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Publication date:
2010

Document Version
Publisher's PDF, also known as Version of record

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Citation (APA):
Seoane, J. M. (2010). *Individual-based analysis and prediction of the fate of plasmids in spatially structured bacterial populations*. Technical University of Denmark. <http://www.er.dtu.dk/publications/fulltext/2010/ENV2010-287.pdf>

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Individual-based analysis and prediction of the fate of plasmids in spatially structured bacterial populations



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PhD Thesis
December 2010

Department of Environmental Engineering
Technical University of Denmark

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Printed by: Vester Kopi
Virum, December 2010

Cover: Torben Dolin

ISBN: 978-87-92654-15-1

Preface

This thesis is made as a partial fulfillment of the requirements to obtain the Ph.D. at the Technical University of Denmark (DTU). The PhD project was supported by the European Commission through a Marie Curie Actions Excellence Grant (MEXT-CT-2005-024004) RaMAda to BFS. It was conducted from September 2007 until December 2010 at the Department of Environmental Engineering, Technical University of Denmark. The internal supervisor was Professor Barth F. Smets, Department of Environmental Engineering (Technical University of Denmark), and co-supervisor was Associate Professor Claus Sternberg, Institute for System Biology (Technical University of Denmark).

The current thesis is composed of a summary of the subject “*Individual-based analysis and prediction of the fate of plasmids on spatially structured bacterial populations*” and four journal papers (2 published and 2 submitted):

- I. **Seoane, J.**, Sin, G., Lardon, L.A., Gernaey, K.V. & Smets B.F. (2010). A new extant respirometric assay to estimate intrinsic growth parameters applied to study plasmid metabolic burden. *Biotechnology and Bioengineering* **105**: 141-149.
- II. **Seoane, J.**, Yankelovich, T., Dechesne, A., Merkey, B.V., Sternberg, C., & Smets, B.F. (2010). An individual-based approach to explain plasmid invasion in bacterial populations. *FEMS Microbiology Ecology*. *Accepted*.
- III. Merkey, B.V., Lardon, L.A., **Seoane, J.**, Kreft, J.U. & Smets, B.F. (2010). Growth dependence of conjugation explains limited plasmid invasion in biofilms: an individual-based modeling study. *Environmental Microbiology*. *Submitted*.
- IV. **Seoane, J.**, Merkey, B.V. & Smets, B.F. (2010). An Individual-based analysis of plasmid spreading in bacterial microcolonies. *Microbiology*. *Submitted*.

The papers are not included in this web-version, but can be obtained from the library at DTU Environment. Contact library@env.dtu.dk or Department of Environmental Engineering, Technical University of Denmark, Miljoevej, Building 113, DK-2800 Kgs. Lyngby, Denmark.

Publications co-authored and closely related to the topic of the thesis, but not explicitly comprised here are listed below and included several presentations at international conferences.

Seoane, J., Merkey, B., Yankelevich, T., Lardon, L., Smets, B. F. (2010); TOL plasmid invasion in *Pseudomonas putida* is contingent on cell growth and transient derepression. Presented at ASM 110th General Meeting, May 23-27, 2010. San Diego, CA, USA.

Seoane, J., Merkey, B., Yankelevich, T., Lardon, L., Smets, B. F. (2010); TOL plasmid invasion in *Pseudomonas putida* is contingent on cell growth and transient derepression. Presented at ISME 13th General Meeting, August 22-27, 2010. Seattle, WA, USA.

Merkey, B.V., Lardon, L., **Seoane, J.**, Kreft, J.U., Smets, B. F. (2009); An Individual-based model to describe horizontal gene transfer in biofilms. Presented at: International Conference Biofilms 2009. Processes in Biofilms: Fundamentals to applications, September 13-16, 2009, Davis, CA, USA (invited).

Seoane, J., Merkey, B., Lauritzen, B. S., D'Alvise, P. W., Lardon, L., Smets, B. F. (2009). TOL PWW0 transfer dynamics in *Pseudomonas putida* KT2440. Presented at: FEMS 2009. 3rd Congress of European Microbiologists, Gothenburg, Sweden, June 28 - July 2.

Smets, B. F., D'Alvise, P. W., Lauritzen, B. S., Merkey, B., Olsen, K. M., **Seoane, J.** (2009). Plasmid invasion and plasmid persistence in *Pseudomonas putida* biofilm Presented at: FEMS 2009. 3rd Congress of European Microbiologists, Gothenburg, Sweden, June 28 - July 2.

Seoane, J. (2008): Individual Based Modeling describing Horizontal Gene Transfer in Biofilms. Euroscience Open Forum 2008. Barcelona 17 and 18 July 2008.

Seoane, J., Lardon, L., El-Azhari, N., Kreft, J. U. , Smets, B. F. 2008. Analysis of an Individual-Based Model Describing Plasmid Transfer in Biofilms. Presented at ASM 108th General Meeting, June 1-5 2008, Boston, MA, USA.

Acknowledgments

The successful completion of the current PhD thesis would have never been achieved without any of the persons I mention below. Therefore, I would like to express my most sincere gratitude to:

- Prof. Barth F. Smets for giving me the opportunity to become a member of his team and for his enthusiastic support and bright guidance during my PhD studies.
- My Co-advisors Post-Doctoral fellows Laurent Lardon and Brian Merkey for excellent scientific discussion, support and guidance during these three years.
- Post-Doctoral fellow Arnaud Dechesne for his time and availability, the enjoyable and inspiring scientific discussions and his invaluable help.
- All the members of my team for the excellent working environment and the memorable moments we spent together and specially to Lene Kirstejn Jensen and Mona Refstrup for the precious assistance they provided me in the lab.
- My former supervisors Jose Luis Rubio, Claire Chenu, Laure Vieublé, and Mylène Durand-Tardiff for their support and patience.
- Elle Zimmer Hansen, Torben Dolin, Anne Harsting, Birgit Jensen and Susanne Wichman for technical and administrative support.
- Emiliano, Flavio, Valèrie, Elena, Ole, Britta, Sanin and Gianluca for making Denmark a better place to live and all my friends in Spain for making me feel so close in despite of the distance.
- My father, the first PhD in our family and certainly the brightest one, for showing me how wonderful science is; my mother, who taught me with her own example to face adversity “con la cabeza bien alta” and the rest of my family in Madrid and Altea, for their love and support.
- Last but the most important one, thanks to my wife Fiorella for leaving the wonderful Paris behind to be by my side during these three long winters. I would have never made it without you! Toujours ensemble, pour toute l’éternité!

*To my love Fiorella, for
seven wonderful years.*

Abstract

Plasmid conjugative transfer is a key process in the evolution and rapid adaptation of prokaryotes to changes in their environment because these mobile genetic elements may confer adaptive traits to their host, such as antibiotic resistance or increased biodegradation abilities. This thesis investigated the development of new methods for the estimation of the main parameters describing conjugative plasmid transfer at the individual cell level and the applicability of Individual-based model to the study of conjugative plasmid transfer in bacterial populations growing on solid surfaces. More specifically, we wanted to investigate both theoretically and experimentally the factors ruling the fate of the TOL plasmid pWW0 in spatially-structured bacterial populations of the model organism *Pseudomonas putida* KT2440.

In order to attain our goals, a new experimental design based on respirometry was developed to measure the metabolic burden associated with plasmid carriage by a host microorganism. This approach was successfully evaluated on *P. putida* carrying the TOL plasmid pWW0. Plasmid presence was found to reduce the bacterial fitness significantly, as reflected by the observed reduction in the yield (11%) and the specific growth rate (17%) when compared to its plasmid-free counterpart. We also explored the effects of expression of additional recombinant proteins from the plasmid, which decreased the specific growth rate by an additional 14%. In addition, identifiability and sensitivity analysis confirmed the robustness of the new approach for obtaining unique and accurate estimates of growth kinetic parameters.

In a second step, we designed and successfully implemented an individual-based experimental framework to identify and estimate the main parameters governing bacterial conjugation at the individual cell scale. Matings were done in a perfusion chamber containing an agarose slab, which allowed us to monitor plasmid spreading on-line within our bacterial populations without disrupting their spatial structure. In 91.1 % of the cases, successful mating pairs occurred through direct cell-to-cell contact (0-1 μm range) which arose randomly from microcolony morphogenesis without occurrence of pulling action (by conjugal pili). In addition, we have also checked the effect of the relative orientation of donor cells vis-a-vis recipient cells during plasmid transfer. Our results suggest that pWW0 plasmid junctions may appear at any point of contact on the surface of donors and that DNA can be transferred to any available location along the recipient membrane. However, some orientations occurred to be more favorable than others: conjugation appeared to be more likely to occur through the lateral wall of the donor than through the poles, which is supported by with previous observations showing that plasmids are situated preferentially at the center or quarter cell position in the cell (Lawley, *et al.*, 2002). Furthermore, plasmid

transfer was not observed before the new transconjugant cells reached an elongation of 60-70% compared to their maximal length (length attained immediately prior to septum appearance and division), and in 75% of cases the cells exceeded 80% elongation. No successful mating pair was detected that comprised recipient cells that did not divide shortly after transfer, suggesting that recipients are more susceptible to receive the plasmid at advanced stages of cell growth cycle. Finally, we have shown experimentally that the lag times needed for newly formed transconjugant cells to transfer pWW0 were significantly shorter than in the case of initial donor cells.

