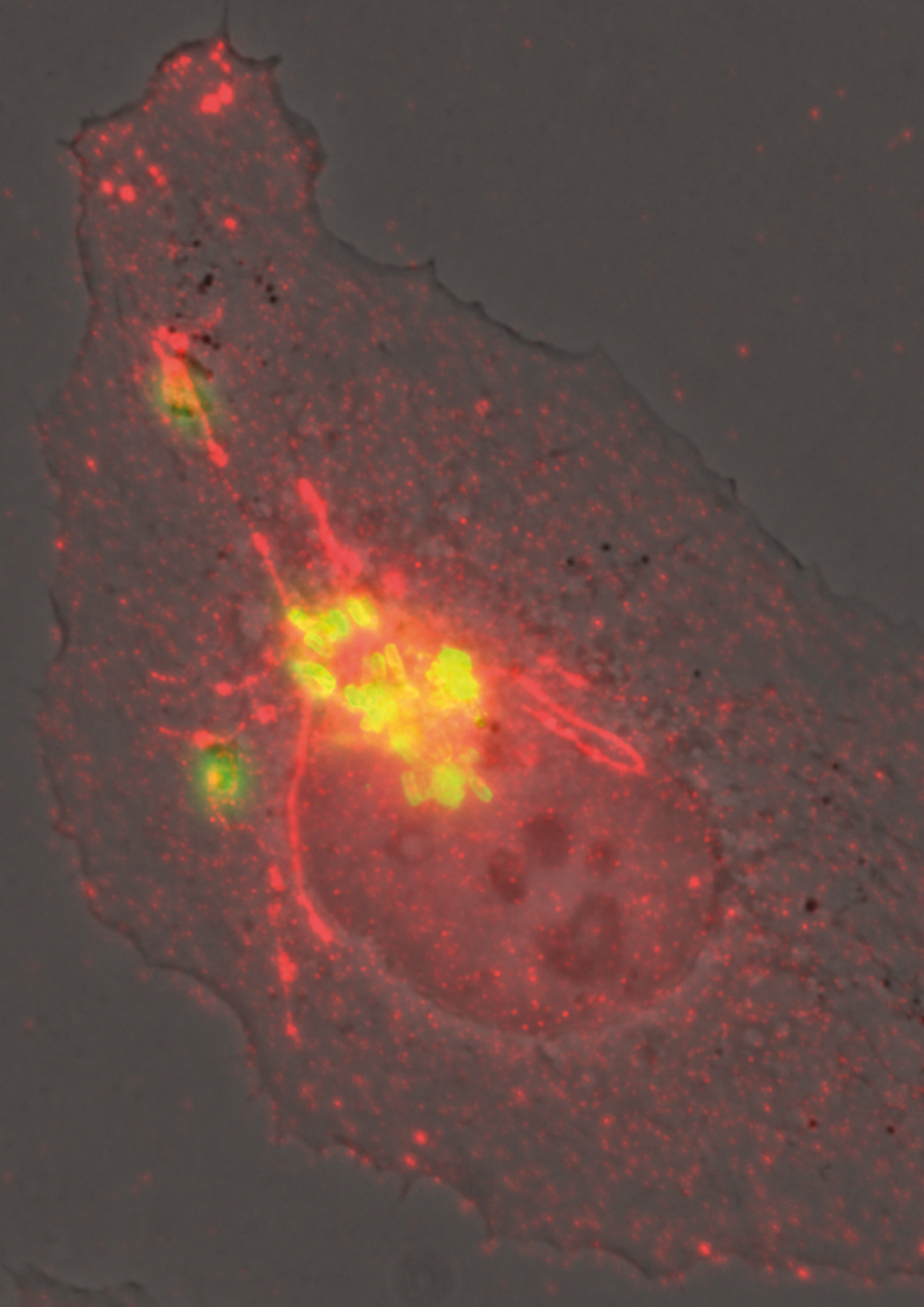

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MICROBIAL BIOTECHNOLOGY

Research on the Department of Microbial Biotechnology is focused on microbes with environmental, industrial or clinical relevance. Work includes several approaches based on molecular genetics, systems and synthetic biology, evolutionary biology, genomics, proteomics and metagenomics. The scientific objectives of the Department are focused on five complementary aspects of microbial biology:

- Environmental microbiology. We aim to characterise the mechanisms underlying the global regulation networks that modulate bacterial metabolism in response to fluctuating environmental conditions. We also study the mechanisms that contribute to horizontal gene transfer in the environment.
- Microbial pathogens. Efforts are directed to understand the host-pathogen interactions occurring in infections caused by different types of microorganisms; the molecular mechanisms underlying the development of bacterial infections are studied as well.
- Microbial resistance to antibiotics. Work aims to understand the evolutionary mechanisms that contribute to bacterial persistence and 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 bacterial infections.
- Microbial responses to hostile environments. Our focus is to understand 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 bacterial strains optimized to obtain products of interest such as antibodies, or to detect and degrade pollutants. In addition, we develop synthetic tools based on amyloids for biotechnological applications.

HEAD OF DEPARTMENT

Silvia Ayora

Secretion of an effector protein by intracellular Salmonella enterica *following infection of an epithelial cell. Shown in red is the signal corresponding to a bacterial effector protein secreted by the type III secretion system encoded in the* Salmonella*-pathogenicity island 2 (SPI-2). In green, signal of lipopolysaccharide (LPS) from intracellular bacteria. In the background, the phase contrast image. Note the translocation of the effector to filamentous structures that are a result of the infection and that are postulated to facilitate nutrient access to the pathogen. (Image from García del Portillo's lab).*

