umcg.nl
Xin	Xu	National University of Singapore	92	xuxin01@nus.edu.sg
Yanyan	Fu	University of Groningen		y.fu@umcg.nl
Yijie	Zhang	Université Paris-Saclay	61	yijie.zhang@i2bc.paris-saclay.fr
Yousef	Alanazi	Newcastle University	99	Y.N.N.Alanazi2@newcastle.ac.uk
Yucheng	Liang	Sorbonne Université	24	lycheer42@gmail.com
Yves	Brun	University of Montreal	12	yves.brun@umontreal.ca