In order to test the main hypotheses explaining the dynamics of conjugal plasmid transfer in surface-associated bacterial populations, we extended a pre-existent individual-based model of microbial growth to include the dynamics of plasmid carriage and transfer by individual cells. Once implemented in our model, the parameter estimates obtained from the previous experimental work allowed us to correctly predict the degree of plasmid invasion in bacterial microcolonies together with the spatial plasmid invasion patterns and other macroscopic aspects such as colony morphology. We used this model to check the main hypothesis explaining the inability of pWW0 plasmid to fully invade tightly packed bacterial structures such as microcolonies or biofilms. Our results have shown that a moderate dependence of plasmid transfer on growth is enough to prevent plasmid invasion in a structured bacterial population, whereas EPS synthesis at biological levels was not able to explain the observed plasmid invasion patterns. In addition, vertical transfer processes were predominant over HGT independently of the selective advantage conferred by the plasmid to their host.

In conclusion, during the current thesis we developed an individual-based experimental framework, which allowed us to identify and estimate the main parameters governing bacterial conjugation at the individual cell scale. From this analysis, we concluded that transient periods of unregulated transfer together with contact mechanics arising from cellular growth and division were determinant in the ability of pWW0 to invade an structured bacterial population. In addition, we also demonstrated that our individual-based model for plasmid conjugative transfer in structured environments adequately predicted spatial patterns of plasmid invasion observed in living microcolonies. Therefore, we expect that the work presented here will facilitate the characterization of the population dynamics of other relevant plasmids and the development of new IbMs for the study of bacterial conjugation and other horizontal gene transfer processes.

Dansk Résumé

Konjugativ plasmidoverførsel er en nøgleproces i udviklingen og hurtig tilvænning af prokaryoter til forandringer i deres miljø, fordi deres bevægelige genetiske elementer kan tillade tilpassende egenskaber til deres vært, som modstandsdygtighed mod antibiotika og forhøjet evne til biologisk nedbrydning. I denne rapport er udviklingen af nye metoder for estimeringen af hovedparametrene behandlet, som beskriver konjugativ plasmid overførsel på et individuelt celle niveau og anvendeligheden af individuel baserede modeller for undersøgelsen af plasmid populationsdynamik i bakteriepopulationer som vokser på hårde overflader. Mere specifikt ville vi både undersøge de teoretiske og eksperimentelle faktorer som bestemmer nedbrydningen af TOL plasmidet pWW0 i rumligt strukturerede bakterie populationer fra model organismen *Pseudomonas putida* KT2440.

For at nå vores mål, er der blevet udviklet et nyt eksperimenterende design baseret på respirometrie, for at måle den metaboliske last sammenhængende med plasmid transporten fra en værtsmikroorganisme. Denne fremgangsmåde blev succesfuldt evalueret på baggrund af *P. Putida* bærende TOL plasmidet pWW0. Der blev fundet frem til, at plasmidforekomster reducerer bakterie bevægeligheden, hvilket kan ses på den observerede reduktion i bakteriehøsten (11%) og den specifikke vækstrate (17%), sammenlignet med den plasmidfrie modprøve. Vi har også undersøgt effekterne af udtrykket for additionelt rekombinerede proteiner fra plasmidet og fandt at det reducere den specifikke vækstrate med yderligere 14 %. Derudover blev robustheden af den nye metode for at frembringe unikke og præcise estimater af vækstkinetiske parametre konfirmeret ved identifikations- og følsomhedsanalyser.

I step 2 designede og implementerede vi succesfuld en individuel baseret eksperimentel ramme for at identificere og estimere nøgleparametrene som styrer bakteriel konjugation på en individuel celle skala. Parringer blev gennemført i et gennemstrømnings- eller perfusionskammer indeholdende en agar lignende overflade, som tillader monitor on-line plasmid spredning inden i vores bakterie populationer, uden at spalte deres rummelige struktur. I 91,1 % af tilfældene, succesfulde parringer fremkom gennem direkte celle til celle kontakt (0-1 μm afstand), som opstår tilfældigt fra mikrokolonial morfogenesis, uden tilstedeværelse af sammentrækning (af conjugal pili). Derudover har vi også undersøgt den relative orientering af donorceller mod recipientceller under plasmid overførsel. Resultaterne viser at pWW0 plasmidforbindelser kan opstå ved hvert kontaktsted på donoroverfladen og at DNA kan blive overført til alle tilgængelige steder på recipient membranen. Men nogle orienteringer viste sig at være mere positiv end andre: konjugation forekom oftere gennem donorens tværvæg end gennem polerne, hvilket er i overensstemmelse med forrige

observationer, som viser at plasmiderne oftest er placeret i det karakteristiske center eller i kvart-celle-positionen i cellen og ikke i polerne. Derudover er plasmid overførsel ikke blevet observeret før de nye transkonjugante celler når en udstrækning på 60-70 % af deres maksimale længde (længde opnået direkte før skillevæggen opstår og celledeling), og i 75 % af tilfældene overskred cellerne 80 % af udstrækningen. Der blev ikke observeret nogen succesfuld parring af modtageceller som ikke opdeltes kort efter overførelsen. Det formodes, at modtagecellerne er mere åbne for at modtage plasmider ved fremskredne stadier af en cellevækstcyklus. Afsluttende har eksperimenterne vist, at tidsdifferencen for nyformede transkonjugante celler for at overføre pWW0 var signifikant kortere end i tilfældet af de oprindelige donorceller.

Ved hjælp af at integrere parameterestimerne uddraget fra det foregående eksperimentelle arbejde, i vores model, kunne vi forudsige graden af plasmidinvasionen i bakterielle mikrokolonier sammen med det rummelige plasmid invasionsmønster og andre makroskopiske aspekter som f.eks. kolonimorfologi. Modellen blev brugt, for at afprøve hovedhypotesen og forklare at pWW0 plasmider ikke fuldt ud kunne invadere tætpakkede bakteriestrukturer som mikrokolonier eller biofilm. Hovedkonklusionen fra simuleringerne er, at EPS synteses på biologisk niveau aldrig vil kunne forklare de observerede plasmid invasionsmønstre. Disse kan kun genskabes, hvis der i modellen introduceres en moderat afhængighed af plasmidinvasionen på bakterievæksten.

I det nærværende PhD speciale har vi udviklet en individuelbaseret eksperimentstruktur som kan bruges til at identificere og estimere hovedparametrene som styrer bakteriekonjugation på en individuel celle skala. Ud fra denne analyse konkluderer vi, at flygtige perioder af ureguleret overførsel sammen med kontaktmekanismer fra cellevækst og splittelse er bestemmende for muligheden af pWW0 for at trænge ind i en struktureret bakteriepopulation. Derudover har vi også demonstreret, at vores individuelle baserede model for konjugativ plasmidoverførsel i strukturerede omgivelser passende forudsagde rummelige mønstre af plasmidindtrængen observeret i levende mikrokolonier. Derfor forventer vi at det præsenterede arbejde vil forenkle beskrivelsen af populationsdynamikken af andre forskningsrelevante plasmider og udviklingen af nye IBM'er for undersøgelsen af bakteriologisk konjugation og andre horisontale gentransfer processer.

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Abbreviations and symbols

CA.....	Cellular Automata
<i>COD</i>	Chemical Oxygen Demand
CP.....	Coupling Protein
CPU.....	Central Processing Unit
CTns.....	Conjugative Transposons
DNA Pol I.....	DNA Polymerase I
DsRed.....	Red fluorescent protein
<i>Dtr</i>	Duplication and transfer system
Eex.....	Entry exclusion system
EPS.....	Extracellular Polymeric Substances
GFP.....	Green Fluorescent Protein
GIs.....	Genetic Islands
GPU.....	Graphics Processing Unit
HGT.....	Horizontal Gene Transfer
IbM.....	Individual-based Modeling
ICEs.....	Integrative and Conjugative Elements
Inc.....	Incompatibility group
MGEs.....	Mobile Genetic Elements
<i>mpf</i>	Mating pore formation genes
<i>mps</i>	Mating pair stabilization
<i>mob</i>	Mobility genes (encoding for Dtr)
MTns.....	Mobilizable Transposons
<i>mrs</i>	Multimer resolution system
LPS.....	Lipopolysaccharides
OD ₆₀₀	Optical density 600 nm
ORF.....	Open reading frames
oriT.....	Origin of transfer
PBRT.....	PCR-based replicon typing
PDE.....	Partial differential equations
pWW0.....	TOL plasmid wildtype
RaMaDa.....	Rapid Microbial Adaptation
rep.....	Replicon
res.....	Plasmid resolution site
T2SS.....	Type 2 secretion system
T4P.....	Type 4 pili
T4SS.....	Type 4 secretion system or transferosome
VGT.....	Vertical Gene Transfer
A_{BF}	Surface of carrier potentially covered by biofilm (μm^2)
b	Biomass maintenance rate (h^{-1})
b_p	Plasmid maintenance rate (h^{-1})
$d_{1,2}$	Distances between agents 1 & 2
D	Dilution rate, (h^{-1})
D (Stewart & Levin).....	Initial influent substrate concentration, (g L^{-1})
D_s	Solute diffusion coefficient, ($\mu\text{m}^2 \text{h}^{-1}$)