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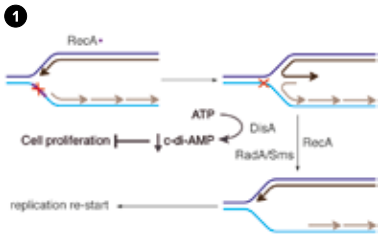
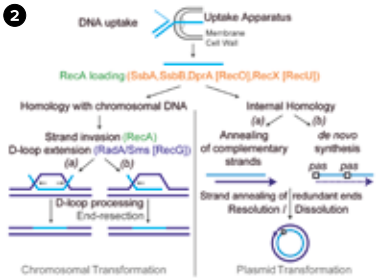
Genetic stability

Our research focuses on the study of the molecular mechanisms that secure genomic stability, promote horizontal gene transfer and control cell proliferation using *Bacillus subtilis* (a representative bacteria of the Firmicutes phylum) as a model. We have shown that the DNA damage response recruits different complex molecular machineries depending on the type of DNA damage and the growth conditions. When the replisome encounters a lesion on the template, the fork stalls and it needs to be stabilised to prevent fork collapse and genome instability. Here, DNA damage tolerance (DDT) subpathways assist fork progression, promoting replication fork reversal, template switching, lesion bypass or translesion DNA synthesis, and finally replication re-initiation. In the presence of a stalled (that mimics a displaced loop [D-loop]) or a reversed (a Holliday junction [HJ]-like structure) replication fork, the recombinase RecA binds to the lesion-containing DNA gap and loads the DNA damage checkpoint protein DisA and the fork remodeller RadA/Sms or RuvAB. DisA recognises and binds D-loop or HJ DNA and suppresses the synthesis of c-di-AMP, that in turn halts cell proliferation until the DNA damage is repaired. Moreover, it contributes to DDT pathways and prevents fork breakage (Fig. 1).

Horizontal gene transfer is a major prokaryotic evolution factor owing to its adaptive value and its power to restore genes inactivated by mutations. Thereby, it prevents the irreversible deterioration of genomes (known as Muller's ratchet). *B. subtilis* cells develop natural competence, with DprA (RecO in a Δ*dprA* strain, SsbA, SsbB, RecX (RecU in Δ*recX* cells), RadA/Sms (RecG in Δ*radA* cells) proteins helping RecA to promote the acquisition of exogenous DNA. Studying the functions that control RecA activities, we are addressing how mediators, modulators and D-loop remodellers contribute to the maintenance of the species and to the acquisition of HGT genes via natural plasmid transformation or viral transfection (Fig 2).





1 *A DDT mechanism. An unrepaired lagging-strand lesion (red cross) causes replication fork stalling. RecA-bound to the lesion-containing gap suppresses DisA dynamic movement and facilitates fork reversal. DisA bound to HJ DNA decreases c-di-AMP synthesis indirectly inhibiting cell proliferation. RecA bound to the nascent leading-strand loads RadA/Sms on the complementary nascent lagging-strand. RadA/Sms unwinds the nascent lagging-strand to provide a substrate for replication re-initiation, with DisA limiting RecA and RadA/Sms activities.*

2 *The DNA uptake apparatus of competent cells takes up linear single-stranded (ss) DNA. RecA, with the help of the mediators (DprA and SsbA) and modulator (RecX) forms a dynamic nucleoprotein filament on the incoming SsbA- and SsbB-coated ssDNA. The RecA filament searches for DNA homology. Once found, RecA initiates strand invasion to form D-loop intermediates (a, b) that are processed by a DNA helicase (RadA/Sms). The integrated strand is ligated, leading to a chromosomal transformant. RecA filamented on heterologous oligomeric plasmid ssDNA undergoes unsuccessful homology search and it must be disassembled. DprA (or RecO) interacts with other independently uptaken complementary strand and catalyses strand annealing, rendering tailed duplex DNA. An intramolecular recombination reaction carried out by DprA (or RecO) circularises the oligomeric plasmid molecule and after DNA replication, leads to plasmid transformation.*

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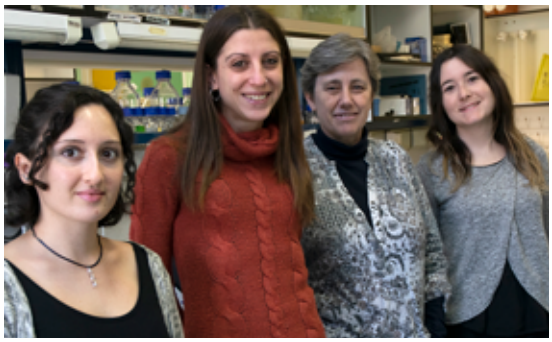

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Romero H, Torres R, Hernández-Tamayo R, Carrasco B, Ayora S, *et al.* Bacillus subtilis RarA acts at the interplay between DNA replication and repair-by-recombination. DNA Repair 2019; 78: 27-36.

Serrano E, Ramos C, Ayora S, Alonso JC. Viral SPP1 DNA is infectious in naturally competent Bacillus subtilis cells: inter- and intramolecular recombination pathways Environ Microbiol 2020; 22: 714-725.

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Recombination-dependent DNA replication

Our research focuses on the study of DNA replication mechanisms, specially on those that cells use to continue DNA replication when this process encounters impediments, which may stall or collapse the replication fork. Replication restart is then mediated by proteins that were initially identified by their roles in homologous recombination. We use *Bacillus subtilis* and its bacteriophage SPP1 as model systems, and biophysics, molecular biology and genetic techniques to study the recombination mechanisms that contribute to genome stability.

In the last years, we have focused on the study of conserved recombination proteins, as an AAA+ ATPase conserved from bacteria to humans. The WRNIP1/RarA AAA+ ATPases play a poorly understood role in the cellular response to blocked replication forks in pro- and eukaryotes. We have observed that RarA is sometimes associated with the replication fork even in the absence of DNA damage, performing a fork protection and regulatory role (Fig 1). We have also started to study the RecD2 helicase, which is the bacterial counterpart of the human helicase B, is also associated with the replisome, and interacts with WRNIP1/RarA.

