04 Microbial Biotechnology

The Department of Microbial Biotechnology integrates research to gain knowledge of key aspects of microbial biology with environmental, clinical or biotechnological relevance. The department hosts ten groups that use several complementary aspects of microbial biology, with approaches that include molecular genetics, genomics, proteomics and metagenomics. The subjects studied include:

Environmental microbiology. We aim to understand how microorganisms degrade organic compounds that generate environmental problems, as well as why a microorganism that can efficiently degrade a compound of interest in laboratory conditions does not usually perform so well in natural conditions. We also study the rhizobial communities of transgenic plants and the effect of herbicides on these communities.

Microbial responses to hostile environments. The focus is to understand bacterial responses to stressful environments, including the reaction to host defence responses and to agents that cause DNA damage. We study how microbial pathogens avoid host defence mechanisms, and how bacteria and fungi replicate DNA and repair DNA damage to improve genome stability.

Microbial pathogens. Efforts are directed to understand how microbial pathogens infect eukaryotic hosts, with emphasis on host-pathogen interactions in infections caused by intracellular bacteria and by opportunistic pathogens. This will facilitate discovery of new targets to combat infections.

Microbial resistance to antibiotics and search for new antimicrobials. We focus on mechanisms of bacterial resistance to antibiotics and analyse the complex responses elicited by exposure of microbes to sublethal concentrations of antibiotics. In addition, we search for novel targets in essential functions to develop drugs to fight pathogens.

Microbial engineering. The purpose is to generate bacterial strains optimised to obtain products of interest, such as recombinant antibodies or hydrolytic enzymes, or to detect and degrade pollutants. We also work to understand the mechanisms bacteria use to export and secrete proteins, which could help in the development of bacterial recombinant strains to be used as delivery systems in the treatment of human diseases.

These topics are highly interconnected. While most established pathogens normally cope with stress conditions by developing efficient adaptive responses, the opportunistic pathogens are metabolically very versatile, making them efficient biodegraders of pollutants. Fighting against pathogenic microorganisms requires deep understanding of their behaviour during infection and of how resistance develops as pathogens are challenged by antibiotics.

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SELECTED PUBLICATIONS

Soberón NE, Lioy VS, Pratto F, Volante A, Alonso JC. Molecular anatomy of the Streptococcus pyogenes pSM19035 partition and segrosome complexes. Nucleic Acids Res. 2011. 39(7):2624-37

Büttner CR, Chechik M, Ortiz-Lombardía M, Smits C, Ebong IO, Chechik V, Jeschke G, Dykeman E, Benini S, Robinson CV, Alonso JC, Antson AA. Structural basis for DNA recognition and loading into a viral packaging motor. Proc Natl Acad Sci USA. 2012. 109(3):811-6

Chiesa M, Cardenas PP, Otón F, Martinez J, Mas-Torrent M, Garcia F, Alonso JC, Rovira C, Garcia R. Detection of the early stage of recombinational DNA repair by silicon nanowire transistors. Nano Lett. 2012. 12(3):1275-81

Yadav T, Carrasco B, Myers AR, George NP, Keck JL, Alonso JC. Genetic recombination in *Bacillus subtilis*: a division of labor between two single-strand DNA-binding proteins. Nucleic Acids Res. 2012. 40(12):5546-59

Cárdenas PP, Carrasco B, Defeu Soufo C, César CE, Herr K, Kaufenstein M, Graumann PL, Alonso JC. RecX facilitates homologous recombination by modulating RecA activities. PLoS Genet. 2012. 8(12):e1003126

Genetic stability

Our research focusses on the study of the mechanisms used by bacteria of the phylum Firmicutes to secure the structural and segregational stability of genetic information. *Bacillus subtilis* cells are used to study repair by recombination and plasmid segregational stability during vegetative growth, and genetic recombination during natural competence. Using DNA repair by recombination, we have shown that:

1. the DNA damage response recruits complex molecular machinery; among the first responders is the RecN protein, which in concert with PNPase promotes the dynamic recruitment of DNA ends

2. AddAB or RecJ, together with the RecQ or RecS helicase and SsbA, create a 3' single-stranded (ss) DNA tailed duplex at the DNA breaks, and RecN recruits recombination proteins to form a repair centre

3. different accessory proteins (*e.g.,* RecFOR, SsbA) regulate RecA filament formation to repair the DNA breaks and the branch migration helicase RuvAB, in concert with the resolvase (RecU), processes the recombination intermediates.

Using genetic recombination, we have shown that:

1. different mediators (*e.g.,* RecO, DprA) regulate RecA nucleation onto the taken up ssDNA, coated by SsbA and/or SsbB during chromosomal transformation

2. RecO- or DprA-mediated strand annealing contributes to circularisation of the incoming plasmid ssDNA during plasmid transformation

3. different RecA modulators (*e.g.,* RecF, RecX, RecU) shut off RecA-mediated DNA strand exchange.

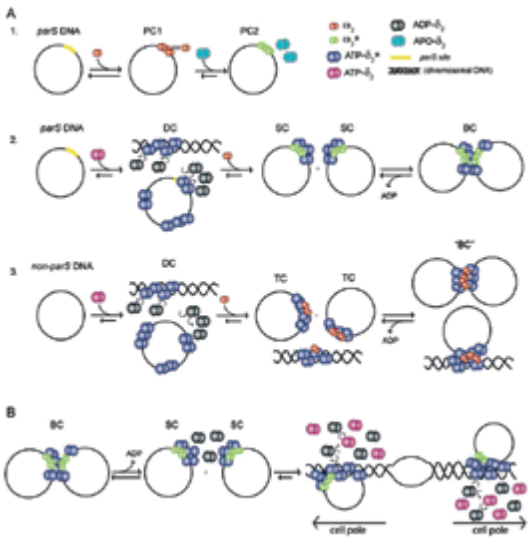
Using the segregation model, we have shown that:

1. the pSM19035 global regulator ω (ω2) controls the fine-tuning of plasmid copy number, plasmid segregation, and the toxin-antitoxin (TA) system

2. the partitioning protein ω2 binds to a centromeric-like region (PC1 and PC2) and the dimeric δ (δ2) ATPase facilitates ω2 recruitment onto parS DNA, leading to a segrosome complex (SC), and this complex facilitates the formation of bridging complexes (BC) and δ2-mediated hydrolysis of ATP

3. the ATP hydrolysis leads to δ2 disassembly from DNA, and the dynamic assembly/disassembly moves the plasmid molecule towards the cell poles to guarantee faithful segregation

4. when partition fails, the TA system halts proliferation of plasmid-free cells, with subsequent overgrowth of plasmid-bearing cells.