d_p	Pilus reach distance, (μm)
K_{La}	Oxygen diffusion rate, (h^{-1})
$K_{shoving}$	Shoving factor, (unitless)
k_S	Half saturation constant, ($\text{mg}_{\text{COD}}\text{L}^{-1}$)
m	Mass, (mg)
P	Biomass pressure, ($\text{N}\cdot\text{m}^{-2} = \text{kg}\cdot\text{m}^{-1}\cdot\text{s}^{-2}$)
p	Transfer proficiency, (probability of success, unitless)
p_{loss}	Probability of segregative loss during cell division, (unitless)
r (Stewart & Levin)	Volumetric reaction rates, (h^{-1})
r_S	Local solute reaction rate, (h^{-1})
S (Stewart & Levin)	Limiting solute concentration, (mass/volume or moles/volume)
S_B	Solute concentration in the bulk compartment, ($\text{g}\cdot\text{L}^{-1}$)
$T_{regulated}$	Transfer lag during transfer regulated periods, (h)
$T_{unregulated}$	Transfer lag during transfer unregulated periods, (h)
\bar{u}	Biomass advective velocity, ($\mu\text{m}\cdot\text{h}^{-1}$)
X_D (Stewart & Levin)	Biomass donor cells, (number of cells/volume)
X_R (Stewart & Levin)	Biomass recipient cells, (number of cells/volume)
X_T (Stewart & Levin)	Biomass transconjugant cells, (number of cells/volume)
$X_{D,o}$ (Stewart & Levin)	Initial conditions for X_D
$X_{R,o}$ (Stewart & Levin)	Initial conditions for X_R
$X_{T,o}$ (Stewart & Levin)	Initial conditions for X_T
V	Volume, (m^3)
v_s	Conjugal pilus scan speed, (h^{-1})
Y_h	Yield, ($\text{g}_{\text{COD}}(\text{X})/\text{g}_{\text{COD}}(\text{S})$)
α (Stewart & Levin)	Metabolic burden, (%)
γ (Stewart & Levin)	Plasmid transfer rate coefficient, ($\text{vol cell}^{-1}\text{h}^{-1}$)
γ	Plasmid transfer rate coefficient, (h^{-1})
δ	Degree of overlap between two adjacent agents, (μm)
λ	Darcy parameter ($\text{m}^3\text{s kg}^{-1}$)
μ	Biomass specific growth rate, (h^{-1})
μ_{max}	Biomass maximum growth rate, (h^{-1})
ρ_j	Agent density, (g / volume)
σ_R	Specific surface area of the reactor, (m^2/m^3)
τ (Stewart & Levin)	Probability of plasmid loss from one of the cell types, (%)
$\varphi_{j, cell}$	Radius of the cell including all intracellular compartments, (μm)
$\varphi_{j, total}$	Radius of the entire agent (including capsular components), (μm)

1 Introduction and Objectives

Recent genomic analysis indicates that Horizontal Gene Transfer (HGT) is a major force impacting the adaptative evolution and rapid adaptation of prokaryotes (Gogarten, *et al.*, 2002, Daubin, *et al.*, 2003). HGT may occur via the processes of transformation, conjugation and transduction. In all cases, it involves the transfer of genetic material from one bacterium to another and may occur within and between different species. This genetic exchange within cells may involve important amounts of DNA including complete genes and operons, resulting in the rapid acquisition of new phenotypic traits in the recipient bacteria.

Prior to the development of the modern molecular techniques, the role of HGT in bacterial adaptation and evolution was not considered to be relevant when compared with the contribution of Vertical Gene Transfer (VGT) (i.e. alterations produced by mutations transmitted to subsequent generations by cell division). The rapid and global emergence of multidrug-resistant bacteria during the 40's (Roberts, 1996) provided the first indications of the real effects of HGT in bacterial adaptation (Davies, 1996). However, it was not until the late 90's when the first complete genome sequences were available and HGT impacts in microorganism's evolution could be appropriately estimated. These analyses were based in the particularities shown by horizontally acquired genes such as the presence of atypical nucleotide composition and in some cases restricted phylogenetic distribution between related strain and species. In addition, it was also possible to find similarities between these genes and the donor sequences, allowing to deduce the route of acquisition (Lawrence & Ochman, 1998, Ochman, *et al.*, 2000). Ochman and co-workers found between 2.7% and 16.6% of estimated foreign DNA in fifteen out of 19 genomes analyzed, providing an illustrative example of the extant of HGT contribution to bacterial evolution.

However, in despite of the main contributions of the phylogenetic approach, these analyses were constrained to those recombination and integration events that were successfully conserved over the evolutionary history of the bacterial chromosome as studied. Because of this, complementary approaches have focused on the direct quantification and analysis of the factors controlling HGT between bacteria living in a wide range of systems, such as rhizosphere and phyllosphere environments (Knudsen, *et al.*, 1988), liquid cultures (Simonsen, 1991), and in biofilms (Arango Pinedo & Smets, 2005).

In this sense, the development of appropriate tools to quantify and predict mobile DNA spreading and its associated functions has become crucial to estimate HGT-associated risks (i.e. antibiotic resistance) and to provide new insights into the evolution of microorganisms (Birge, 1994, Maloy *et al.*, 1994, Davison, 1999).

Mathematical modeling is a very powerful approach for this purpose since it facilitates the organization of previous knowledge and the inference of biological trends while overcoming experimental limitations. Mass-action-based models, firstly applied to the study of conjugative gene transfer in the late 1970's (Stewart & Levin, 1977), have been used successfully to describe plasmid transfer in homogeneously-mixed planktonic populations (Simonsen, 1991, Smets, *et al.*, 1994).

However, perfect mixing cannot be assumed in spatially structured environments such as solid surfaces. Therefore, there is a need for a different modeling approach able to account for environmental conditions in surface-attached bacterial communities. In this sense, Individual-based Modeling (IbM) has been suggested as the most suitable method for describing conjugative gene transfer in solid surfaces (Sorensen, *et al.*, 2005), since this can be considered as discrete event occurring between two individual cells.

Unfortunately, this methodology is not exempt of limitations. The lack of individual-based observations together with the high computational demands inherent to these models makes difficult its application to HGT studies in practice. The aim of this research was therefore:

- (i) to design and develop robust procedures to identify and estimate the main parameters describing bacterial growth and conjugational plasmid transfer in bacterial populations growing on solid surfaces (Appendix I and II).
- (ii) to develop and validate an individual-based model (iDynoMiCs) describing microbial growth and conjugative gene transfer dynamics in colonies and biofilms (Appendix III and IV).

2 Theoretical background

2.1 Modes of Horizontal Gene Transfer

HGT processes in bacteria may occur by three different mechanisms: Transformation, transduction and conjugation. All the three processes share a common characteristic: the transfer of genetic material from one cell to another. Recent discoveries have shown that the nature of these genetic materials, known as mobile genetic elements (MGE), may be very diverse since they are formed from a combination of functional modules derived from phage, plasmids and transposons. MGE classification is therefore extremely complex, being currently possible to distinguish up to 10 MGE general groups (Roberts, *et al.*, 2008).

2.1.1 Transformation

HGT by transformation refers to the uptake and stable integration of free DNA into the bacterial genome. The fraction of cells within a bacterial population that is able to incorporate new phenotypic traits through this mechanism is designed as competent for DNA transformation. This is the only prokaryotic HGT process that relies uniquely in the physiological status of the host and have been demonstrated in approximately 90 bacterial species (Sorensen, *et al.*, 2005). *Acitenobacter sp.* in soils (Nielsen & van Elsas, 2001), *Bacillus subtilis* in foodstuffs (Bräutigam, *et al.*, 1997) and aquatic systems (Matsui, *et al.*, 2003), or *Helicobacter pylori* in humans (Smeets & Kusters, 2002) are just some examples. Relatively little is still known, however, about the prevalence and phylogenetic distribution of organisms possessing this property. Thus, whether natural transformation only benefits a limited number of species or has a large impact on lateral gene flow in nature remains unknown (Johnsborg, *et al.*, 2007).