Recombination also leads to evolution, and we have studied how recombination proteins may contribute to the acquisition of viral DNA or DNA from related species during natural transformation. A proteolysed bacteriophage might release its DNA into the environment. We have observed that RecD2 is required to resurrect an infective lytic phage from inactive environmental viral DNA. This protein, together with DprA, RadA, RecJ and RecX facilitates RecA-dependent gene acquisition from bacteria of related species (Fig 2).

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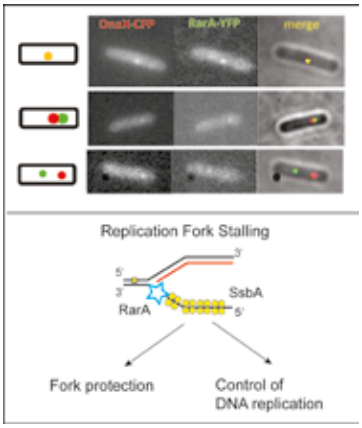

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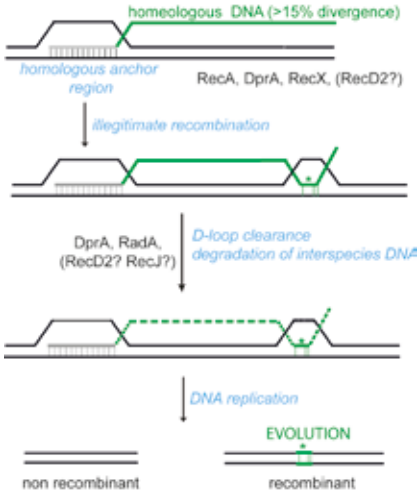

1 *Role of WRNIP1/RarA proteins. Epifluorescence microscopy shows colocalisation of the RarA protein with the clamp loader (DnaX) of the B. subtilis replisome in some cells. The lower image shows the model of RarA action at blocked forks. RarA and the singles-stranded binding protein SsbA bind to the collapsed forks and protect it from undesired recombination and control DNA replication restart.*

2 *Proposed mechanism of acquisition of DNA from related species during natural transformation. When B. subtilis competent cells acquire DNA with high sequence divergence from related species only some regions are fully homologous to the host chromosome. RecA, with the help of some accessory proteins uses this region to catalyse strand exchange and this recombination intermediate is used as an anchor region to facilitate an illegitimate recombination event in another region of the chromosome which leads to the acquisition of some nucleotides from the interspecies DNA. How the RecD2 helicase participates in this process is under study.*

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Stress and bacterial evolution

Our main goal is to understand the genetic mechanisms involved in bacterial genome stability and their roles in evolution and adaptation. We study the genetic basis of both stable and induced hyper-mutation/hyper-recombination as bacterial "strategies" to speed adaptation to stress, particularly to antibiotic stress. Recently we have described a novel non-canonical mismatch repair system in prokaryotes (present in some Archaea and most Actinobacteria), responsible for maintaining genome stability. Disentangle its genetic and biochemical bases in *Mycobacterium* and *Streptomyces* and its relation with the frequency of antibiotic resistance development in *Mycobacterium tuberculosis* is our commitment. This knowledge will be applied to i) understand and prevent the development of antibiotic resistance in this deadly bacterial pathogen and ii) improve prokaryotic species of biotechnological interest.

We collaborate in the development and analysis of new inhibitors of tolerance/resistance to antibiotics in different *Mycobacterium* species. Studies on new molecules to avoid antibiotic-mediated SOS mutagenesis in other bacteria (such as *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and on inhibitors of β-lactamase activity are also being developed.

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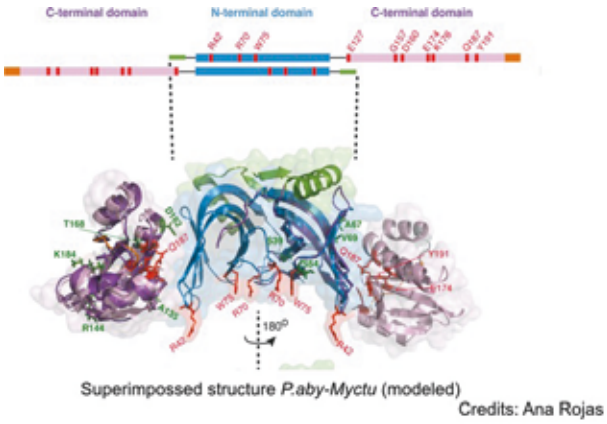
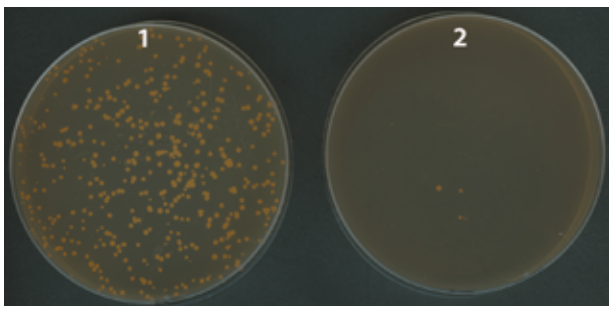


Superimpossed structure *P.aby-Myctu* (modeled)
Credits: Ana Rojas

❷



❶ *Domain characterisation of* M. tuberculosis *NucS, the key protein of the novel non-canonical mismatch repair system. Credits: Ana Rojas.*

❷ *NucS is a guardian of the genome stability in* Mycobacterium. *Increased production of mutant clones resistant to rifampicin in a* Mycobacterium smegmatis *strain lacking nucS gene (1) in comparison with the nucS-proficient strain (2).*

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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 recombinant antibodies and the design of bacteria for diagnostic and therapeutic use. We study protein secretion systems found in pathogenic *E. coli* strains, such as enteropathogenic *E. coli* (EPEC), and engineer them to develop protein nanomachines that can be applied for selection of recombinant antibodies and the delivery of therapeutic proteins by non-pathogenic *E. coli* strains. Among the recombinant antibodies, we employ camelid single-domain antibodies, called nanobodies, which are the smallest antibody fragments with full antigen-binding capacity. We use synthetic biology approaches and genome engineering to combine the expression of these modular parts in the designed bacteria.

