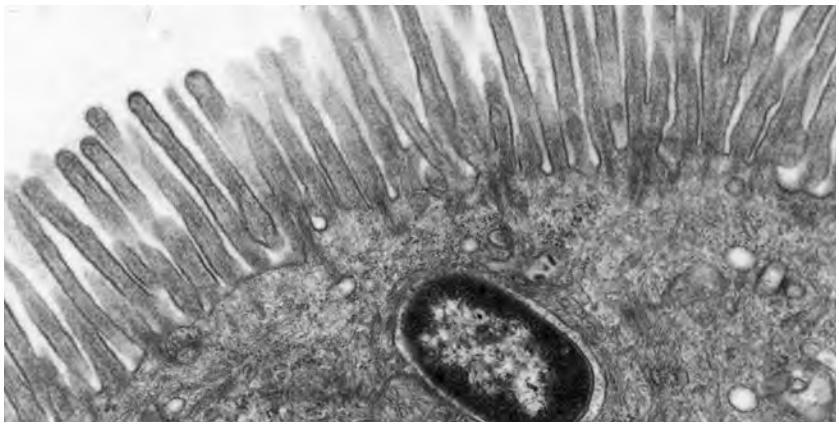

Microbial Biotechnology

Research in the Department of Microbial Biotechnology focuses on the molecular aspects of the biology of bacteria, their interaction with the environment or the host during infection, the spread of antibiotic resistance in the clinic, and on exploiting their biotechnological potential for biomedical and environmental applications. Our work includes multiple approaches using molecular genetics, systems and synthetic biology, evolutionary biology, genomics, proteomics and metagenomics. The scientific objectives of the Department are grouped on five complementary aspects of microbial biology:

- Environmental microbiology. We aim to characterise the mechanisms underlying the global regulatory networks that modulate bacterial metabolism in response to changes in environmental conditions, which can compromise biotechnological applications. We also study the the assembly of microbial communities and the mechanisms that contribute to horizontal gene transfer among environmental and pathogenic microbes.
- Microbial pathogenesis. Efforts are directed to understand the molecular mechanisms underlying extracellular and intracellular infections, from tissue colonisation to cell invasion and persistent infections in different host cell types.
- Microbial resistance to antibiotics. Work aims to understand the evolutionary mechanisms that contribute to antibiotic resistance in bacteria, among them, the impact of plasmids and antibiotic-polluted ecosystems. In addition, we study basic processes of microbial physiology, as cell division, which may define antimicrobial targets, and nanobody based therapies to combat infections.
- Microbial responses to hostile environments. Our work focuses on the understanding of bacterial responses to stressful environments, including general stress responses. We study how bacteria replicate and repair damaged DNA.
- Microbial engineering. Our purpose is to generate engineered bacterial strains optimised to obtain products of interest such as nanobodies, and able to combat tumors, pathogens or environmental pollutants. In addition, we develop synthetic tools based on bacterial proteins, including amyloids, for biotechnological applications.

HEAD

Luis Ángel Fernández

Figure Legend: Salmonella enterica *in the interior of an enterocyte following invasion of the intestinal epithelial barrier. The micrograph was taken in a sample obtained from a mouse challenged with the pathogen per the oral route (Francisco García-Portillo´s lab).*

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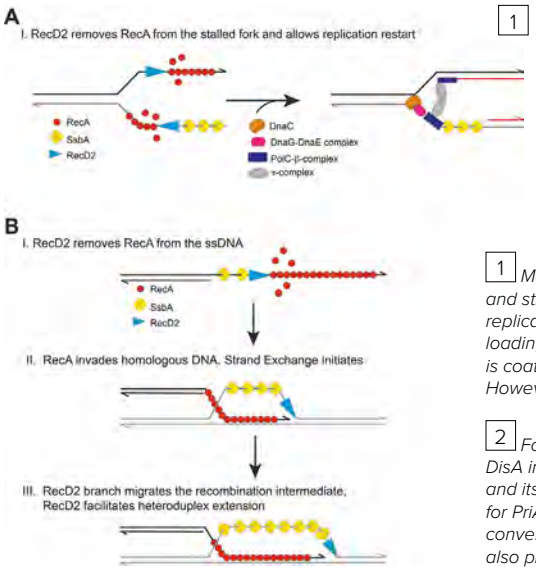
Genetic stability

We investigate the factors that govern genetic stability in bacteria and the mechanisms of horizontal gene transfer, using *Bacillus subtilis* as the model.