2.1.2 Transduction

Transduction involves the incorporation of foreign cellular DNA in the chromosome of the host cell as an indirect consequence of the infection by bacteriophage virus. This phenomenon generally arises from errors in DNA packaging or prophage excision and can occur in the natural environment where phages are abundant and genetically and morphologically diverse. Surprisingly, only a few works have studied transducing phages in terrestrial habitats, particularly those infecting *Streptomyces sp.* (Hodgson, 2000) and *Lysteria sp* (Wommack & Colwell, 2000). By contrast, phage-mediated DNA transfer is a main subject in marine microbiology because ocean waters contain virus concentrations as high as 10^7 particles/ml (most of them probably phages) (Wommack & Colwell, 2000). Therefore, phages virus outnumber bacteria in oceans by a factor of ten (Brüssow & Hendrix, 2002). If we assume a successful

transduction frequency of 10^{-8} per plaque forming unit for marine phages (Jiang & Paul, 1998), we could expect phage-mediated gene transfer to occur at a rate of about 20 million time per seconds in the oceans (Bushman, 2002). This rate is probably not so high in the practice if we consider that transformation processes are extremely sensitive to environmental changes and infection is highly bacteria-phage specific (Bergh, *et al.*, 1989), although this needs to be confirmed. Only a few marine phages have been sequenced from the 4000 to 7000 viral types estimated in a 100 liter water sample (Canchaya, *et al.*, 2003), suggesting that further research is needed before making conclusions about the genomics of marine bacteria and their phages.

2.1.3 Conjugation

The third HGT mechanism, known as bacterial conjugation, refers to the direct transfer of DNA between bacterial cells during cell-to-cell contact. Between the three classical mechanisms of horizontal gene transfer, new evidence suggests that conjugation is quantitatively the most important (Halary, *et al.*, 2010). This is because transduction is mediated by phages, which have restricted hosts and small cargo regions whereas some plasmids can conjugate between remotely related organisms belonging even to different kingdoms (Heinemann, 1991).

Within the different MGEs able to transfer through conjugation we can find conjugative genomic islands (a subtype of genomic island or GIs), conjugative transposons (CTNs), mobilizable transposons (MTNs) and plasmids. However, the highly mosaic composition of MGEs make difficult to establish the boundaries between the different groups of elements. It has been proposed to retain the term CTNs for those transposons exhibiting random or semi-random integration in the chromosome of their host, while those conjugative integrating elements which have distinct site-preference should be referred as conjugative GIs. The third group, called mobilizable transposons (MTNs), would cover MGE that integrate into the chromosome of their host and can be mobilized via conjugation to other cells, but lack a conjugation system on their own (Osborn & Böltner, 2002, Roberts, *et al.*, 2008). However, there is an increasing tendency in the current literature to gather these three MGE groups into the term “integrative and conjugative elements” (ICEs). This trend is supported by recent experimental evidence suggesting that probably the autonomous plasmid-like replication mechanism is a common property of all ICEs and contributes to the stability and maintenance of these mobile genetic elements in bacterial populations (Lee, *et al.*, 2010). ICEs contribution to bacterial evolution and diversity have been reviewed comprehensible by others (van der Meer & Sentschilo, 2003, Didelot & Maiden, 2010) while conjugation mediated by plasmids will be further describe in the next sections.

2.2 Plasmid life style

It was J. Lederberg who proposed for the first time the term plasmid (an hybrid of cytoplasm or plasmagene and the term “-id” as in plastid or chromatid) to refer to extranuclear structures that are able to reproduce in an autonomous state but were also part of the genetic constitution of the cell (Lederberg, 1952, Lederberg, 1998). However, during the following decade plasmids were often confused with episomes, which are defined as DNA fragments that can exist independently of the main body of genetic material at some times, while at other times is able to integrate into the chromosome (i.e. transposons and insertion sequences) (Jacob. & Wollman, 1958). These ubiquitous entities are present in cells of all kingdoms of life and all ecosystems. They also display an amazing diversity of characteristics, such as mechanism of transmission, host range specificity or genetic composition. In addition, many plasmids contain a huge variety of different genes encoding for phenotypic traits that confers adaptative advantages to their host cells such as antibiotic resistance or new biodegradation pathways (Smets & Barkay, 2005, Sorensen, *et al.*, 2005).

2.2.1 Plasmid replication and maintenance

As we have mentioned before, conjugative systems can greatly contribute to plasmid spread within bacterial populations. However, it is less obvious to understand why conjugative systems appear so often associated with plasmids in nature. Theoretically, a whole chromosome could be transferred by conjugation as long as it contains the genetic unit that initiates the movement of DNA between mating pairs (origin of transfer or *oriT*), but this is a very rare event. If we consider that DNA transfer into a recipient cell proceeds at about 45 kb min⁻¹ at 37 °C (Lawley, *et al.*, 2004), transferring *E. coli* whole chromosome would take more than one hour (Thomas & Nielsen, 2005) and mating junctions would break down before successful completion of the whole process. In this sense, we could expect natural selection to promote those systems that are contained in smaller pieces of foreign DNA such as plasmids, since these transfers and establish faster in the host cell. However, the same reasoning applies to other ICEs that are much less frequently observed in nature, such as conjugative transposons. A more detailed analysis of the plasmid life style shows how being contained within a plasmid may provide additional advantages. Natural plasmids, such as pWW0, are very poorly lost under laboratory conditions (Duetz & Van Andel, 1991, Smets, *et al.*, 1994). This is because, being plasmids completely independent extrachromosomal elements, they have developed different strategies aiming to optimize their establishment in a bacterial population. This is particularly relevant in large low-copy plasmids, which usually contain sequences encoding for mechanisms specifically dedicated to minimize physical plasmid loss via vegetative segregation, such as tightly regulated replication systems, copy-number control, active partitioning and post-segregational killing.

Plasmid replication systems

One of the most important characteristics of plasmids is that they replicate in an autonomous and self-controlled way. Replication is central to the control of a number of important plasmid properties such as copy number, host range, incompatibility and mobility. Although the mechanisms involved in plasmid replication have been thoroughly reviewed elsewhere (del Solar, *et al.*, 1998), we will briefly describe them here. In most of plasmids, replication occurs via two possible ways: theta and strand displacement or rolling circle. Between these two, replication by the theta-type system is the most well known among the prototype circular plasmids of gram-negative bacteria such as ColE1, RK2 and F. It involves melting of the parental strands, synthesis of a primer RNA (pRNA), and initiation of DNA synthesis by covalent extension of the pRNA. Synthesis can start from one or from several origins although DNA synthesis is continuous on one of the strands (leading strand) and discontinuous on the other (lagging strand). Replication can be either uni or bidirectional. Most of plasmids using the theta mechanism of replication require a plasmid-encoded Rep initiator protein and, in some cases, replicons may require the host DNA Polymerase I (DNA Pol I) during the early stages of leading-strand synthesis.

Replication by the rolling circle mechanism is widespread among small (10 kb) multicopy plasmids from the *Archaea* and *Bacteria* such as pE194/pLS1, pT181 or pC194/pUB110 (Khan, 2005). The DNA substrate for the Rep proteins mediated nicking has to be in a single-stranded configuration. Leading-strand synthesis is terminated by various specific strand transfer reactions, also mediated by the Rep protein. After completion of leading-strand synthesis, the Rep protein is inactivated and plasmid ssDNA intermediates are produced. RNAP-directed synthesis of a short RNA primer initiates lagging-strand replication.

Plasmid maintenance strategies: copy-number control

Naturally occurring plasmids are usually stably maintained in their bacterial hosts. This stability often must be accomplished in spite of a very low number of plasmid copies per cell. Replication-control mechanisms play a very important role here by ensuring a constant number of plasmid copies per chromosome for segregation to each daughter cell (Nordström, *et al.*, 1984, Gerdes, *et al.*, 2000). Plasmid copy-number can vary from 1 (the F plasmid) to over a hundred (pUC18), being a defining property of the plasmid itself which depends on the replication mechanism. In general, these control systems maintain the rate of replication in steady state at an average of one replicative event per plasmid copy and cell cycle. Deviations from the average copy number in individual cells are corrected through replication inhibitors encoded by the plasmid itself and acting at initial replication steps. In this way, concentration of these negative regulators in the new host is negligible allowing plasmids to attain their typical copy number very fast. Once the characteristic plasmid copy number is reached, the control

system increase or decrease the rate of replication per plasmid copy and cell cycle by randomly selecting individual plasmid copies for replication. Consequently, two plasmids with identical replicons (isologous) cannot be distinguished by the control system and fluctuation arising because of the random selection of individual copies for replication and partitioning cannot be corrected. This leads to segregation of plasmids within the host population, a probabilistic phenomenon known as plasmid incompatibility (Novick, 1987) (Figure 1). Archetypical plasmids F (Kline, 1985), R1 (Nordström, *et al.*, 1984) and P1 (Sengupta, *et al.*, 2010) replication/maintenance functions and incompatibility have been comprehensively reviewed elsewhere.