In these two years we have been working in the following projects:

1) Expression and selection of nanobodies against pathogens and cancer. We have used *E. coli* surface display and protein secretion systems to screen immune libraries of nanobodies and to select high-affinity clones that, for instance, inhibit the adhesion of enterohemorrhagic *E. coli* (EHEC) to human intestinal cells. We have also selected nanobodies binding relevant antigens in cancer (e.g., EGFR, PD-L1). Further, we started an ongoing work to obtain neutralising nanobodies against SARS-CoV-2.

2) Engineering *E. coli* bacteria as anti-tumour agents. We have continued this synthetic biology project to modify a non-pathogenic *E. coli* chassis with synthetic adhesins and a type III protein secretion system (T3SS) to obtain bacteria with specific anti-tumour activities.

3) Accelerating protein evolution *in vivo*. We have developed a novel *in vivo* mutagenesis system in *E. coli*, called T7-DIVA, based on the recruitment of base deaminases to a target gene with T7 RNA polymerase. This system enables us to accelerate the directed evolution of proteins of interest, such as enzymes and antibodies.

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Álvarez B, Mencía M, de Lorenzo V, Fernández LA. *In vivo* diversification of target genomic sites using processive base deaminase fusions blocked by dCas9. Nature Commun 2020; 11: 6436.

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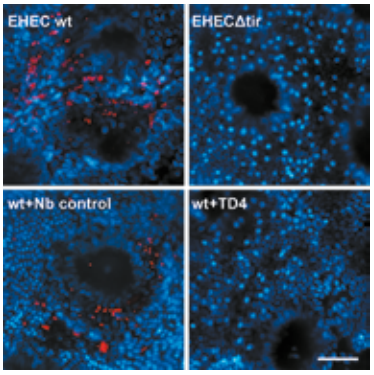

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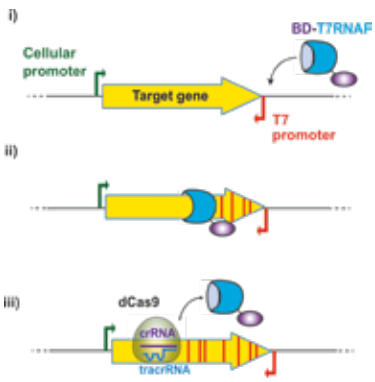

❶ *Attachment of enterohemorrhagic* E. coli *(EHEC) to human colonic biopsies is inhibited by nanobody TD4. Biopsy samples from the transverse colon were infected for 8 h with EHEC wild-type alone (EHEC wt) or in the presence of a nanobody against the extracellular domain of EHEC translocated intimin receptor TirM (wt + TD4) or a control nanobody binding amylase (wt + Vamy). Incubations with mutant EHECΔtir were included as negative control. Tissue samples were stained for EHEC (red) and cell nuclei (blue), bar = 50 µm.*

❷ *Schematic representation of the T7-DIVA in vivo mutagenesis system. The T7 RNA polymerase fusion to a base deaminase (BD-T7RNAP) binds the T7 promoter (i), initiating the transcription and moving along the target gene (yellow filled arrow) introducing mutations (red stripes) in the gene (ii). The fusion stops and detaches from the DNA when encounters a dCas9 molecule bound to a specific sequence determined by the CRISPR RNA (crRNA)(iii).*

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Laboratory of intracellular bacterial pathogens

Our lab is interested in deciphering how an important intestinal pathogen, *Salmonella enterica*, evolved to establish long lasting infections inside eukaryotic cells.

One of our major aims is to understand the changes in the cell wall that take place upon colonisation of the intracellular niche by this pathogen. We are especially interested in *Salmonella* proteins absent in non-pathogenic bacteria bearing activities that alter peptidoglycan structure. Although the peptidoglycan is sensed as a danger signal by the immune system, specific structural modifications may have the opposite effect and facilitate *Salmonella* persistence in the host, a common outcome in infections caused by this and other intracellular pathogens. Therefore, dissecting structural changes of the peptidoglycan triggered in response to eukaryotic signals as well as the responsible enzymes, it is of outmost relevance for designing new anti-infective strategies. In this line, we recently discovered new *Salmonella*-specific peptidoglycan synthases that promote cell elongation and division in the intracellular niche. Remarkably, these enzymes "replace" those that the same bacterium uses to elongate and divide outside the host cell. Such enzymatic switch illustrates the uniqueness of the *Salmonella* intracellular lifestyle in comparison to what it is normally observed in bacteria growing in artificial laboratory media. Some of these pathogen-specific enzymes, not detected in standard growth conditions and only visible *in vivo* in bacteria colonising host tissues, have low affinity for the antibiotics used in clinics.

Our future aims include:

- To unravel how *Salmonella* regulates the switch of peptidoglycan enzymes.
- The search for new drugs targeting these pathogen-specific enzymes.
- The identification of new peptidoglycan enzymes responding to intracellular cues.
- To study the evolution of distinct families of peptidoglycan enzymes.

In collaboration with Drs. Pucciarelli and Ortega, we are also pursuing studies focused on understanding regulation of the adaptative response of *Listeria monocytogenes* to cold.