1 *Dynamic assembly of different types of protein·DNA complexes*

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SELECTED PUBLICATIONS

Ayora S, Carrasco B, Cárdenas PP, César CE, Cañas C, Yadav T, Marchisone C, Alonso JC. Double-strand break repair in bacteria: a view from *Bacillus subtilis*. FEMS Microbiol Rev. 2011 Nov;35(6):1055-81

Cañas C, Carrasco B, García-Tirado E, Rafferty JB, Alonso JC, Ayora S. The stalk region of the RecU resolvase is essential for Holliday junction recognition and distortion. J Mol Biol. 2011 Jul 1;410(1):39-49

Lo Piano, A., Martínez- Jiménez, M.I., Zecchi, L., & Ayora S. (2011). Recombination- dependent concatemeric viral DNA replication. Virus Research, 160, 1 – 14

Zecchi L, Lo Piano A, Suzuki Y, Cañas C, Takeyasu K, Ayora S. Characterisation of the Holliday junction resolving enzyme encoded by the *Bacillus subtilis* bacteriophage SPP1. PLoS One. 2012;7(10):e48440

Kidane D, Ayora S, Sweasy JB, Graumann PL, Alonso JC. The cell pole: the site of cross talk between the DNA uptake and genetic recombination machinery. Crit Rev Biochem Mol Biol. 2012 Nov-Dec;47(6):531-55

Recombination-dependent DNA replication

Our research focusses on the mechanisms that cells use to continue replication progress when this process encounters impediments to DNA replication, which may eventually collapse the fork, producing a broken DNA end. Replication restart is then mediated by proteins that were initially identified by their roles in homologous recombination and repair of DNA double-strand breaks (DSB). Accurate repair of DSBs is essential to life. Study of the recombination mechanisms has shown the complexity of the recombination process, due to the large numbers of proteins involved. We thus use a simple model system, *Bacillus subtilis* and its bacteriophage SPP1, and several biophysics, structural and molecular biology techniques to decipher these complex mechanisms (Ayora *et al.*, 2011; Lo Piano *et al.,* 2011).

One process we study in depth is the resolution of a central homologous recombination intermediate, the Holliday junction structure. In the last two years, we explored the role of the stalk region in the RecU Holliday junction resolvase, and show that it is essential for interaction with RecA and for recognition and specific binding to a Holliday junction structure (Cañas *et al.*, 2011 J. Mol. Biol., 410:34-49). Resolution of a Holliday junction is also an essential step during the shift from theta to concatemeric replication in the phage SPP1. We characterised the SPP1-encoded Holliday junction resolvase. Our analyses showed that G44P, which has limited similarity to other Holliday junction resolvases, cleaves Holliday junctions and replicated D-loops, and might participate in recombination-dependent DNA replication (Zecchi *et al.*, 2012 PLoS ONE, 7, e48440).

One goal of our group is the *in vitro* reconstitution, using purified SPP1 replication and recombination proteins, of the mechanisms that operate at a stalled replication fork and lead to replication restart.

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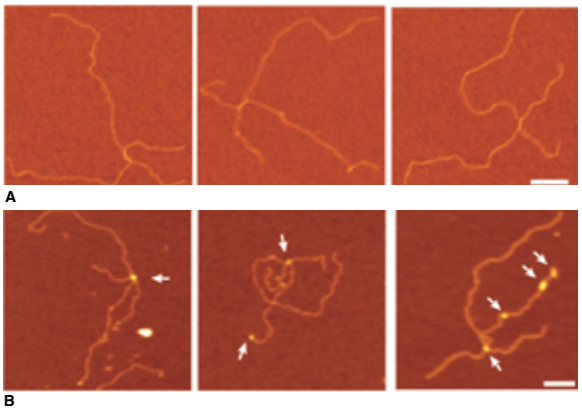

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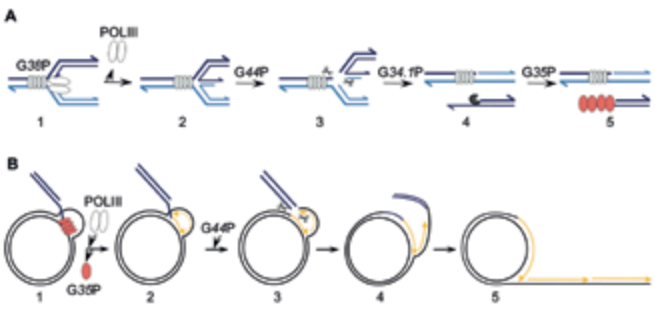

1 *Visualisation by atomic force microscopy of the Holliday junction structure (A) and binding of the G44P protein to this recombination intermediate (B). G44P bound to the junction as well as to the dsDNA arms. Arrows denote protein-DNA complexes. Scale bar = 100 nm.*

2 *Recombination-dependent DNA replication (RDR) in the SPP1 virus generates concatemeric linear DNA. (A) The generation of a double-strand break (DSB) triggers RDR (B).*

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SELECTED PUBLICATIONS

Thi TD, López E, Rodríguez-Rojas A, Rodríguez-Beltrán J, Couce A, Guelfo JR, Castañeda-García A, Blázquez J. The effect of recA inactivation on mutagenesis of *Escherichia coli* exposed to sub-lethal concentrations of antimicrobials. J Antimicrob Chemother. 2011 Mar;66(3):531-8