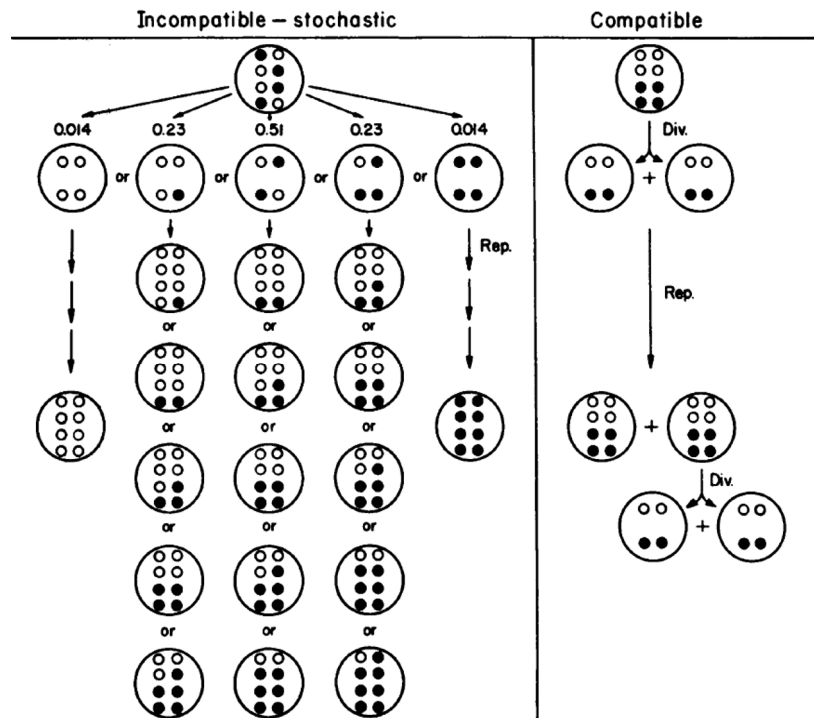


Figure 1: Segregational incompatibility. Black and white symbols represent differentially marked plasmids that replicate according to a 4-8-4 cycle. In the left half, the two plasmids have isologous replicons and show segregational incompatibility. At the top the various combinations that can arise via random assortment with equipartitioning are modeled; numbers represent the binomial probabilities for each combination. Beneath the partitioning diagram are given all of the possible combinations that can result from random selection for replication. In the right half, the two plasmids have heterologous replicons and are compatible. These two patterns represent the haploid and diploid modes of inheritance as applied to multicopy plasmids. Reprinted from Novick *et al.*, 1987.

In addition to tightly controlled replication systems, bacterial plasmids employ various strategies to guarantee their proper maintenance, such as active partitioning, post-segregational killing mechanisms and multimer resolution systems.

Plasmid maintenance strategies: active partitioning

Partition systems are, in general, mechanisms that actively determine plasmid specific localization within the bacterial cell. These are essential for the stability and thus the survival of low-copy-number plasmids in growing bacterial populations (Funnell, 2005). In addition, partition systems also determine incompatibility, which is independent from the replication-mediated incompatibility discussed in the previous section. In this sense, two different plasmids (i.e. with compatible replicons) cannot stably coexist in the same cell if they compete for the same partitioning system (Austin & Nordstrom, 1990, Ebersbach, *et al.*, 2005).

The development of fluorescence microscopy during the last decade has allowed researchers to perform *in vivo* analysis of plasmid localization in bacterial cells during the different stages of cell division (Lawley, *et al.*, 2002, Gordon, *et al.*, 2004, Ebersbach, *et al.*, 2005). Plasmids with active partition systems seem to occupy specific locations thorough the cell cycle: P1, F and RK2 are usually localized at or near the quarter and three quarter positions except in the youngest cells, where they are often localized at mid-cell (Niki & Hiraga, 1997, Pogliano, *et al.*, 2001, Gordon, *et al.*, 2004). However plasmid R1, which has a different partition system, appears to move from the cell center to localize at the poles (Jensen & Gerdes, 1999). This plasmid and cell location specificity has been shown to be dependent on their partition systems (Niki & Hiraga, 1997). Another interesting observation is that the number of fluorescent foci detected is usually smaller than the number of plasmid copies in the cell, which suggest that plasmids copies cluster together in pairs or groups at a limiting number of attaching sites (Gordon, *et al.*, 1997, Gordon, *et al.*, 2004). Finally, different types of plasmid (e.g., RP4 vs F) occupies different positions within the same cells, indicating the existence of different partitioning signals (Ho, *et al.*, 2002). These results supported the replicon model for intracellular positioning proposed long time before (Jacob, *et al.*, 1963): plasmid copies would be placed at the mid-cell in young cells but would replicate and relocate in the quarter and three-quarter positions which, after cell division, would in turn become the midcell of the daughter cells (Figure 2).

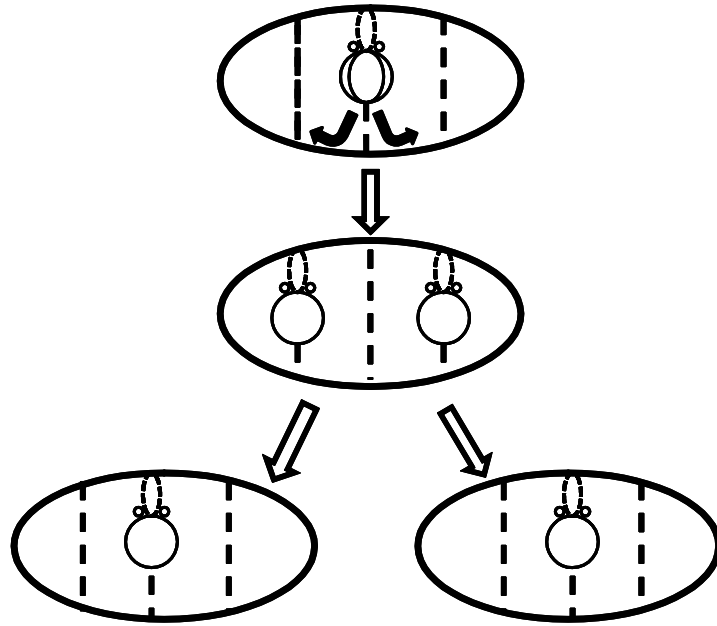


Figure 2: General Scheme depicting a plasmid partition reaction. Newly replicated plasmids are relocated in the quarter-cell positions prior to cell division, which becomes the mid-cell region afterwards. Adapted from Funnell and Phillips, 2004.

Without such a partition system, a basic plasmid is unstably maintained in a growing bacterial population, resulting in the formation of plasmid-free cells in a frequency that is dependent upon the plasmid copy number. The loss rate (L , the probability of formation of a plasmidless cell per cell and cell generation) is $L = (1/2)^{2n}$, where n is the plasmid copy number per cell at birth of the cell. The rate decreases rapidly with increasing copy number and L becomes 10^{-6} at $n=10$ (Nordström & Gerdes, 2003).

Plasmid maintenance strategies: postsegregational killing

Post-segregational killing systems (also called toxin-antitoxin systems) aim to facilitate plasmid establishment in bacterial populations by plasmid-mediated selective elimination or growth impairment of cells that have failed to acquire a plasmid copy. These mechanisms confer an advantage on plasmid-retaining cells by reducing the competitiveness of their plasmid-free counterparts, thereby ensuring the retention of the plasmid in the population (Jaffe, *et al.*, 1985, Gerdes, *et al.*, 1986).

The general mechanism of action (Hayes, 2003) involves the production of a stable protein by the toxic gene, whereas the antitoxin is either a labile protein or an untranslated, antisense RNA species. The toxin is neutralized by inhibition of toxin translation when the antitoxin is an RNA (type I), or by binding of the partner antitoxin when the latter is a protein (type II). When due to a failure on replication or other defect a plasmid-free variant is produced, the new cell will

still inherit the toxin-antitoxin complex. Since the antitoxin component is degraded more rapidly by host enzymes than the toxin (which is not replenished without the presence of the plasmid), the toxin rapidly attains concentration levels that cause death or growth restriction of the plasmid-free cell. Well-characterized *psk* systems include the *hok-sok* locus of *E. coli* plasmid R1 and the *ccdB-ccdA* locus from the *E. coli* F plasmid. Post-segregational cell-killing genes are used in systems such as cloning vectors that include the *ccdB* gene to ensure that only plasmids disrupted with a cloned insert are propagated (Sorensen, *et al.*, 2005).

Plasmid maintenance strategies: multimer resolution systems

The third mechanism involved in stable plasmid maintenance is the multimer resolution system (*mrs*). Plasmid replication involves very often the appearance of multimers or catenanes. If unresolved, some daughter cells will receive more plasmid copies than expected and thereby increase the chance of one of the daughter cells becoming plasmid-free. Because of this, almost all plasmids and chromosomes have genes that encode enzymes with resolvase activity. A well-characterised example is given by the *mrs* system encoded by the *parCBA* operon of plasmid R2K. Here, the *parA* gene encodes a resolvase, which acts on the plasmid resolution site (*res*) to resolve plasmid multimers ensuring that plasmids are separated from each other to become separate entities (Sorensen, *et al.*, 2005).