In the presence of endogenous threads, the replisome disassembles roughly five times during each cell cycle, leaving single-stranded DNA (ssDNA) regions coated by a single-stranded binding protein (SsbA). RecA recombinase may assemble at this ssDNA region and inhibit DNA replication restart. We found that the RecD2 helicase avoids this, and facilitates thereby replication restart. Another helicase, PcrA, also modulates RecA activities. In addition, we analysed how the damage checkpoint (DisA) is implicated in the preservation of stalled replication forks and genome integrity in *B. subtilis*. DisA catalyses the formation of c-di-AMP, and we found that levels of this essential second messenger are altered by replication stalling. RecA interacts with and loads DisA at the stalled fork. DisA-mediated synthesis of c-di-AMP is suppressed upon DisA binding to DNA structures that mimic stalled or reversed forks (gapped forks or Holliday junctions [HJ]) and c-di-AMP synthesis is blocked in the presence of helicases RecG, RuvAB and RadA/Sms bound to these structures. Low c-di-AMP levels indirectly inhibit DNA replication by inhibiting DNA primase activity. *In vitro* assays also revealed that DisA assembled at stalled or reversed forks limits RecG- and RuvAB-mediated branch migration and fork remodeling. Finally, how these helicases that act on recombination intermediates (RecG, RuvAB, RecD2 and RadA/Sms) contribute to the acquisition of divergent sequence during natural competence was analysed.



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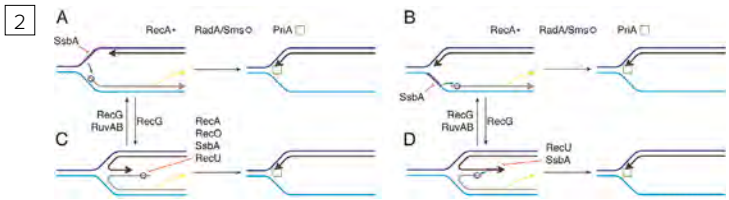

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1 *Model depicting how RecD2 helicase may modulate RecA during replication restart and strand exchange. (A) On a stalled fork, RecA may bind to the ssDNA regions inhibiting replication restart. RecD2 removes RecA from the stalled fork and thereby, facilitates replisome loading and replication restart. (B) During homologous recombination the invading strand is coated with RecA. RecD2 may translocate 5´➔3´ along the ssDNA and remove RecA. However, once RecA initiates strand exchange it may accelerate heteroduplex extension.*

2 *Fork remodelling after DisA detection of stalled leading- (A) or lagging-strand (B). The DisA interacting protein RadA/Sms is loaded on the stalled fork in coordination with RecA and its mediators and unwinds the nascent lagging-strand to yield the optimal substrate for PriA binding and replication restart (A and B). Alternatively, the RecG remodeller converts the stalled forks into Holliday junction-like structures (C and D), which can be also processed by the RadA/sms helicase facilitating PriA replication restart.*

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Stress and bacterial evolution

The research of the "Stress and Bacterial Evolution" group is focused on the genetic mechanisms involved in genome stability and their roles in bacterial evolution and adaptation. The group´s interest is to understand the genetic basis of both stable and induced hyper-mutation/hyper-recombination as bacterial "strategies" to speed adaptation to stress, particularly to antibiotic challenge. Recently, the group described the genetic characteristics of a novel non-canonical mismatch repair (nc-MMR) system in some Prokaryotes, including Mycobacteria and *Streptomyces*, a key pathway for maintaining their genome stability. Currently, we are trying to disentangle the possible relationship between genome stability/instability and antibiotic resistance development in pathogenic Mycobacteria, such as *M. tuberculosis, M. abscessus* and *M. avium*.

From a practical perspective, the lab wants to apply this knowledge to i) prevent and fight antibiotic resistance in bacterial pathogens by searching for new antibiotics (such as anti-mutagenic molecules and inhibitors of *β*-lactamases) and new target genes, and ii) engineer prokaryotic species (eg *Streptomyces*) to improve their biotechnological features.

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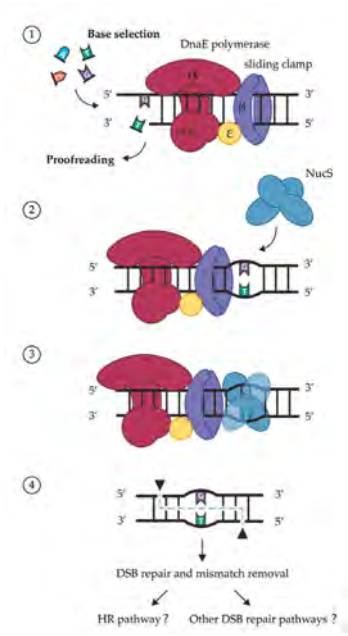