Rodríguez-Rojas A, Oliver A, Blázquez J. Intrinsic and environmental mutagenesis are key components of Pseudomonas aeruginosa chronic lung infections. J Infect Dis. 2012;205 (1):121-7

Rodríguez-Beltrán J, Rodríguez-Rojas A, Guelfo JR, Couce A, Blázquez J. The *Escherichia coli* SOS gene dinF protects against oxidative stress and bile salts. PLoS One. 2012;7(4):e34791

Blázquez J, Couce A, Rodríguez-Beltrán J, Rodríguez-Rojas A. Antimicrobials as promoters of genetic variation. Curr Opin Microbiol. 2012 Oct;15(5):561-9

Couce A, Guelfo JR, Blázquez J. Mutational spectrum drives the rise of mutator bacteria. PLoS Genet. 2013 Jan;9(1):e1003167

Stress and bacterial evolution

The major interest of the group is to understand the bacterial responses to stress. We specifically study hypermutation and hyper-recombination as "bacterial strategies" to speed adaptation to environmental stresses. One of the models used here is antibiotic stress and the development of antibiotic resistance. Our work is focussed on both stable and inducible hypermutation/hyper-recombination in *E. coli*, *P. aeruginosa* and *M. smegmatis/tuberculosis*.

We are currently studying:

1. Compensation of stable hypermutation. Once adapted, hypermutable bacteria must decrease (compensate) the high mutation rate to avoid accumulation of deleterious mutations. Our study aims to determine the molecular mechanisms involved in this compensation.

2. Regulation of stress responses and inducible hypermutation

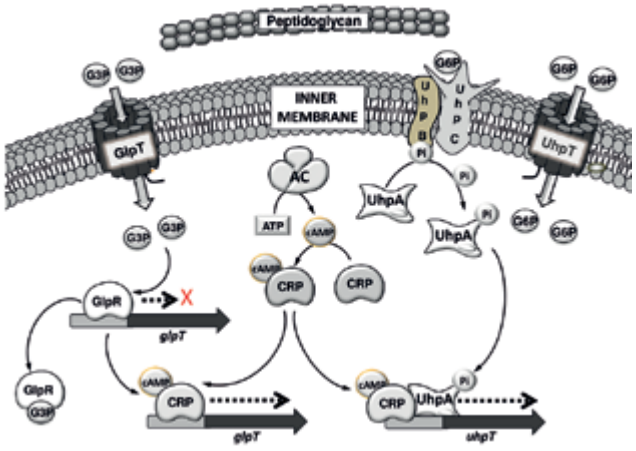
- Environmental regulation of mutagenesis
- Transcriptional regulation of specialised DNA polymerases (belonging to the SOS regulon)
- Effect of antibiotics on mutation and recombination: are antibiotics promoters of antibiotic resistance?

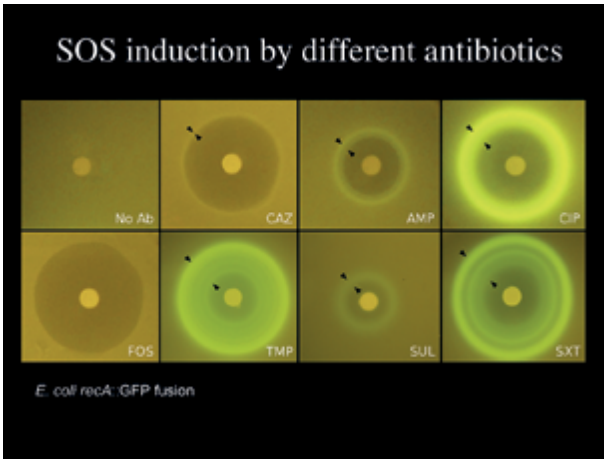
3. Hypermutation in bacteria lacking a DNA mismatch repair system (MMR) such as Mycobacterium and Streptomyces. This will allow the use of hypermutant/hyper-recombinant bacteria of industrial interest as biotechnological tools to produce modified biosynthetic pathways.

4. Evolution of resistance to beta-lactam antibiotics and the development of new inhibitors of beta-lactamases

5 The molecular basis of bacterial evolution. Combatting antibiotic resistance by preventing evolution (mutation, recombination and horizontal transfer).

1 *Regulation of GlpT and UhpT.*





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SELECTED PUBLICATIONS

Merchan S, Pedelini L, Hueso G, Calzada A, Serrano R, Yenush L. Genetic alterations leading to increases in internal potassium concentrations are detrimental for DNA integrity in *Saccharomyces cerevisiae*. Genes Cells (2011) vol. 16 (2) pp. 152-65

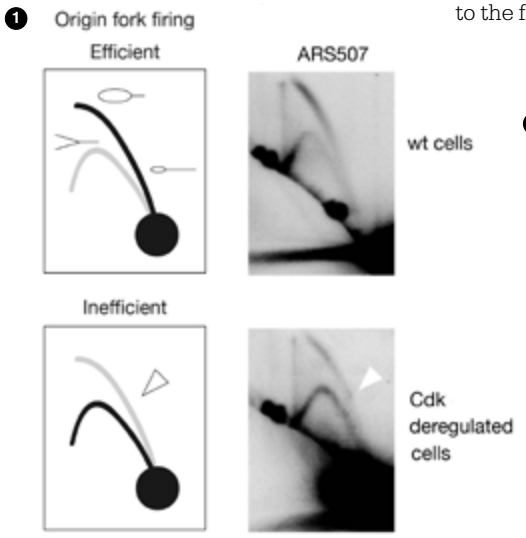
Cell cycle, DNA replication and genome stability in eukaryotes