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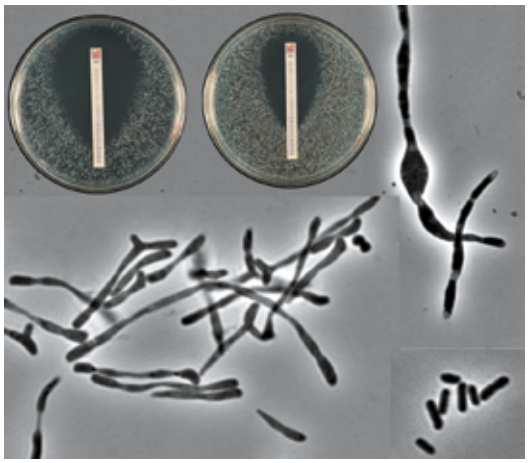

1 *Morphological alterations in* Salmonella enterica *serovar* Typhimurium *mutants lacking elements involved in the switch of PBP2 / PBP3 by the pathogen-specific enzymes PBP2SAL / PBP3SAL. Some of these mutants display increased resistance to antibiotics when grown in minimal media mimicking intracellular conditions*

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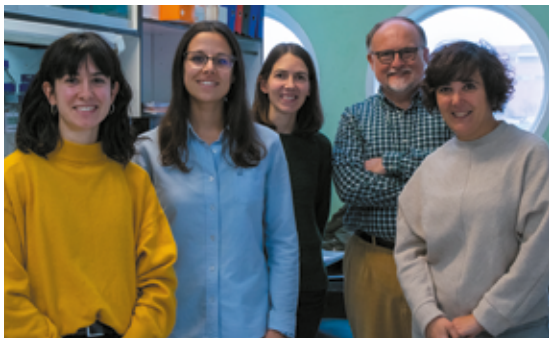

Synthetic bacterial amyloids

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

RepA is a protein from a bacterial plasmid whose WH1 domain undergoes conformational changes capacitating it as a transcriptional repressor, or as a DNA replication initiator or, through assembling amyloid oligomers, to hinder 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 two strains with distinct appearance and cytotoxicity, modulated by the Hsp70 chaperone DnaK. 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.

We have used RepA-WH1 as a benchmark for the design of synthetic tools to probe protein amyloidogenesis, including gold nanoparticles-based sensors, screening devices exploiting amyloid-promoted overriding of translation termination, both in yeast or in bacteria, and *in vitro* expression devices to address amyloidosis within cytomimetic lipid vesicles. Recently, control on RepA-WH1 amyloidogenesis has also 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. We are now exploring these devices as novel antimicrobials ('optobiotics').

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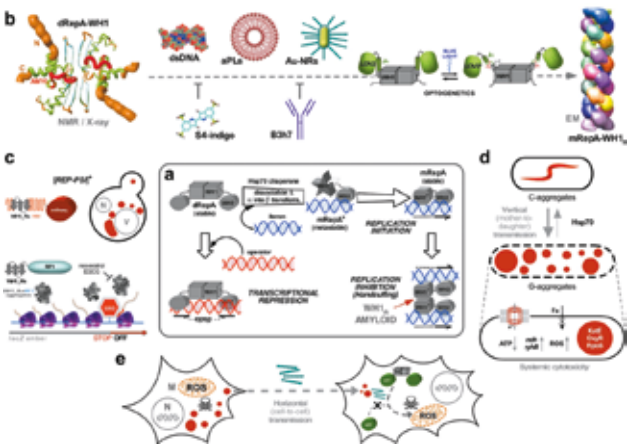

1 *A 'generic' synthetic model of amyloidosis engineered from bacterial RepA.*
(a) RepA is a dimeric transcriptional repressor that dissociates as monomers to initiate plasmid replication. Finally, through its WH1 domain assembles post-replicative, inhibitory amyloid oligomers.
(b) Fraying terminal helices in RepA-WH1 dimers prime RepA-WH1 dissociation and the assembly of the monomers as filaments, involving an amyloidogenic loop (red). Amyloidogenesis can be driven by DNA and acidic phospholipids (aPLs) or by gold nanoparticles (Au-NRs), and inhibited by S4-indigo, or by a conformation-specific antibody (B3h7). Fusion of a plant photosensor domain (LOV2) to the N-terminal helix in RepA-WH1 enables optogenetic modulation of amyloidogenesis: blue light illumination generates cytotoxic oligomers.
(c) The amyloidogenic stretch in RepA-WH1 can functionally replace prionogenic NM sequences in the yeast prion [PSI+]. The same repeats fused to E. coli RF1 enable stop codon read-through by ribosomes, counteracted by anti-amyloid compounds.
(d) RepA-WH1 is vertically inherited in E. coli *as two distinct amyloid strains: cytotoxic globular (G), or harmless comet-shaped (C) particles. Hsp70 chaperone detoxifies aggregates by favoring the C strain. G oligomers make pores at the inner membrane and enhance oxidative stress.*
(e) Horizontal spread of RepA-WH1 can be achieved in mammalian cells, but it is restricted by the need of its heterologous expression.

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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. My laboratory is a key laboratory in the field of functional membrane microdomain bacterial compartmentalization 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 resistance to antibiotic treatments. To do this, we work in the interface of molecular and cellular biology with other scientific disciplines, such as structural, infection, synthetic and systems biology. This interactive and multidisciplinary environment provides to my laboratory a 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.

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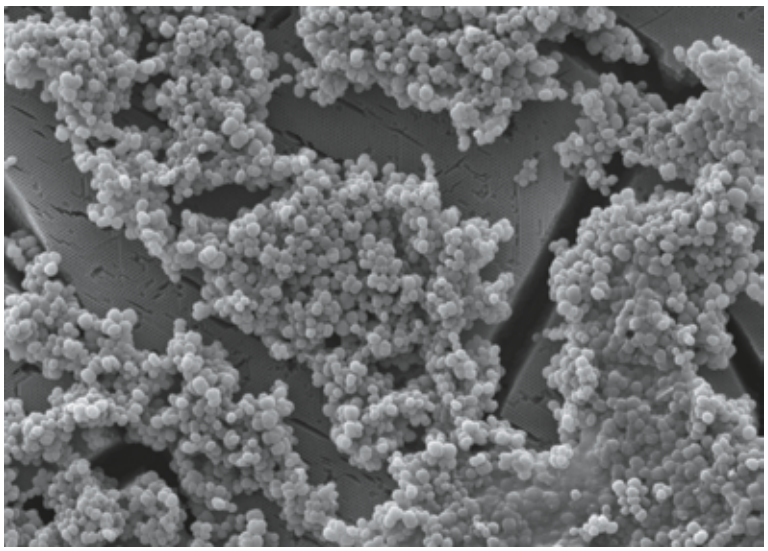
❶



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

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SELECTED PUBLICATIONS

Hernando-Amado S, Sanz-Garcia F, Martinez JL. Antibiotic resistance evolution is contingent on the quorum sensing response in *Pseudomonas aeruginosa*. Mol Biol Evol. 2019; 36 (10): 2238–2251.