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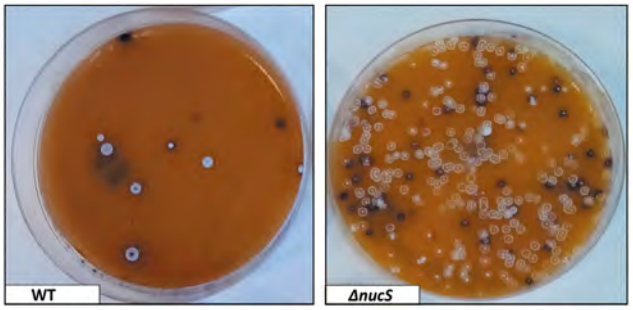

1 *Model of action of the non-canonical MMR pathway in Actinobacteria. DnaE core polymerase (α subunit, red; ε subunit, yellow), sliding clamp (β subunits, purple) and NucS dimer (blue). (1) During replication DnaE polymerase performs base selection and, through its PHP domain, proofreading activity (3'–5' exonuclease). In mycobacteria, ε subunit has no proofreading activity. (2) The mismatches that escape these correction processes are the substrate of NucS. (3) NucS binds to the dsDNA containing a mismatch and its activity is stimulated by interaction with the sliding clamp. (4) NucS nicks both strands around the mismatch leaving a DSB. Finally, the DSB and the mismatch may be repaired through either HR pathway or other DSB repair mechanisms. Source: Cebrián-Sastre. Cells. 2021. 10(6):1314.*

2 *A* Streptomyces coelicolor *nucS mutant (right) produces a high number of mutants resistant to the antibiotic (rifampicin) in comparison with the wild type strain (left).*

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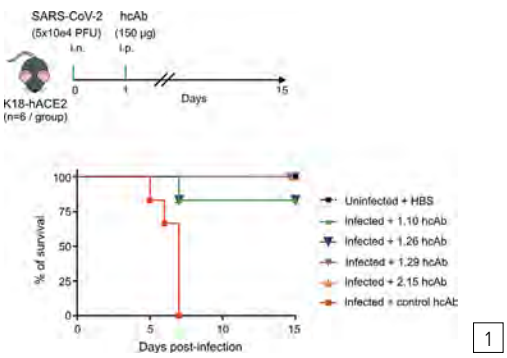
Bacterial engineering for biomedical applications

Our research is aimed to engineer *E. coli* bacteria for biomedical applications, including the selection of single-domain antibodies (nanobodies) and the design of synthetic bacteria for diagnostic and therapeutic use. Nanobodies are the smallest recombinant antibody fragments with full antigen-binding capacity, and are derived from heavy-chain-only antibodies found in camelids (e.g. dromedaries, llamas). We study protein secretion systems found in pathogenic *E. coli* strains, such as enteropathogenic *E. coli* (EPEC), and engineer them to produce protein nanomachines that can be applied in the selection and expression of nanobodies, or the delivery of therapeutic proteins into tumour cells. We use synthetic biology and genome engineering to combine the expression of these modular parts in our engineered bacteria.

These years our work has focused on: 1) The expression and selection of nanobodies that neutralise SARS-CoV-2 infection in vivo; 2) The engineering *E. coli* bacteria as anti-tumour agents able to bind to tumour cells and deliver a protein cargo; 3) The directed evolution of proteins with a novel *in vivo* mutagenesis system in *E. coli*, called T7-DIVA.



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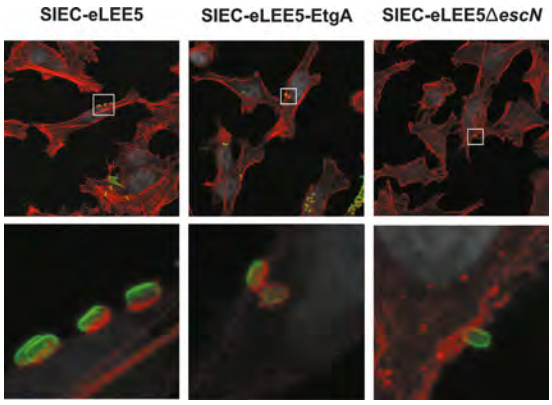

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1 *Protection of hACE2-trangenic mice after a lethal SARS-CoV-2 infection by selected nanobodies. Groups of K18-hACE2 mice (n=6/group) were either infected intranasally (i.n) with a lethal dose of SARS-CoV-2 (infected groups) or mock infected with PBS (uninfected group). On day 1 postinfection, 150 µg of nanobodies 1.10, 1.26, 1.29, 2.15 or control, fused to human IgG1 Fc domain (hcAbs) were administered intraperitoneally (i.p.) to animals in the infected groups. The uninfected group was treated i.p. with buffer (HBS). Graph represents the percentage of daily mice survival in each experimental group up to 15 d.p.i.*