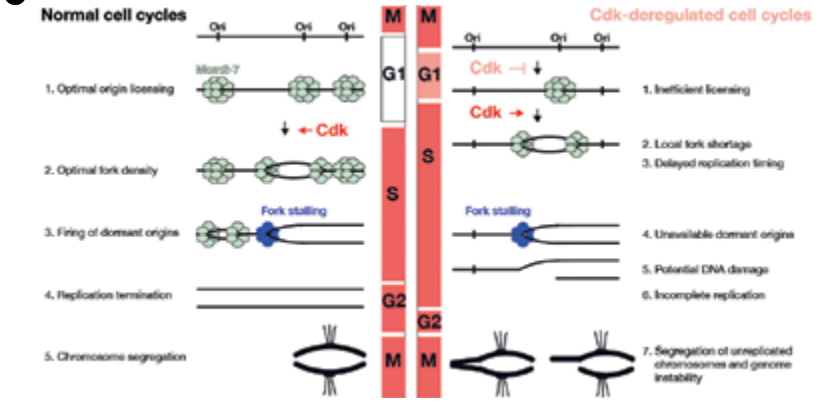
We study the regulation of chromosomal replication, the process that duplicates the genome during each cell division, by the cell cycle machinery that ensures correct division of the mother cell into two daughter cells. Both processes must be synchronised for stable genome transmission to progeny, and deregulation during DNA replication or other errors are mutagenic and have disease-causing potential. We use the model eukaryotic organism *Saccharomyces cerevisiae*, which shows conservation of numerous cell biology processes throughout eukaryotic evolution.

How replication is regulated to terminate correctly, and what molecular aberrations occur during abnormal, disease-causing cell cycles is largely unknown. Both questions are the focus of our research. Cells prepare DNA replication during the preceding G1 phase, in which they test the environmental conditions and enter the cell cycle or remain arrested. Impairing this schedule is deleterious for cells and increases genome instability, thought to be mediated by S phase abnormalities. We found that altering potassium homeostasis impairs normal cell cycle progression in G1, reduces the efficiency of DNA replication, requires the DNA damage response pathway, and is detrimental to genome integrity. This also applies during tumourigenic cell cycles. Cyclin-dependent kinases (Cdk) regulate progression through G1 and are frequently deregulated in cancer cells. These cells show chromosome instability and unscheduled G1/S transition and S phase progression, but the molecular aberrations during replication and how they contribute to instability is little known. We studied these questions in Cdk-deregulated yeast cells and found decreased distribution and density of origins at a chromosome arm. Origins should be widespread in large numbers along chromosomes for complete, stress-resistant DNA replication and prevention of genome instability; consistent with this, we found that the decreased origin density correlates directly with chromosome instability in the region. Origin activity is also altered in p27$^{-/-}$ MEF (murine embryonic fibroblasts). Finally, in both eukaryotes, origins are differentially compromised by Cdk deregulation, which supports non-uniform chromosome instability during tumorigenesis. Origin shortage might thus contribute to the fragility of certain chromosome regions frequently observed during oncogenesis.

1 *Two-dimensional DNA (2D) gels of replication intermediates to measure the activity and efficiency of DNA.*

2 *Schedule of cell cycle progression, and dynamics of replication and segregation of a given chromosome region in normal and Cdk-deregulated cells, as suggested by our work.*





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SELECTED PUBLICATIONS

Palomino C, Marín E, Fernández LÁ. The fimbrial usher FimD follows the SurA-BamB pathway for its assembly in the outer membrane of *Escherichia* coli. J Bacteriol. 2011 Oct;193(19):5222-30

Fernández LÁ, Muyldermans S. Recent developments in engineering and delivery of protein and antibody therapeutics. Curr Opin Biotechnol. 2011 Dec;22(6):839-42

Jurado P, Fernández LA, de Lorenzo V. Production and characterisation of a recombinant single-chain antibody (scFv) for tracing the sigma 54 factor of *Pseudomonas putida*. J Biotechnol. 2012 Jul 31;160(1-2):33-41

Bodelón G, Palomino C, Fernández LÁ. Immunoglobulin domains in *E. coli* and other enterobacteria: from pathogenesis to applications in antibody technologies FEMS Microbiol Rev. 2012 Mar;37(2):204-50

Protein secretion and antibody expression

Our group conducts both basic and biotech-oriented research to understand and exploit protein secretion in *E. coli* and other Gram-negative bacteria (*proteobacteria*). Our basic research centres on the molecular mechanisms that bacterial pathogens use to secrete proteins (cytotoxins, proteases, adhesins) and to assemble cell surface organelles (fimbriae) that participate in bacterial virulence. We focus especially on those proteins and surface organelles secreted and assembled by (EPEC), enterohemorragic (EHEC) and uropathogenic (UPEC) *E. coli* strains. The biotechnological projects exploit these protein secretion systems to develop novel expression and selection technologies for recombinant antibodies in non-pathogenic commensal and laboratory *E. coli* strains. Among the recombinant antibody formats available (single-chain Fv, Fab, Fc-fusions, etc.), we focus on single-domain antibodies (sdAb) or nanobodies, the smallest antibody fragment known to date (~15 kDa) with full antigen-binding capacity. Nanobodies are based on a single VH domain obtained by recombinant DNA technology from heavy-chain-only antibodies from camelids (dromedaries, llamas). Despite the lack of a paired VL domain, nanobodies show high affinity and specificity for their cognate antigens. In addition, they are very similar to human VH3 sequences, making them excellent candidates for many applications, including therapy.

In the last few years, we demonstrated the capacity of the bacterial type V secretion system (T5SS) for bacterial display of single-domain antibodies, and are exploiting their translocator domains for selection of nanobodies to antigens of interest in biomedicine. We have also studied *E. coli* type 1 fimbriae by analysing the biogenesis of the fimbrial usher FimD, its interaction with periplasmic chaperones, and the Bam complex and its mechanism of activation by FimH. Finally, we continued our studies using the type III protein secretion system (T3SS) from EPEC strains to deliver single-domain antibodies from *E. coli* cells into the cytosol of human cells.