2.2.2 Plasmid transmissibility

Mobility, on the contrary of replication, is not an essential characteristic of plasmids. Recent evidence have shown that most plasmids larger than 300 kb are non-mobilizable (Figure 3, A) and, given their size, they are unlikely to be transferred by transformation or transduction. In addition, many large plasmids carry essential RNA genes, such as tRNA and rRNA (Figure 3, B) (Smillie, *et al.*, 2010), which have led some researchers to consider them as secondary chromosomes named chromids (Harrison, *et al.*, 2010). Usually these secondary chromosomes are smaller than the main chromosome, contain only a few essential genes and code for niche-specific functions (Egan, *et al.*, 2005, Slater, *et al.*, 2009, Harrison, *et al.*, 2010). Some examples of multichromosomal bacteria are found among different clades such as *Rhizobium*, *Bulkhoderia* and *Vibrio* (Ochman, 2002). In addition, some chromids contain plasmid-like origin of replication, e.g. chromosome 2 in *Vibrio cholerae* is very similar to the *oriVs* of P1 and F plasmids (Egan & Waldor, 2003) and replicates in a different phase of the cell cycle (Rasmussen, *et al.*, 2007).

Between the different integrative conjugative elements (ICEs), mobile plasmids are considered major players in conjugative processes. In spite of their diversity, all the conjugative systems known until the date share the same modular structure with two functional subsets: the DNA transfer and replication

system (*Dtr*) (also called MOB genes, for mobility) and the Type 4 secretion system (*T4SS*), also called transferosome or mating pore formation complex (*mpf*) (Willetts & Crowther, 1981). The *Dtr* system is essential for conjugation and is responsible for plasmid replication and processing of the conjugative plasmid into a transfer-competent intermediate (a protein–DNA conjugate known as the relaxosome). *T4SS* is essential for production of exocellular pili and formation of a trans-envelope channel structure presumably serving as a conduit for protein and DNA substrates. *Dtr* and *T4SS* are linked through the coupling protein (CP, VirD4). The CP first acts as a pilot protein guiding the DNA-protein complex generated by the *Dtr* to the entry of the *Mpf* channel and then probably participate in the active secretion of the substrate (Funnell & Phillips, 2004).

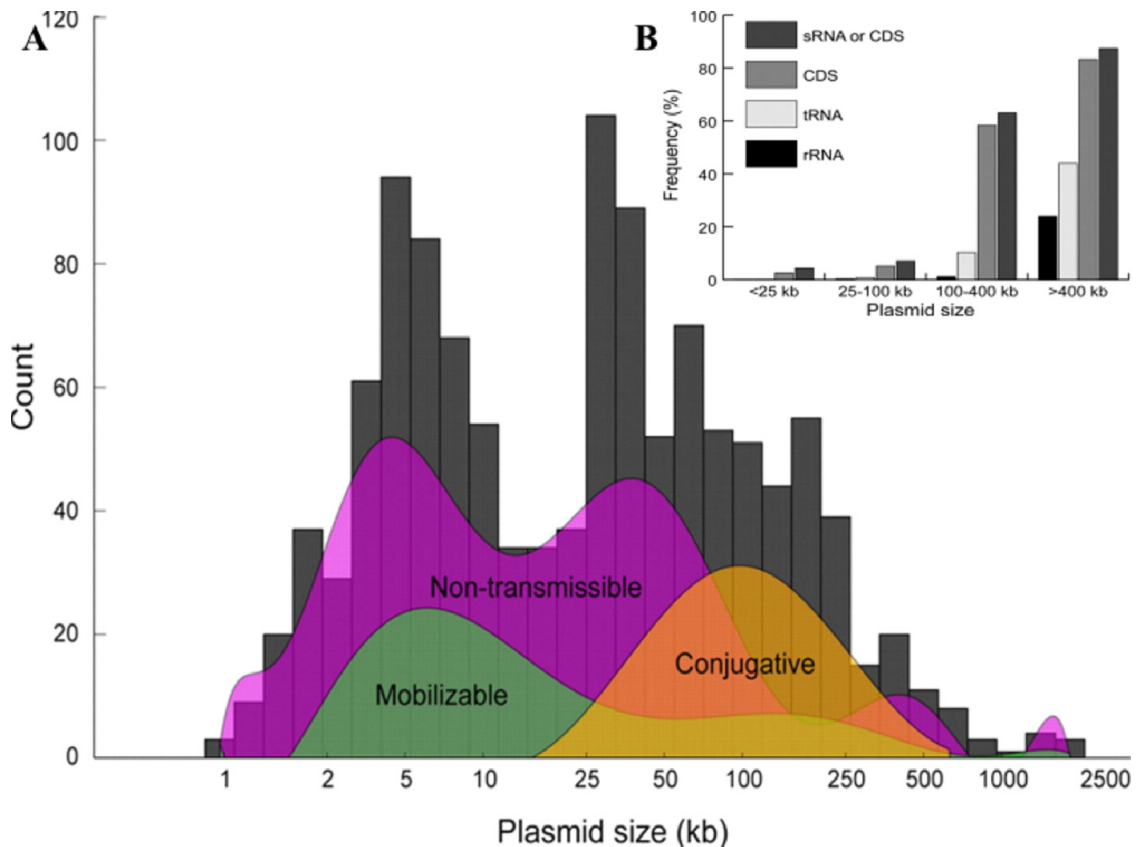


Figure 3: (A) Distribution of conjugative, mobilizable, and nonconjugative plasmids according to plasmid size (curves were created from a polynomial interpolation of the histograms of each class). (B) Presence of tRNA, rRNA, or protein-encoding genes best homologous to *E. coli* or *B. subtilis* essential genes in plasmids classified according to genome size. Small plasmids (<25 kb) rarely contain such genes, whereas very large plasmids (>400 kb) often contain them. Reprinted from Smillie et al., 2010

The modular organization of conjugative systems in functional subunits provides high flexibility in their structure. Particularly interesting cases are those plasmids that lack an *Mpf* system but encode their own *Dtr* and, optionally, their own CP. Such MGE, known as mobilizable plasmids (Mob), could be transferred from one bacterium to another if an *Mpf* system able to interact with the *Dtr* system of the Mob plasmid is present in the same donor bacterium (encoded either on a second plasmid or on the chromosome). Hence, plasmids can be classified into three categories according to mobility: conjugative, mobilizable, and non-transmissible.

The DNA transfer and replication system

The *Dtr* proteins include a relaxase and one or more accessory factors, and when bound to *oriT*, the resulting DNA-protein complex is termed the relaxosome. Detailed characterization of relaxosomes has been carried out for representatives self-transmissible broad plasmid such as RP4 (Pansegrau, *et al.*, 1993); narrow host range plasmids such as F (Frost, *et al.*, 1994); as well as mobilizable plasmids such as R1162 (Scholz, *et al.*, 1989). The term relaxosome originated through the discovery that upon the relaxase-mediated nicking of the DNA strand destined for translocation (hereafter termed the T strand), supercoiled plasmid DNA is converted to the relaxed, open circular form. When a mating pair has formed, a signal is transmitted from the transferosome to the *Dtr* through the CP protein. Following this signal, a cascade of processes occurs (Figure 4, left). *Dtr* proteins initiate processing by binding to a cognate origin-of-transfer (*oriT*) sequence. Recognition of the *oriT* is attained by the specific binding of dedicated *oriT*-specific auxiliary factors within relaxosomes. Typically, multiple binding sites for one or frequently more plasmid-specific initiator proteins are encoded near an *oriT* region that is characterized additionally by regions of thermal instability. The relaxase cleaves a specific site within *oriT*, and this step initiates conjugation. Accompanying the nicking reaction, relaxase remains bound to the 5' end of the T strand, which is displaced by an ongoing conjugative DNA replication process. At this point, the relaxase interacts with the T4CP and then with other components of the *T4SS*, guiding the T strand through the translocation channel. Once in the recipient cell, it catalyzes the recircularization of the T strand and may also participate in second-strand synthesis or recombination into the chromosome (Alvarez-Martinez & Christie, 2009). This DNA processing reaction is common for nearly all known conjugation systems (> 1000) (Garcillán-Barcia, *et al.*, 2009, de la Cruz, *et al.*, 2010).