Hernando-Amado S, Coque TM, Baquero F, Martinez JL. Defining and combating antibiotic resistance from One Health and Global Health perspectives. Nat Microbiol 2019; 4 (9): 1432-1442.

Ruppe E, Ghozlane A, Tap J, Pons N, Alvarez AS, *et al.* Prediction of the intestinal resistome by a novel 3D-based method. Nat Microbiol 2019; 4: 112-123.

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Ecology and evolution of antibiotic resistance

We study the biology of opportunistic pathogens, focusing on the networks and the evolutionary processes that connect resistance and virulence. In the last years, we have standardised some tools, based on experimental evolution, whole-genome sequencing and functional assays, for predicting the evolution of antibiotic resistance and the consequences of acquiring such resistance for bacterial physiology. Using these approaches, we characterised mechanisms of resistance to last-generation antibiotics and combination of them. Notably, acquisition of resistance is linked to changes in the susceptibility of other antibiotics besides those used for selection. In this regard, we have determined the networks of cross-resistance and collateral susceptibility associated to the acquisition of resistance to different antibiotics.

One important element in our studies is determining the elements that modulate the robustness and predictability of evolutionary trajectories towards antibiotic resistance of bacterial pathogens. Robustness is particularly relevant for exploiting the information concerning collateral susceptibility in order to implement more efficient therapeutic strategies based in antibiotic combinations or cycling. We have been working in this topic and have found some robust collateral susceptibility networks that will be explored in clinical strains in the next future. Among the elements that drive the evolution of antibiotic resistance from stochasticity to determinism, we are particularly interested in the epistatic interactions between elements involved in antibiotic resistance and virulence of bacterial pathogens. Besides mutation-driven resistance, we are studying compounds or conditions that can lead to transient resistance, as well as those that can increase the susceptibility to antibiotics and could hence be used as co-adjuvants in therapy.

A final aspect of our work concerns the One Health and Global Health aspects of antibiotic resistance. We contributed to the development of novel tools and metagenomic analyses for analysing the role of different non-clinical habitats in the evolution and spread of antibiotic resistance.

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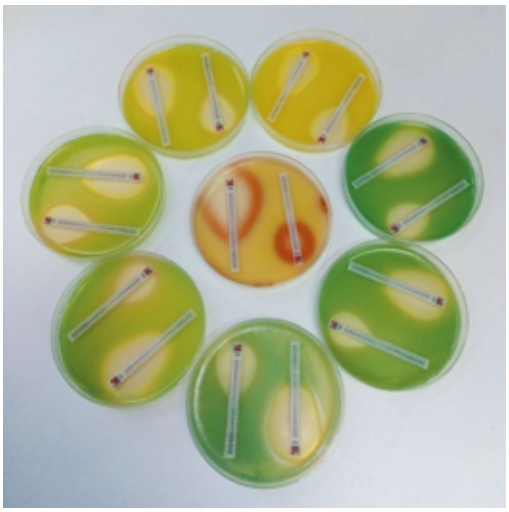

1 *Radiative evolution of antibiotic resistance in presence of antibiotics. Evolution is generally considered a very slow process. However, microorganisms, and bacteria in particular, reach a large number of generations in a very short period of time. This makes it possible to use Adaptive Laboratory Evolution (ALE) experiments to predict bacterial evolution in the presence of different selective pressures, such as antibiotics. This photograph shows the different changes in pigmentation and antibiotic resistance level (measured with E-Test strips), of the opportunistic pathogen* Pseudomonas aeruginosa, *after only 21 days of ALE in the presence of different concentrations of tobramycin.*

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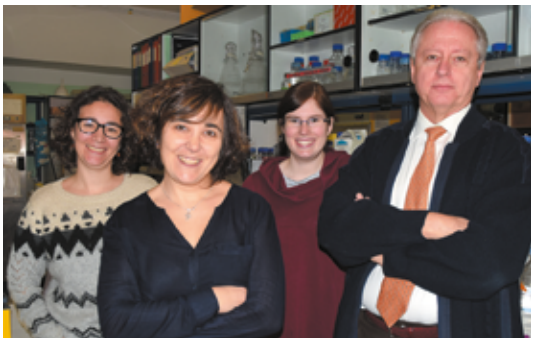

Heterologous gene expression and secretion in Gram-positive bacteria with industrial applications

Our group has a long-standing interest in the physiological and molecular characterisation of the protein secretory routes of the soil Gram-positive bacteria *Streptomyces lividans*, a well-known efficient producer of extracellular hydrolytic enzymes and other compounds of industrial application.

We have described and characterised the bacterial proteins (peptidyl-prolyl *cis-trans* isomerases and thiol-disulphide oxidoreductases) that are involved in the production of extracellular mature active proteins in both secretory pathways, the major Sec secretory pathway, and the minor Tat secretory pathway, which release unfolded and folded proteins respectively.

Four thiol-disulphide oxidoreductases are necessary for the formation of disulphide bonds when protein contains several disulphide bonds and surprisingly two of them are necessary in a protein devoid of disulphide bonds (Tat-dependent agarase) when it is overproduced, supporting the role of Sli-DsbA as a chaperone in the production of active agarase [Gullón S *et al*, 2019]. Additionally, we identified and characterised a Tat-dependent *S. lividans* FKBP-like lipoprotein, Sli-FKBP, that is involved in the folding of secretory proteins when they are overproduced, even in the proteins that are exported by the Tat pathway, so adjusting the level of expression of *sli-fkbp* may facilitate folding of dependent proteins [Vicente RL *et al*, 2019; Figure 1].