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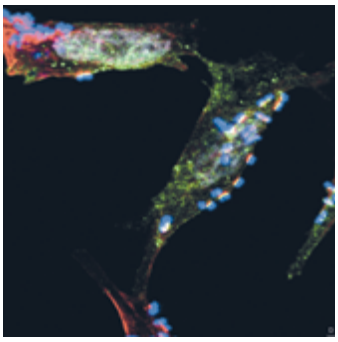

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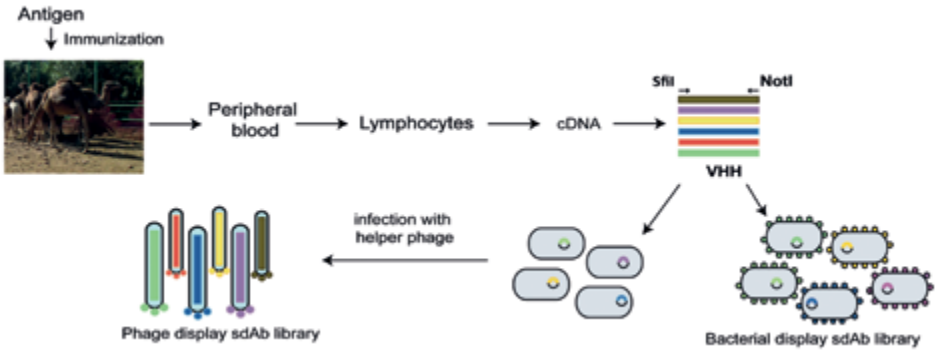

1 *Confocal microscopy of human HeLa cells cultured in vitro and infected with EHEC (anti-O157 monoclonal antibody, blue). Extracellular EHEC bacteria inject the translocated intimin receptor (Tir) into the HeLa cell cytoplasm, triggering actin pedestal formation beneath the adhered bacteria. Tir is stained green with anti-Tir rabbit polyclonal antibody and actin is stained red with phalloidin; yellow indicates Tir and actin colocalisation*

2 *Cloning of single-domain antibodies from immunised camelids and their expression on the E. coli cell surface (bacterial display) or filamentous bacteriophages (phage display) for screening and isolation of specific binders of an antigen of interest.*

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SELECTED PUBLICATIONS

Ortega A, Gonzalo-Asensio J, García-Del Portillo F. Dynamics of *Salmonella* small RNA expression in non-growing bacteria located inside eukaryotic cells. RNA Biol. 2012 Apr;9(4):469-88

García-del Portillo F, Cossart P. A new view to intracellular pathogens and host responses in the South of Spain. EMBO Mol Med. 2012 Mar;4(3):160-4

García-Del Portillo F, Pucciarelli MG. Remodeling of the *Listeria monocytogenes* cell wall inside eukaryotic cells. Commun Integr Biol. 2012 Mar 1;5(2):160-2

García-del Portillo F, Calvo E, D'Orazio V, Pucciarelli MG. Association of ActA to peptidoglycan revealed by cell wall proteomics of intracellular *Listeria monocytogenes*. J Biol Chem. 2011 Oct 7;286(40):34675-89

Paradela A, Mariscotti JF, Navajas R, Ramos-Fernández A, Albar JP, García-del Portillo F. Inverse regulation in the metabolic genes pckA and metE revealed by proteomic analysis of the *Salmonella* RcsCDB regulon. J Proteome Res. 2011 Aug 5;10(8):3386-98

Intracellular bacterial pathogens

Our group is interested in deciphering the cues that sustain the ability of certain bacterial pathogens to successfully colonise the intracellular niche of eukaryotic cells. As models, we use two intracellular bacterial pathogens with marked differences in their envelope structure, the Gram-negative bacterium *Salmonella enterica* serovar Typhimurium and the Gram-positive bacterium *Listeria monocytogenes*.

A largely unexplored area of the biology of *S. enterica* serovar Typhimurium involves the mechanisms it uses to establish a dormant non-growing state within the eukaryotic cell. *In vivo* and *in vitro* experiments support the idea that *S. enterica* serovar Typhimurium has evolved to spend most of its "intracellular life" in a state of "limited proliferation". Our studies have uncovered a set of small regulatory RNA (sRNA) that are upregulated by dormant intracellular *Salmonella* upon entry into the host cell. Transcriptomic analyses also showed a profound change in the expression prolife of numerous pathogen genes, including marked upregulation of genes that map to the virulence plasmid pSLT. Most of these plasmid genes were known to be silent in laboratory conditions. In dormant non-growing intracellular bacteria, we are currently analysing the mechanisms that activate important virulence regulators such as the PhoP-PhoQ system and the *Salmonella* pathogenicity islands 1 (SPI-1) and 2 (SPI-2). Our future efforts will focus on understanding how the bacterial envelope is modified when the pathogens adapts to this unique non-growing lifestyle within the eukaryotic cell.

In the research devoted to *Listeria monocytogenes*, we have progressed in the elucidation of changes in the cell wall when this pathogen infects epithelial cells. Proteomic studies identified the surface proteins bearing the C-terminal sorting motif LPXTG that are synthesised by the pathogen during the proliferation phase in the eukaryotic cytosol. We also characterised a unique mode of association of the ActA surface protein to the peptidoglycan when the pathogen adapts to the intracellular lifestyle. Our ongoing investigation focusses on new *L. monocytogenes* LPXTG family surface proteins of unknown function that might have a role in stabilisation of the cell wall architecture and in host cell invasion.

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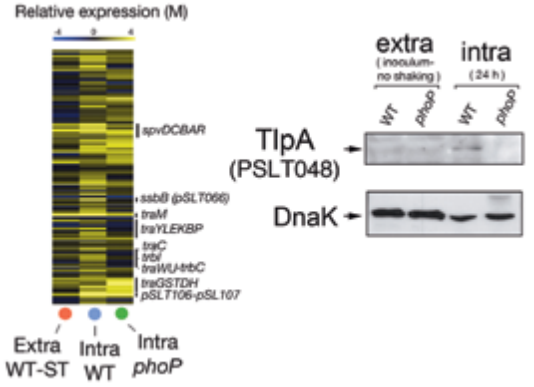
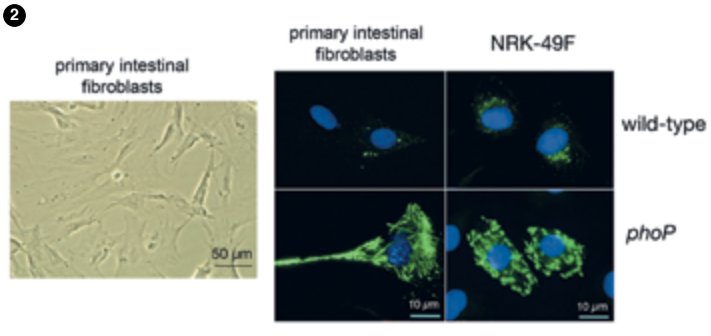