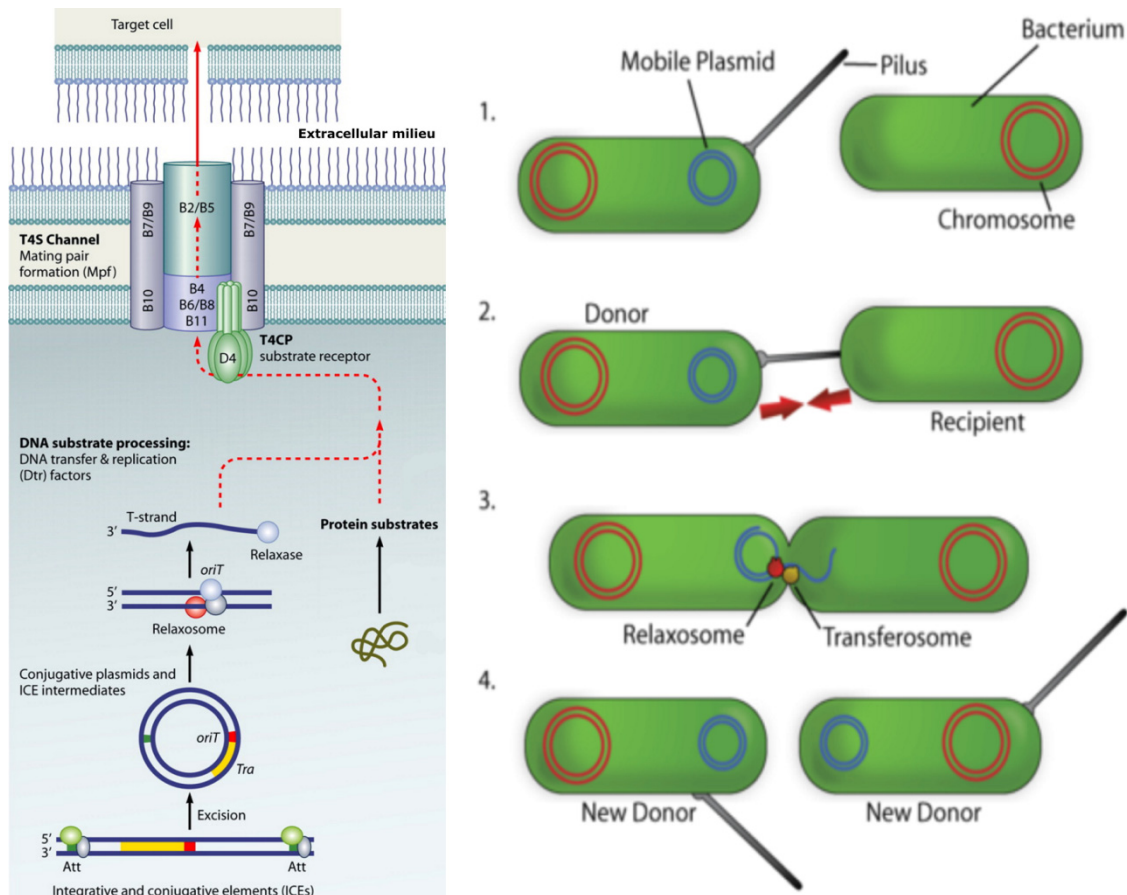


Figure 4: **Left:** Mechanism of T4SS. DNA substrates are composed of conjugative plasmids and *ICEs*. For conjugative transfer, DNA substrates are processed by (i) excision from the chromosome by excisionase / integrase enzymes or DDE transposasas (for *ICEs*), (ii) processing of the plasmid or *ICE* circular transfer intermediate at the origin-of-transfer sequence (*oriT*) by the *Dtr* factors (the *Dtr-oriT* complex is termed a relaxosome), (iii) recruitment of the relaxase-T-strand intermediate to the T4CP, and (iv) translocation through the *T4SS* channel. Figure modified from Alvarez-Martinez *et al.* (2009). **Right:** Conjugative transfer at the individual cell level. The beginning of a conjugative event is given by the recognition and binding of the recipient cell surface by the donor pilus tip (1). After binding, the F-pilus retracts (2) and mating pair stabilization (*mps*) results in a stable association between donor and recipient cells (3) which favors the successful completion of the conjugation process (4).

The transferosome

The most obvious indicator of the existence of a conjugative *T4SSs* in a bacterium cell is the synthesis of conjugative pili. In this sense, the first pili classification system (Ottow, 1975) divided pili into six groups with Group 1 (type 1) pili being the adhesive fimbriae characterized by the related properties of hemagglutination and mannose sensitivity; Group 2 pili were the conjugative pili whereas group 4 (type IV) pili were associated with twitching motility and adhesion. This nomenclature has proven its robustness and remains in use to this day. Later work demonstrated the presence of the complex secretion systems that were responsible for the assembly and function of these pili, with Type 2 secretion systems (*T2SS*) assembling Type IV pili (*T4P*) and Type 4 secretion systems (*T4SS*) assembling conjugative or group 2 pili (Funnell & Phillips, 2004).

T4SS normally involves mechanisms requiring direct contact with a recipient cell to translocate DNA and protein substrates across the recipient envelope. There are three types of *T4SSs* described in the literature: (i) conjugative systems, defined as the translocation of DNA to the recipient cells by a contact-dependent process; (ii) effector-translocator systems, functioning to deliver proteins or other effectors molecules to eukaryotic target cells; and (iii) DNA release or uptake systems that translocate DNA to or from the extracellular milieu (Cascales & Christie, 2003). Since pili biosynthesis is out of the scope of the current PhD thesis and has been extensively reviewed elsewhere (Hazes & Frost, 2008, Alvarez-Martinez & Christie, 2009), in the current PhD thesis we will focus in *T4SSs* conjugative systems, and more specifically in their role in the conjugative transfer process.

Bacterial conjugation starts when the pilus tip of a donor cell recognizes the cell surface of a recipient cell (Figure 4, right), possibly by interacting with lipopolysaccharides (LPS) or, based on the promiscuity of some conjugative transfer systems, a generalized negative charge on the cell surface (Manchak, *et al.*, 2002). In the IncII plasmid R64, recipient recognition and binding is mediated by expression of seven plasmid-encoded PilV adhesins, which are thought to be located at the tips of the thin R64 pili in the donor cell (Ishiwa & Komano, 2003). Interestingly, recent results on R388 plasmid transfer in *E. coli* have shown that recipient cells cannot avoid to be used as recipients in bacterial conjugation (Perez-Mendoza & de la Cruz, 2009). After binding, the pilus retracts probably because of the existence of constant outgrowth and retraction (Clarke, *et al.*, 2008). However, it is not well-established whether the pilus is used to transfer genetic material (Harrington & Rogerson, 1990), or just to pull recipients into the proximity of the donor cells before a separate conjugative junction is formed by fusion of a portion of the cell membranes (Panicker & Minkley, 1985). Recent evidence suggests that in F plasmid, pili may serve as a channel for DNA transfer during conjugation although in a very small frequency (Babic, *et al.*, 2008). These results are in agreement with the large amount of available data for the F plasmid transfer system indicating that efficient transfer requires direct cell-to-cell contact (Dürrenberger, *et al.*, 1991, Samuels, *et al.*, 2000). Similarly, in P-like systems the isolation of "uncoupling" mutations that block detectable pilus production while permitting efficient DNA transfer suggest that conjugative pili are dispensable for DNA transfer (Jakubowski, *et al.*, 2003, Jakubowski, *et al.*, 2005).

The mating pore

In both F and P gram-negative conjugative systems, when a donor and a recipient cell come into close contact they form "mating junctions". One single donor cell may form junctions with several different recipient cells (Samuels, *et al.*, 2000, Gilmour, *et al.*, 2001, Lawley, *et al.*, 2002), which are visible in thin-section electron micrographs (Samuels, *et al.*, 2000). Although using this technique allowed the observation of electron-dense regions probably composed of proteins,

it was not possible to visualize precisely the mating pore embedded within the mating junctions.

New advances in microscopy are allowing researchers to discern *T4SS* architecture and how substrates may be transferred across the gram-negative cell wall. Fronzes and co-workers have recently unveiled for the first time the spatial structure of the *T4SS* core complex encoded by the plasmid pKM101 in the archetypal *Agrobacterium tumefaciens* using cryo-electron microscopy (Figure 5) (Chandran, *et al.*, 2009, Fronzes, *et al.*, 2009).

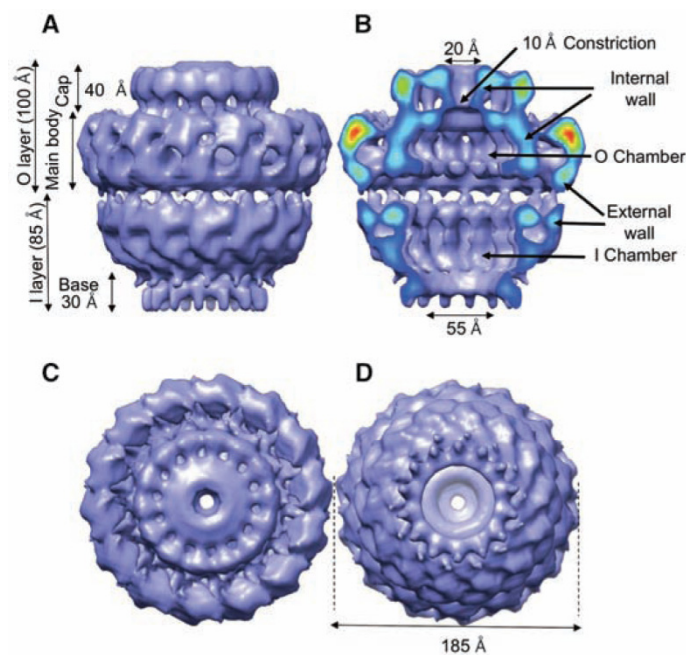


Figure 5: Cryo-EM structure of the T4SS secretion system. (A) Side view. (B) Cut-away side view. Electron density is color-coded from red to blue to indicate regions of strong to weaker density, retrospectively. (C) Top view from the outside of the cell. (D) Bottom view (view from the cytoplasm). Reprinted from Fronzes *et al.* (2009).