2 *Translocation of Tir protein into mammalian cells by synthetic injector* E. coli *(SIEC) strains. Confocal fluorescence microscopy images of HeLa cells infected with the indicated bacterial strains SIEC-eLEE5, SIEC-eLEE5-eEtgA and SIEC-eLEE5ΔescN (T3SS mutant). F-actin in cells is stained red, and DNA and nuclei are stained grey. Bacteria are stained in green. The F-actin accumulations can be visualized as strong red fluorescence signals associated with attached bacteria, except in the ΔescN mutant, indicating the translocation of Tir protein by the T3SS. Bottom images are magnifications of the regions marked with white squares.*

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Intracellular bacterial pathogens

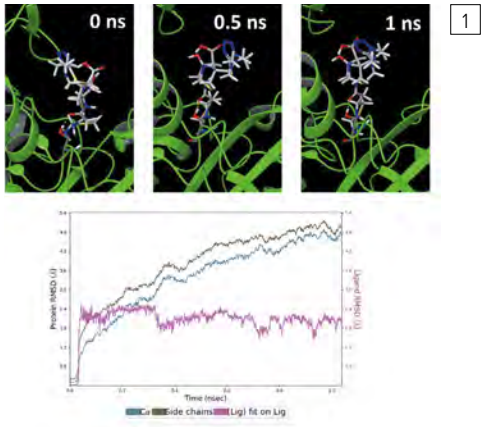
Intracellularity as lifestyle has been selected by an array of successful bacterial pathogens in which *Salmonella* is included. Our lab aims to understand how *Salmonella* evolved to colonise acidic vacuolar compartments of eukaryotic cells and the changes occurring in its cell envelope during the residence in such unique niche. A common phenomenon occurring in this vacuolar compartment is the entry of the pathogen into a state of reduced metabolic activity (latency) that favours persistence. This metabolic switch and in such particular environment represents one of the major obstacles to eradicate the intracellular infections caused by *Salmonella*.

Morphological studies

We have identified several proteins upregulated by intravacuolar *Salmonella* in response to the acidic environment that encounters within the infected host cell. Some of these proteins are peptidoglycan synthases involved in morphogenesis, which are pathogen-specific and "replace" enzymes extensively characterised in bacteria grown in laboratory media. This feature is currently exploited to identify drugs acting selectively on targets expressed by intracellular bacteria. Besides this applied investigation, we are much interested in determining at the molecular and cellular level how *Salmonella* morphogenesis is regulated within the phagosomal compartment, considering the spatial constrains that are imposed by the surrounding phagosomal membrane. We have found unique morphogenetic complexes involved in cell elongation and division of intracellular *Salmonella* and our aim is to characterise the involved components. We also seek to understand how these new morphogenetic systems interact with the virulence factors that promote survival and proliferation of intracellular bacteria.

Evolutionary studies

We are starting studies focused on the analysis of enzymatic activities of uncultivated bacteria predicted to be linked to peptidoglycan metabolism. These studies are offering new insights into what it could be the origin of the cell wall in unicellular microbes, a major event that marked the split of the Archaea and Bacteria domains.



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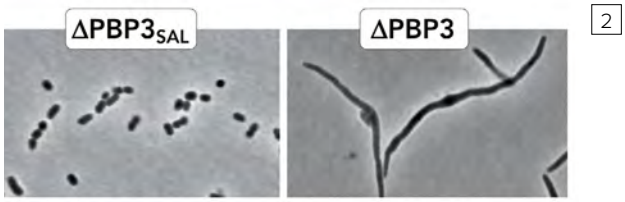

2

1 *Molecular dynamics showing binding of a drug to the morphogenetic protein PBP3SAL upregulated by intracellular Salmonella. ns = nanosecond.*

2 *Morphological alteration caused by a drug that shows higher affinity to PBP3SAL than to PBP3 as inferred by the response of the respective mutants.*

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Dessaux C, Pucciarelli MG, Guerreiro DN, O'Byrne CP, García-Del Portillo F. Activation of the *Listeria monocytogenes* stressosome in the intracellular eukaryotic environment. Appl Environ Microbiol 2021, 87(12), e0039721.