1 *Expression profiles in extracellular and intracellular bacteria of Salmonella enterica serovar Typhimurium genes that map to the virulence plasmid pSLT*

2



2 *Demonstration of the phenotype similarity of S. enterica serovar Typhimurium in cultured fibroblasts (rat cell line NRK) and in primary fibroblasts isolated from mouse intestine*

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SELECTED PUBLICATIONS

Olivares J, Alvarez-Ortega C, Linares JF, Rojo F, Köhler T, Martínez JL. Overproduction of the multidrug efflux pump MexEF-OprN does not impair *Pseudomonas aeruginosa* fitness in competition tests, but produces specific changes in bacterial regulatory networks. Environ Microbiol. 2012 Aug;14(8):1968-81

Sánchez MB, Martínez JL. Differential epigenetic compatibility of qnr antibiotic resistance determinants with the chromosome of *Escherichia* coli. PLoS One. 2012;7(5):e35149. doi

Martínez JL, Baquero F, Andersson DI. Beyond serial passages: new methods for predicting the emergence of resistance to novel antibiotics. Curr Opin Pharmacol. 2011 Oct;11(5):439-45

Hernández A, Ruiz FM, Romero A, Martínez JL. The binding of triclosan to SmeT, the repressor of the multidrug efflux pump SmeDEF, induces antibiotic resistance in *Stenotrophomonas maltophilia*. PLoS Pathog. 2011 Jun;7(6):e1002103

Martínez JL, Rojo F. Metabolic regulation of antibiotic resistance. FEMS Microbiol Rev. 2011 Sep;35(5):768-89

Opportunistic pathogens

Nosocomial infections due to opportunistic pathogens constitute a relevant health problem. As models for understanding the mechanisms involved in the pathogenic process of these microorganisms, we are using *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. These organisms are free-living bacteria with a characteristic phenotype of intrinsic resistance to antibiotics. Acquisition of resistance is the consequence of millions of years of evolution in natural, non-clinical ecosystems, long before humans began to use antibiotics for therapy. We recently expanded our interest to the study of *Klebsiella pneumoniae*. We aim to understand the biology of opportunistic pathogens, focussing on the networks and the evolutionary processes that connect resistance and virulence. This includes understanding the role of non-clinical natural ecosystems in the acquisition and evolution of antibiotic resistance and virulence, as well as how bacteria evolve during infection (mainly in chronic infection) and the differential host responses triggered by these pathogens. Our work might also provide information on novel targets in the search for drugs of use for reducing resistance to antibiotics.

Among these potential targets, we are especially interested in studying multidrug efflux pumps. These resistance elements are present in all live beings and contribute to many processes, including resistance to anticancer chemotherapy in humans and antibiotic resistance in bacteria. Nevertheless, whilst these pumps can expel these drugs, they have a different original function in nature. We found that the substrate of the MexEF-OprN *P. aeruginosa* efflux pump is L-kynurenine, an intermediate in the biosynthesis of the quorum-sensing molecule PQS. Expression of efflux pumps is usually downregulated; these elements can nonetheless be overexpressed in the presence of an effector. This is the case of the *S. maltophilia* efflux pump SmeDEF, whose expression is triggered by the biocide triclosan, suggesting that commonly used biocides might select for antibiotic-resistant microorganisms.

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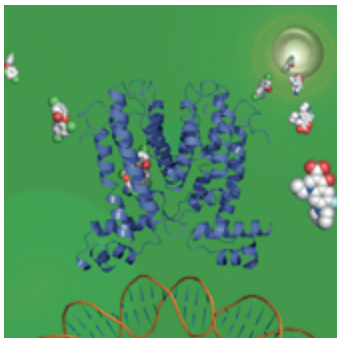

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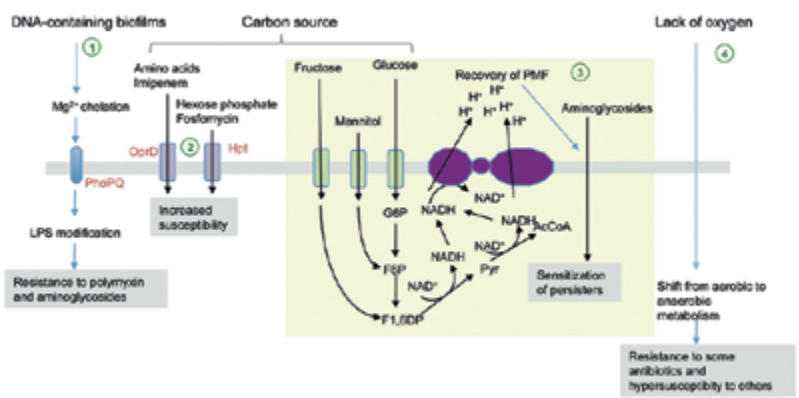
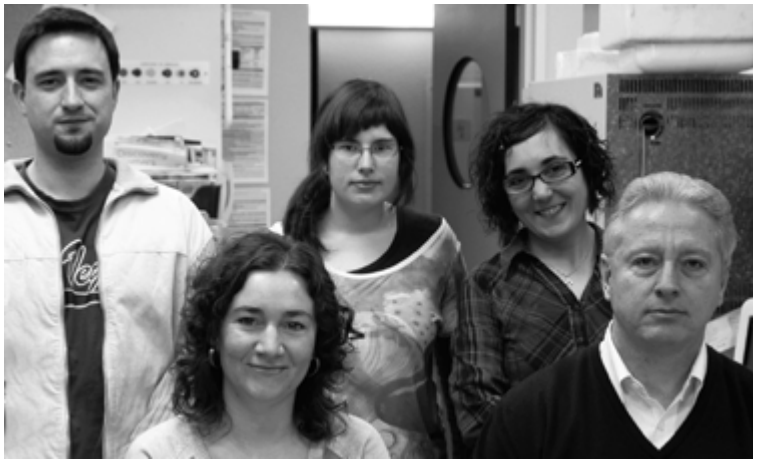