Additionally, we have described a dynamic flux balance analysis (DFBA) that adapt to the non-uniform time-dependent patterns that occurs in the protein secretion to study the metabolic changes induced by secretory protein overproduction [Valverde JR *et al*, 2019]. This will allow us to estimate the metabolic cost of that overproduction which, in turn, would enable us to design secretory protein production processes.

The obtained results would be applied first-hand at an industrial level for optimising scaling up secretory protein production, as well as favouring the design and construction of new and efficient secretory strains in *S. lividans*.

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Gullón S, Marín S, Mellado RP. Four thiol-oxidoreductases involved in the formation of disulphide bonds in the *Streptomyces lividans* TK21 secretory proteins. Microb Cell Fact 2019; 18: 126.

Vicente RL, Marín S, Valverde JR, Palomino C, Mellado RP, Gullón S. Functional identification of a *Streptomyces lividans* FKBP-like protein involved in the folding of overproduced secreted proteins. Open Biol 2019; 9: 190201.

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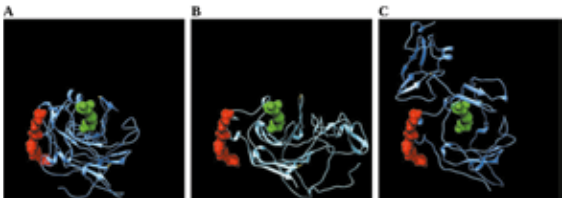

1 *Mature agarase with P127 and P183: both in cis conformation A) P125 in trans, P183 in cis B) both in trans conformation C). Agarose bound to the active site (green) and the allosteric site (red) are indicated. Between trans and cis forms exist differences in dimensions and surface charge. This modelling could explain the increase in agarase activity and the shift in SDS-PAGE mobility when Sli-FKBP is overproduced. Credits: JR Valverde*

IN MEMORIAM

Rafael Pérez Mellado (1950-2019), died suddenly the 27th of March 2019. Rafael was an innovative scientist, passionate about applying science as a way to improve society. He always displayed great intellectual concern that made him a visionary and led him to passionately explore all the activities he undertook.

After a long period at the Centre for Molecular Biology (CBM), he embarked on the beginning of the 80's, together with Victor Rubio and Francisco Malpartida, in the creation of the CNB and especially the Department of Microbial Biotechnology. He was Technical Vice Director for many years and had the difficult mission of organising the development of the CNB as one of the most important and influential center for innovation and translational research in Biology in Southern Europe.

Over the years, his laboratory was involved in a number of important applied projects, including the use of *Streptomyces lividans* as a cell factory. Additionally, he had active participation in numerous national and international commissions where his opinions were heard, and in many cases followed by the competent authorities. Particularly important in the last years was his work in the Ministry of Foreign Affairs and Cooperation as an recognised authority on biological security. In these lines, we want to pay homage to one of the legends of the CNB.

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Regulation of gene expression and metabolism in bacteria

To be competitive in the environments they colonise, bacteria must optimise metabolism to attain maximum gain from available nutrients. Not all potential carbon sources are equally effective in this respect. For this reason, when confronted with a mixture of potentially assimilable compounds at sufficient concentrations, many bacteria preferentially use one of them, leaving others aside until the preferred one is consumed. This implies a complex regulatory process termed catabolite repression. Unravelling the molecular mechanisms involved helps understanding how bacteria coordinate their metabolism and gene expression programs and optimise growth. It also aids in the design and optimisation of biotechnological processes and to understand how bacteria degrade compounds in Nature.

The regulators and molecular mechanisms responsible for catabolite repression differ among microorganisms. Our work is focused on *Pseudomonas putida*, a bacterium with a versatile and robust metabolism much used in biotechnology. Catabolite repression relies on a complex regulatory network that includes the Crc and Hfq proteins, which inhibit translation of mRNAs containing a specific A-rich sequence motif within their translation initiation region. Two small RNAs, CrcZ and CrcY, the levels of which vary greatly depending on growth conditions, antagonise the inhibitory effect of Hfq and Crc. Our aim is to characterise the influence of Crc, Hfq, CrcZ and CrcY in the physiology of *P. putida*, the signals to which they respond, and the molecular mechanisms by which they regulate gene expression.

In addition, we have analysed the role of Hfq in other processes such as iron homeostasis and the regulation of ISPpu9, an insertion sequence of *P. putida* KT2440 in which we have observed that translation of the transposase gene mRNA 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 RNAs.

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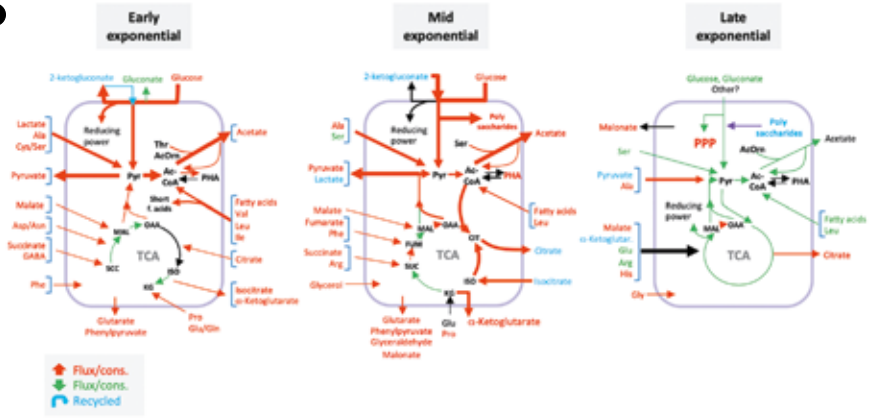

1 *Effect of inactivating the crc gene on the configuration of the metabolite fluxes related to central carbon metabolism during early, mid and late exponential growth. The fluxes that increased (in red), decreased (in green), or remained unchanged (in black) in the Crc-null strain as compared to the wild type, are highlighted. Compounds that were released to the medium and later recycled are indicated in blue.*

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SELECTED PUBLICATIONS

RC MacLean & A San Millan. The evolution of antibiotic resistance. Science 2019; 365(6458): 1082-1083.