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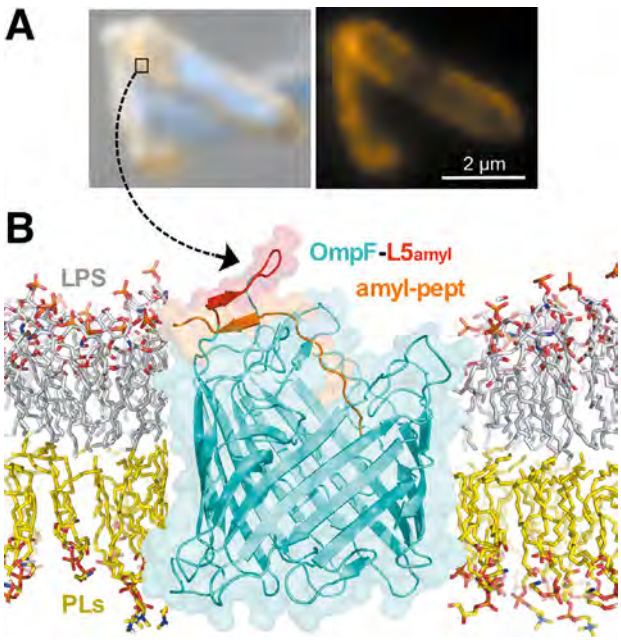
Synthetic bacterial amyloids (SynBAmyl)

Functional amyloids are protein assemblies that enable the epigenetic inheritance of phenotypes. However, when generated by protein misfolding, amyloids can trigger human diseases. We create, through bottom-up Synthetic Biology, bio-resources with two major aims: i) understanding the molecular determinants of the shift between function and toxicity in natural amyloids; and ii) generating new tools based on amyloids for Biotechnology and Biomedicine.

RepA is a protein from a bacterial plasmid whose WH1 domain undergoes conformational changes enabling it as a transcriptional repressor, or as a DNA replication initiator or inhibitor, the latter by assembling a functional amyloid, that hinders premature re-replication rounds. RepA-WH1 dimers become metastable monomers upon allosteric binding to plasmid-specific dsDNA sequences or acidic phospholipids, thus triggering amyloidogenesis. We engineered RepA-WH1 to become a biosafe prion-like protein (prionoid) that is transmitted from mother-to-daughter *Escherichia coli* cells, causing a synthetic 'generic' amyloid proteinopathy. RepA-WH1 aggregates propagate as strains with distinct appearance and cytotoxicity, modulated by an Hsp70 chaperone. RepA-WH1 amyloidosis recapitulates in bacteria the hallmarks of mitochondrial routes associated with human amyloid diseases, including the formation of oligomeric pores at the internal membrane and the generation of reactive oxygen species.

Recently, control on RepA-WH1 amyloidogenesis has been achieved through optogenetics, i.e., the fusion of a blue light-responsive plant domain (LOV2) to the N-terminus of WH1. Expressing LOV2-WH1-mCherry in *E. coli* under blue light illumination leads to the assembly of oligomers that hamper bacterial growth ('optobiotics').

Along the last two years, we have engineered the bacterial outer membrane porin OmpF by grafting its extracellular loops with an amyloidogenic peptide sequence, thus enabling homotypic self-recognition of the same sequence when either presented in solution or displayed on functionalized surfaces. We are exploring the potential of such synthetic bacterial devices as biosensors for environmental amyloids, to then further expand their abilities to achieve amyloid clearance.



Engineering the E. coli *outer membrane (OM) porin OmpF as a scavenger of amyloid peptides and proteins. (A) Rhodamine-labelled amyloid RepA-WH1 peptide probe (orange) is selectively titrated on the envelope of bacteria expressing OmpF having the same amyloidogenic sequence grafted into its extracellular loop 5 (L5). (B) 3D model of the OmpF β-barrel (cyan) embedded in the OM with the target amyloidogenic peptide (orange) docked, forming a β-sheet with the amyloid-grafted L5 in the porin (red).*

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Wegrzyn K, Zabrocka E, Bury K, Tomiczek B, Wieczor M, *et al*. Defining a novel domain that provides an essential contribution to site-specific interaction of Rep protein with DNA. Nucleic Acids Res 2021, 49, 3394-408.