1 *Overexpression of efflux pumps can be achieved when an effector binds the negative transcriptional regulator of the system, which is released from its operator. As a consequence, the pump is overexpressed and extrudes different compounds, including antibiotics.*

2 *The phenotype of susceptibility to antibiotics can change under different conditions: 1. The presence of extracellular DNA at biofilms chelates cations and induce the pathway of resistance to antimicrobial peptides. 2. The presence of a given carbon source may induce the expression of its transporter. If the transporter is used as well by an antibiotic, its induction makes bacteria hypersusceptible. 3. The use of specific carbon sources can rescue bacteria from an antibiotic-resistant persistent phenotype. 4. Hypoxia, which is a common situation at the deepest zones of bacterial biofilms, alters the susceptibiity to antibiotics (From FEMS Microbiol Rev (2011) 35: 768).*



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SELECTED PUBLICATIONS

Barriuso J, Marín S, Mellado RP. Potential accumulative effect of the herbicide glyphosate on glyphosate-tolerant maize rhizobacterial communities over a three-year cultivation period. PLoS One. 2011;6(11):e27558

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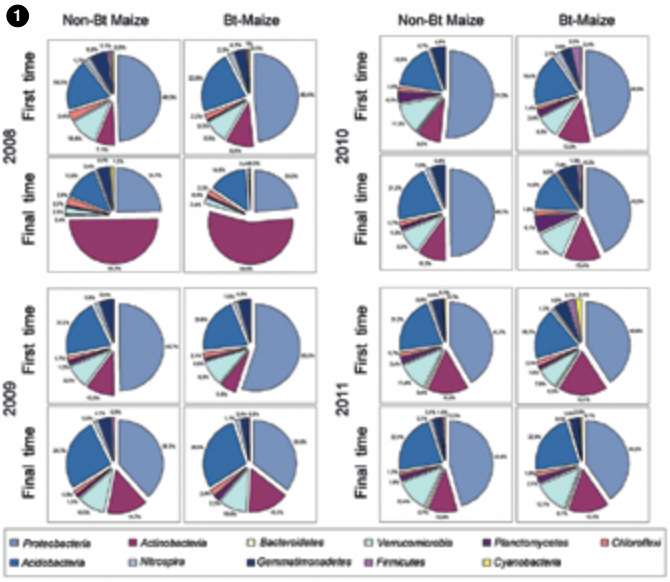
Heterologous gene expression and secretion in Gram-positive bacteria of industrial application

We traditionally focus our research on the physiological and molecular characterisation of the main protein secretion mechanism (Sec system) of soil Gram-positive bacteria of the *Streptomyces* genus, namely *S. lividans* and *S. coelicolor*. These are widely used in industry as efficient producers of extracellular hydrolytic enzymes and other compounds of industrial interest.

Deficiency in the translocase complex (SecG mutant strain) or the major type I signal peptidase (SipY mutant strain) function in *S. lividans* results in a group of genes seemingly regulated in the same way, including the absence of secretory protein production in both cases. These genes can be linked directly or indirectly to the *bld* cascade, suggesting its involvement in the cell response to the secretory defect of both mutant strains. Proteomic and transcriptomic analyses have determined that a newly identified *S. coelicolor* two-component system influences various processes characterised by the transition from primary to secondary metabolism, eliciting a partial stringent response and altered patterns of secretory proteins and antibiotics. Overproduction of alpha-amylase in *S. lividans* causes secretion stress and permitted identification of a two-component CssRS-like system that regulates three HtrA-like proteases, which appear to be involved in the degradation of misfolded secretory proteins.

We recently compared the effect of glyphosate (RoundupPlus), a post-emergency applied herbicide, and Harness GTZ, a pre-emergency applied herbicide, on the rhizobacterial communities of genetically modified NK603 glyphosate-tolerant maize. The effect was monitored by high throughput DNA pyrosequencing (Next Generation Sequencing, NGS) of the bacterial DNA coding for the 16S rRNA hypervariable V6 region. The results strongly suggest that glyphosate is environmentally less aggressive over long-term continuous cultivation periods. The rhizobacterial communities of transgenic maize engineered to express the *Bacillus thuringensis* Cry toxin (Bt maize) were equally monitored for a number of years. We tested several simple and complex workflows to analyse NGS from rhizobacterial community experimental data, using a variety of available tools, and determined their accuracy and efficiency under various conditions. We have identified which method-combination workflow is more attractive depending on sequence variability number and length.



1 *Taxonomic breakdown of certain rhizobacterial community phyla in cultures of Bt-maize over a four-year period. The percentages of Proteobacteria, Actinobacteria, Bacteroidetes, Verrucomicrobia, Planctomycetes, Chloroflexi, Acidobacteria, Nitrospira, Gemmatimonadetes, Firmicutes and Cyanobacteria are indicated and do not include the unassigned sequences. Unclassified sequences were not included, as they were of no taxonomic use.*

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SELECTED PUBLICATIONS

Fonseca P, Moreno R, Rojo F. Growth of *Pseudomonas putida* at low temperature: global transcriptomic and proteomic analyses. Environ. Microbiol. Rep. 2011, 3: 329-339