Rodríguez-Beltrán J, Sørum V, Toll-Riera M, de la Vega C, Peña-Miller R, San Millan A. Genetic dominance governs the evolution and spread of mobile genetic elements in bacteria. Proc Natl Acad Sci USA. 2020; 117 (27): 15755-15762.

JH DelaFuente, J Rodríguez-Beltrán, A San Millán. Methods to study fitness and compensatory adaptation in plasmid-carrying bacteria. Methods Mol Biol 2020; 2075: 371-382.

Plasmid biology and evolution

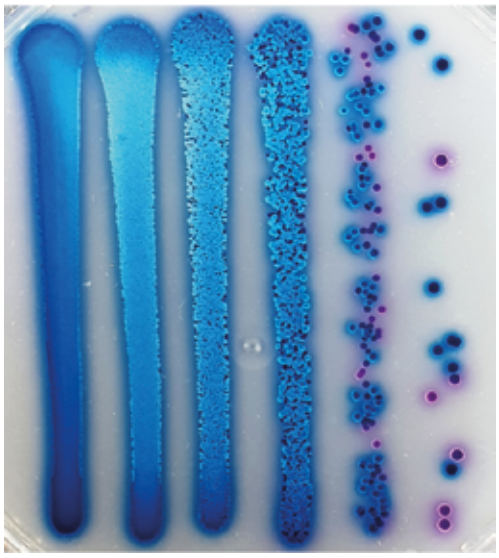
We are interested in the evolutionary forces that drive plasmid dynamics in bacterial populations as well as in the impact of plasmids in bacterial ecology and evolution.

Plasmids play a crucial role in bacterial evolution because they can transfer genes horizontally between different cells. 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.

The evolutionary impact of plasmids goes beyond horizontal gene transfer. Plasmids are usually kept at multiple copies per bacterial cell, producing islands of polyploidy in the genome. In the Plasmid Biology and Evolution lab, we are extremely interested in understanding how the multicopy nature of plasmids affects bacterial evolution. Our recent works revealed that multicopy plasmids are able to accelerate gene evolution and maintain allelic diversity, acting as catalysts of bacterial evolution.

This group joined the CNB in July 2020.

❶



❶ *Carbapenem-resistant* Klebsiella pneumoniae *(blue) and* E. coli *(pink) colonies growing on selective agar.*

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SELECTED PUBLICATIONS

Natale P, and Vicente M. (2021) Bacterial Cell Division. In: eLS. John Wiley & Sons, Ltd. Chichester doi.org/10.1002/9780470015902.a0000294.pub3

Genetic control of the cell cycle

The reconstruction of the divisome, the machinery responsible for bacterial division, in the test tube serves to verify that the description of the mechanisms that ensure proliferation is correct and also to use it in the design of new drugs to stop infections. The assembly of the divisome begins with the positioning in the middle of the cell length of a proto-ring in which ZipA contributes, together with FtsA, to place FtsZ, a protein that forms a contractile division ring, in the right place. The reconstruction of ZipA and FtsZ complexes inside artificial vesicles mimics the contractile property of the proto-ring, showing that the vesicles shrink when a GTP analog, an essential compound for the polymerisation of FtsZ, is added.

In addition to providing anchoring of FtsZ to the membrane, ZipA has a role to prevent its degradation.

In *Escherichia coli*, FtsA and FtsZ are encoded by genes located adjacently within the division and cell wall *dcw* cluster. Some peculiar genetic regulatory mechanisms including transcription from a sigma S dependent gearbox promoter operate in the cluster to produce proteins as FtsA and FtsZ that are used to assemble the proto-ring once per cell cycle. On the other hand, the *zipA* gene maps at a different chromosomal region, at min 54.54. We find that the expression of *zipA* is under the control of a housekeeper, and not a gearbox, promoter. The housekeeper regulation suggests that ZipA may play additional roles besides the anchoring of FtsZ to the cytoplasmic membrane. In fact, in addition to anchoring and stabilising FtsZ, ZipA forms part of the cytoplasmic membrane where it needs to be inserted in precise amounts to avoid adverse effects. Furthermore, the disruption of this promoter reduces ZipA protein production by 60% and leads to cell filamentation.

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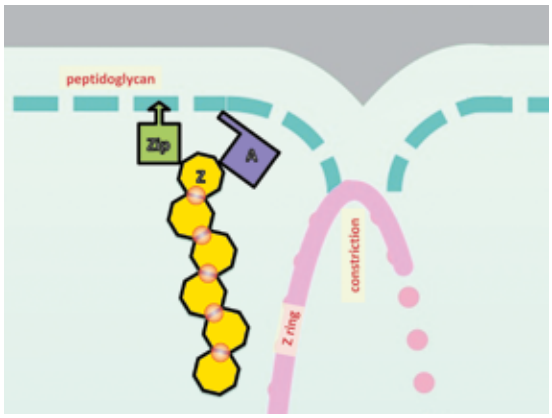

1 *The* E. coli *proto-ring. The interactions of FtsZ (Z), a cytoplasmic protein, with the proto-ring anchors, FtsA (A) and ZipA (Zip) serve to associate it to the cytoplasmic membrane forming the proto-ring, the initial precursor of the division machinery. The FtsZ monomers form polymers in which GTPase active sites, represented as red circles, are formed in the intersection between two monomers. The hydrolytic activity is needed for the function of the FtsZ ring in septation, probably by fuelling constriction of the envelope. The discontinuous blue line represents the cytoplasmic membrane. The division ring is depicted in pink. The location of the peptidoglycan layer is marked but not depicted.*