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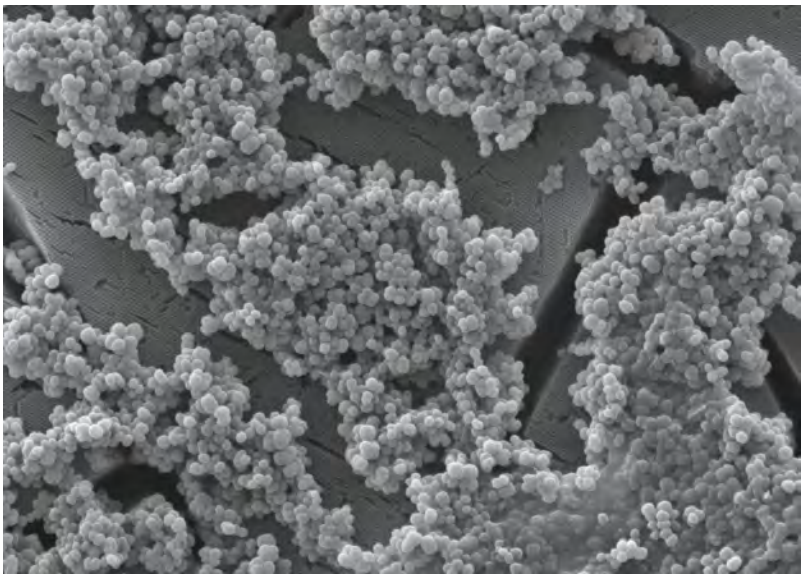
Molecular infection biology

A number of bacterial cell processes are confined in platforms termed functional membrane microdomains, some of whose organizational and functional features resemble those of lipid rafts of eukaryotic cells. How bacteria organise these intricate platforms and their biological significance remains an important question. This laboratory is a key group in the field of functional membrane microdomain bacterial compartmentalisation and its role during infections, using MRSA (Methicillin-resistance *Staphylococcus aureus*) as model organisms. Our research is supported by competitive funding, such as ERC-StG-2013 or H2020 RIA Biotech-03-2016.

We aim to identify the structure and molecular mechanisms that leads to bacterial membrane compartmentalisation and their role during staphylococcal infections that are resistant to antibiotic treatments. To do this, we work in the interface of molecular and cellular biology with other scientific disciplines, such as structural, infectious diseases, synthetic and systems biology. This interactive and multidisciplinary environment provides to the laboratory means to open new areas to study new mechanisms of bacterial infections and to discover new antimicrobial strategies to fight antibiotic resistance and multi-drug resistance pathogens, with special emphasis on those associated with hospital infections.



Electron microscopy image of S. aureus *cells growing attached to a surface*

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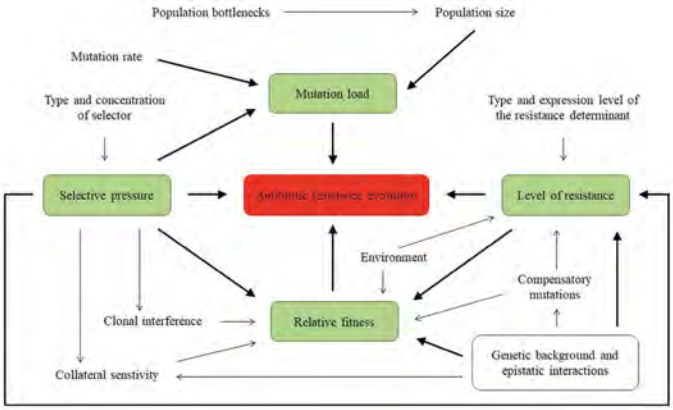
Ecology and evolution of antibiotic resistance

One of the most cumbersome human health problems is the increased prevalence of antibiotic resistant pathogens. As stated by the WHO, this is not just a problem dealing with regular infections. Several therapeutic procedures as transplantation, anti-cancer therapy or surgery require a good prevention and treatment of potential associated infections. Antibiotic resistance can comprise such treatments and, hence several aspects of current clinical practice. With few new antibiotics introduced into the market, a better use of those currently available is mandatory.

Our laboratory has been involved in the development of tools for predicting the pathways of emergence of antibiotic resistance. In addition of their predictive potential, the use of these tools has allowed to determine the emergence of some trade-offs associated to the selection of antibiotic resistance. These trade-offs constitute bacterial weaknesses that could be exploited for improving current anti-bacterial therapeutic strategies. Among them, we have explored collateral sensitivity and fitness costs. Collateral sensitivity consists on the increased susceptibility to one antibiotic when bacteria acquire resistance to a different one. This situation can be exploited to design sequential or combinatory treatments. However, its implementation requires that the phenotype is conserved in different bacteria and in different growing conditions. We have been able to identify some robust collateral sensitivity patterns that could be exploited for treating *Pseudomonas aeruginosa* infections. Regarding fitness costs, it is generally accepted that resistant bacteria can present a growth impairment in such a way that they will be outcompeted by their wild-type susceptible counterparts. However, it was soon evident that resistant bacteria can acquire compensatory mutations that restore their fitness. We have identified antibiotics that select mechanisms of resistance that impose high fitness costs. These fitness costs are compensated when bacteria grow in absence of the antibiotic, but resistance also declines. Further compensatory evolution preserves a conserved collateral sensitivity phenotype in a variety of strains indicating that this phenotype is robust.