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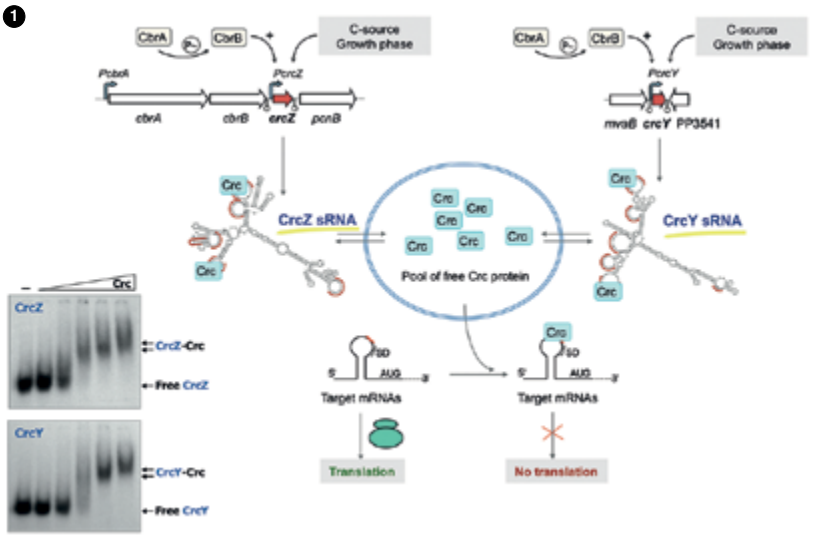
Regulation of the metabolism of hydrocarbons in bacteria

To be competitive in the environments they colonise, bacteria should optimise metabolism to attain maximum gain from the available nutrients at a minimum energetic cost. Not all potential carbon sources are equally effective in this respect. Probably for this reason, when confronted with a mixture of potentially assimilable compounds at sufficient concentrations, many bacteria use one of them preferentially, leaving other non-preferred compounds aside until the preferred one is consumed. This selection implies a complex regulatory process termed catabolite repression control. Unravelling the molecular mechanisms that underlie these regulatory events helps to understand how bacteria coordinate their metabolism and their gene expression programs, to optimise growth. It also helps to design and optimise biotechnological processes and is important for understanding how bacteria degrade compounds in nature. This is particularly true for compounds that are difficult to degrade and accumulate in the environment, posing pollution problems. Hydrocarbons are a particularly relevant example of this kind of non-preferred compounds. The influence of catabolite repression goes beyond the optimisation of metabolism, since it also affects virulence and antibiotic resistance in pathogenic bacteria.

Our aim is to characterise the global regulation networks responsible for catabolite repression, identifying their components, the signals to which they respond, and the molecular mechanisms by which they regulate gene expression. The regulatory proteins involved in these networks are different in distinct microorganisms. We use *Pseudomonas putida* as an experimental model because it has a versatile metabolism, it colonises very diverse habitats and is widely used in biotechnology. We are currently focussed on two catabolite repression networks.

One relies on the Crc protein; our work has shown that Crc binds to an unpaired A-rich sequence located at the translation initiation region of some mRNAs, thereby inhibiting their translation. Crc availability is controlled by two small RNAs, the levels of which vary sharply depending on growth conditions. The other regulatory network under study receives signals from the electron transport chain, thereby coordinating respiration with metabolic needs. Finally, we found that growth temperature influences catabolite repression. At low growth temperatures, repression is relieved in some genes, but not in others. This can be relevant for a number of biotechnological applications.



1 *The CrcZ and CrcY sRNAs control the availability of free Crc protein in P. putida. Crc inhibits the translation of many genes in response to diverse signals.*

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SELECTED PUBLICATIONS

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Genetic control of the cell cycle

What do Luciano Pavarotti and Freddy Mercury have in common? The answer is that although they suffered from serious diseases, pancreatic cancer and AIDS, respectively, both ultimately died of pneumonia, an infectious disease produced by the bacterium *Streptococcus pneumoniae*. Bacterial infections are not a thing of the past, as we were sorely reminded by the 2011 *Escherichia coli* outbreak in Germany, in which over 50 deaths were registered. Another infectious disease that produces a great deal of human suffering and is a major cause of death in poor countries is tuberculosis, a consequence of *Mycobacterium tuberculosis* infection. This microbe infects one-third of the world population and is often found latent in the infected persons.

A common feature of all infections is that antibiotics, the medicines that have been used to treat them, are losing their effectiveness as microbes gradually acquire resistance to those already in use. To maintain our health, new drugs to block the proliferation of bacteria are needed urgently. To find them we study those proteins that most microbes need to multiply.

We have identified details as to how the *E. coli* protein FtsZ, also needed by *S. pneumoniae*, *M. tuberculosis* and many other pathogens, works in the test tube. We then try test new compounds predicted to block its activity, to select those that can be used as antimicrobials. We have also analysed FtsA, another protein needed for microbial proliferation, which we purified from *S. pneumoniae*. We study WhiB5, a *M. tuberculosis* protein implicated in the virulence of this pathogen and in its exit from latency. This could help to develop strategies to avoid reactivation of latent infection.

Together with a third protein called ZipA, FtsZ and FtsA assemble to form the initial machinery needed for *E. coli* multiplication. An important part of our recent and future work is to obtain knowledge that will allow test tube reconstruction of this machinery, called the proto-ring because it first assembles to form a ring at mid-cell. This synthetic biology approach will yield powerful tools to develop and test new antibiotics.

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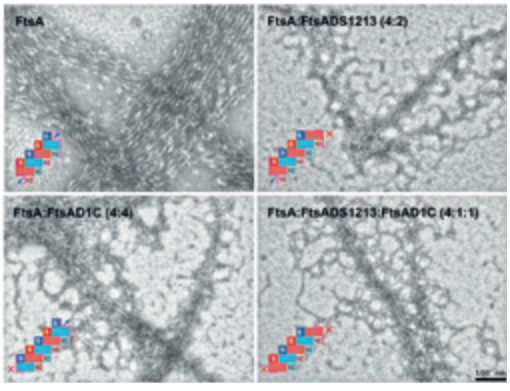

1 *Electron microscopy images of polymers formed by mixtures of streptococcal FtsA protein containing FtsA variants in different proportions*

2 *Polymerisation of FtsZ (green) triggered by GTP, detaches FtsA (red) from its location at the inner side of Escherichia coli membrane vesicles, pulling it to the vesicle lumen where both proteins subsequently colocalise*

2