Conceptual scheme bringing together the factors that constrain the evolution of antibiotic resistance and showing the interplay among them. *Four factors have a central effect on the evolution of antibiotic resistance: the relative fitness of the resistant mutant, the level of resistance conferred by the resistance mechanism, the strength of selection pressure, and the frequency of mutation. Genetic background and epistatic interactions have a major effect on antibiotic resistance evolution: resistance level, as well as the potential genetic modifications to compensate it, fitness cost and collateral sensitivity associated with a specific mutation, are strongly dependent on genetic background. The central factors are also influenced by other aspects, such as the emergence of compensatory mutations or the population size. Reproduced with permission from Pablo Laborda's PhD Thesis.*

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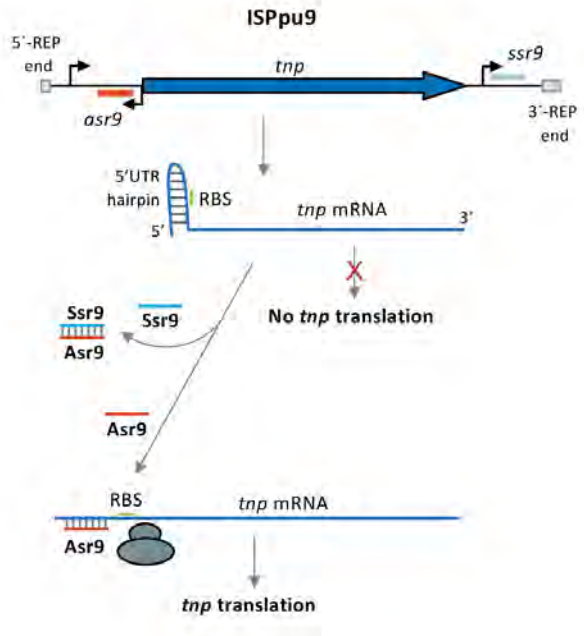
Regulation of gene expression and metabolism in bacteria

Pseudomonas putida KT2440 is a model bacterium that has become a valuable tool in biotechnology. It is not virulent, it is resistant to various types of stress, and has a great metabolic versatility. To optimise its performance, it is necessary to understand in detail the complex regulatory networks that coordinate the expression of its genome and the configuration of its metabolic fluxes. These regulatory processes optimise growth, but also limit the performance of many biotechnological processes. An additional problem derives from the elements that affect the stability of its genome. Strain KT2440 has a large genome with many insertion sequences, genomic islands and repeated sequence elements that can lead to mutations, rearrangements, or the deletion of large DNA segments. Obtaining stable strains useful in biotechnological processes requires understanding how the activity of mobile genetic elements is regulated. Our efforts are directed to address these problems.

The optimisation of metabolic fluxes relies on a regulatory network that includes the Crc and Hfq proteins, and two small RNAs named CrcZ and CrcY. Crc and Hfq inhibit translation of mRNAs containing a specific sequence motif within their translation initiation region, a process that is antagonised by CrcZ and CrcY. We have characterised the influence of these regulatory elements on *P. putida* physiology and the molecular mechanisms involved. Our interest on the Hfq protein led us to analyse its role in other processes such as iron homeostasis and the regulation of ISPpu9, an insertion sequence present in *P. putida* KT2440. We have observed that translation of the mRNA encoding the ISPpu9 transposase is inhibited by a highly structured 5' untranslated region, effect that is counteracted by an antisense small RNA and further modulated by a second small RNA. We are currently studying the influence of Hfq on this regulatory process.



Expression of the transposase gene (tnp) from the insertion sequence ISPpu9 present in Pseudomonas putida *KT2440 is inhibited by a strong secondary structure that blocks the translation of the tnp mRNA. The Asr9 small RNA binds to this region, weakening the secondary structure and facilitating translation. The Ssr9 small RNA likely counteracts Asr9, by binding to it.*

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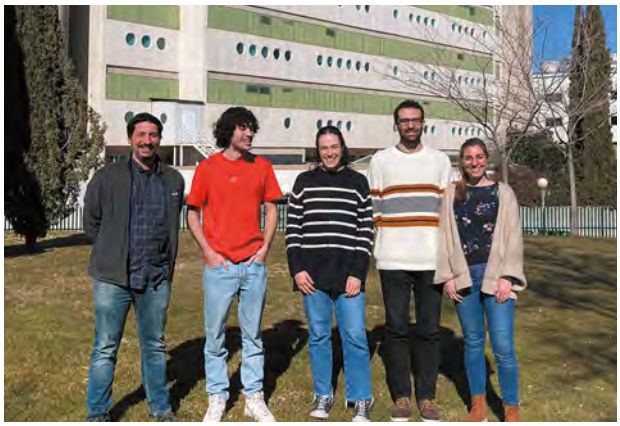

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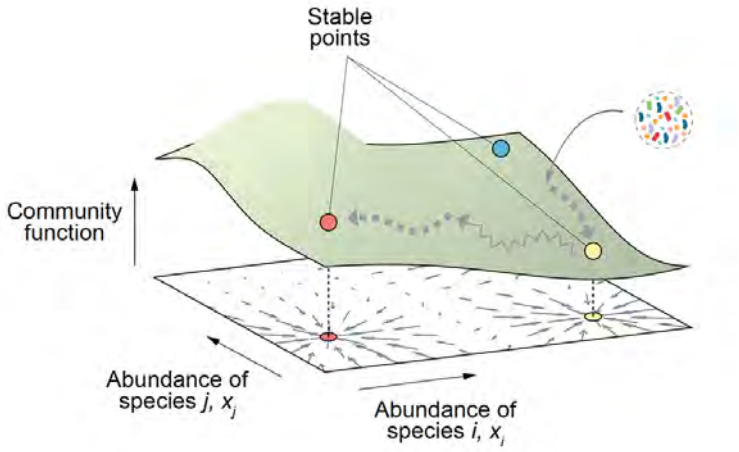
Microbial evolution and ecology

Our research centres on exploring how evolutionary biology may help us make sense, predict, and engineer microbial communities. To this end, we employ a combination of laboratory experiments, computer simulations, and mathematical modeling.

Our laboratory is highly interdisciplinary and quantitative in nature, and both predoctoral and postdoctoral researchers typically combine theory and experiment in their projects, acquiring a broad range of technical skills across the wet-lab / dry-lab divide. They also get exposed to a broad scientific intellectual background in ecology, evolution and systems biology.

We have two main research lines. The first consists of engineering microbial consortia from the top down, using evolutionary engineering. The second one consists of developing quantitative and predictive models linking microbial community composition and function.

This group joined the CNB in Summer 2022.



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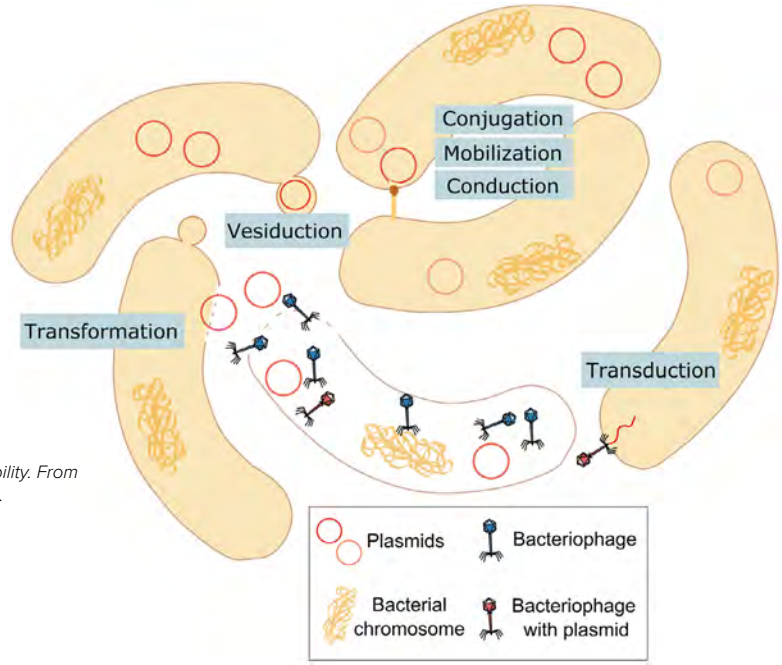
Plasmid biology and evolution

In the PBE lab we are interested in the evolutionary forces that drive plasmid dynamics in bacterial populations, with special focus in the evolution of antibiotic resistance in bacteria. Plasmids play a crucial role in bacterial ecology and evolution because they can transfer genes horizontally between different bacteria. The most striking example of how plasmids drive bacterial evolution is the global spread of plasmid-mediated antibiotic resistance over the last few decades. Plasmids are arguably the main vehicle for the spread of antibiotic resistance genes among clinically relevant bacteria, contributing to the overwhelming antibiotic resistance crisis we are currently facing. In our group we try to understand the population biology of antibiotic resistance plasmids using advanced molecular and evolutionary techniques. Ultimately, we intend to apply the concepts that we learn from the study of the evolution of plasmid-mediated antibiotic resistance to develop more rational intervention strategies to control infectious diseases.



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