These new data have provided very useful information on the study of the mating pore formation and stabilization, which is one of the main factors limiting the amount of DNA that can be transferred by conjugation (Thomas & Nielsen, 2005).

Barriers to Conjugation

Recipients cells that receive a plasmid usually become unavailable for additional conjugation rounds. This phenomenon, known as entry exclusion (Eex), is an essential characteristic of conjugative plasmid biology as suggested by the fact that all conjugative plasmids contain at least one gene encoding for this feature (Garcillán-Barcia & de la Cruz, 2008). The F plasmid contains two different Eex mechanisms, which are accepted as prototypes for all others. The first one involves an outer-membrane Eex protein (TraT), which prevents the bacterium from becoming a recipient for conjugation by being exposed in the outer membrane, interacting with the donor cell and inhibiting the binding of pili of donor bacteria to the cell surface of the recipient (Achtman, *et al.*, 1978, Sukupolvi, *et al.*, 1990). The second mechanism acts inhibiting DNA transfer by the presence of the protein TraS, which is located at the inner membrane of the recipient cell. TraS blocks redundant conjugative DNA synthesis and transport between donor cells, suggesting that it interferes with a signalling pathway required to trigger DNA transfer (Audette, *et al.*, 2007). TraS acts conjointly with TraT to mediate entry exclusion in the F plasmid (Achtman, *et al.*, 1978), while in other systems such as TrbK of plasmid RP4, there is only one Eex component (Haase *et al.*, 1996).

Once the plasmidic DNA has successfully cross the membrane of the recipient cell it still has to avoid being recognize as foreign DNA by the restriction endonucleases of the new host. The fact that at this point plasmidic DNA is single-stranded may provide some protection, as suggested by the much lower transformation frequencies of double-stranded DNA (Lacks & Springhorn, 1984). Nevertheless, the frequency of transconjugants is reduced if the recipient has a restriction system to which the incoming plasmid is susceptible (Arango Pinedo & Smets, 2005). Broad-host-range plasmids such as RP4 seem to have lost most of their restriction sites in order to minimize the effects of such barriers (Wilkins, 2002).

2.2.3 Plasmid diversity

The reports of transfer of multiple antibiotic resistances in between *E. coli* and related bacteria in the 1950's led researchers to make considerable efforts to establish a general plasmid classification system. First attempts were based on phenotypes such as antibiotic resistance, but with the rapid development of the plasmid biology field the need of a more systematic classification system emerged. In this sense, Watanabe and co-workers proposed a classification method based on the ability of a plasmid to inhibit F plasmid transfer (also called F factor, sex factor or fertility factor) when present together in the same host cell (Watanabe, 1969). In this way, plasmids were divided in two main groups, fi^+ (fertility inhibition property plus) and fi^- . A later work (Meynell, *et al.*, 1968) showed a correlation between the fi group and the type of sex pili produced. Accordingly, plasmids were designated as F-like (for fi^+) and I-like (for fi^-). As plasmid biology advanced, some plasmids were shown to be nonconjugative and not to inhibit conjugal transfer. Once again, there was a need to find one universal property of plasmids that could be used to establish the basis of plasmid classification and the most obvious was plasmid replication.

In the early 1970's, a new approach was proposed based in observations showing that when plasmid are closely related (i.e. share common elements involved in plasmid replication and partitioning, see section 2.2.1) they are usually unable to coexist in the same cell (Hedges & Datta, 1973). This phenomenon, known as plasmid incompatibility, allowed classifying plasmids in incompatibility groups (Inc). In order to determine if a new plasmid belong to an specific incompatibility group or not, it was enough to introduce it by conjugation or transformation in a cell where the reference plasmid was already present. The experience was repeated then in the opposite sense. If each of the two experiments led to the elimination of the resident plasmid, then both plasmids were incompatible and belonged to the same Inc group. These groups were named then using letters of the alphabet, with F kept for the F factor and related plasmids: IncI, plasmids producing type I pili susceptible to phage IfI; IncN, N3-related plasmids susceptible to phage IKE; IncF, plasmids producing type F pili susceptible to phage Ff; and IncP, RP4-related plasmids susceptible to the PRR1 phage (Hedges & Datta, 1973). Currently, 27 Inc groups are recognized in *Enterobacteriaceae* by the Plasmid Section of the National Collection of Type Cultures (London, United Kingdom), including six IncF (FII to VII) and three IncI (I1, I7, I2) variants (Carattoli, 2009) (Table I). However, this classification system gets confusing when referring to broad host range plasmids such as those naturally found in *Pseudomonas sp.* (Taylor, *et al.*, 2004). An illustrative example is given by *Pseudomonas areuginosa*, where 14 subgroups are recognize within the global IncP incompatibility group (Table II): plasmids such as RP1 are equivalent to RP4 and RK2 into the P-1 group in *Pseudomonas* and into the P group in *Enterobacteriaceae*. Similarly, IncP-3 plasmids of

Pseudomonas (e.g., RIP64) belong to the IncC group in *Enterobacteriaceae* and IncP-4 plasmids of *Pseudomonas* (e.g., R1162) belong to the IncQ group in *Enterobacteriaceae*.

Table I: Selection of the most relevant plasmid incompatibility groups in *Enterobacteriaceae*

Inc group ^a	Plasmid	Original host	Size (Kb)
FI	R455	<i>Proteus morganii</i>	97
FII	R1	<i>Salmonella enterica</i>	96
FIII	ColB-K98	<i>Escherichia coli</i>	108
FIV	R124	<i>Salmonella enterica</i>	126
I	R46 (5 subgroups)	<i>Salmonella enterica</i>	51
J	R391	<i>Proteus rettgeri</i>	89
N	N3	<i>Shigella spp.</i>	51
P	RP1	<i>Pseudomonas areuginosa</i>	60
Q	R300b	<i>Salmonella enterica</i>	9
T	Rts1	<i>Proteus vulgaris</i>	217
W	S-a	<i>S. flexneri</i>	35

^aPlasmids within the same incompatibility groups are unable to coexist in the same host cell.

Fortunately, the increasing availability of new molecular tools during the last three decades has allowed the development of new approaches to the study of plasmid diversity. In 1988, Couturier and co-workers proposed a genetic plasmid typing scheme based on southern blot hybridization, using cloned replication regions (replicons) as probes (Couturier, *et al.*, 1988). This approach successfully provided classification for both conjugative and nonconjugative plasmids, but the low specificity of the hybridization method underestimated plasmid diversity because of the cross-hybridization reaction among highly related replicons (repI, repB/O, repFII, repFIC). Since 2005, a PCR-based replicon typing (PBRT) scheme has been available, targeting the replicons of the major plasmid families occurring in *Enterobacteriaceae* and also including PCR assays (FrepB and FIIAs PCRs), detecting the FII, FIII, FIV, and FIV variants and the FII replicon of the *Salmonella* virulence plasmids, respectively (Carattoli, *et al.*, 2005).

Table II: Plasmid incompatibility groups in *P. areuginosa*

Inc group^a	Plasmid	Size (Kb)
P-1	RP1	60
P-2	pMG1	481
P-3	RIP64	147
P-4	R1162	8
P-5	Rms163	224
P-6	Rms149	56
P-7	Rms148	224
P-8	Fp2	93
P-9	R2	68
P-10	R91	54
P-11	pMG39	93
P-12	R716	170
P-13	pMG25	102
p-14	pBS222	17

^aPlasmids within the same incompatibility groups are unable to coexist in the same host cell.

However, the PBRT scheme still showed several limitations, since the proposed classification was still based on plasmids belonging to the classic Inc groups and failed to identify divergent or novel replicons. Alternatively, Francia and co-workers proposed to compare the differences between the amino acid sequences of conjugative plasmid relaxases (which are contained in all the conjugative systems known until the date, see section 2.2.1) establishing a new classification scheme for mobilizable plasmids (Francia, *et al.*, 2004). This methodology has been recently extended to T4CPs and T4SSs in an outstanding work carried out within the same research group, allowing the monitoring of the evolutionary patterns of plasmids in phylogenetic trees and the classification of 98% of all the diversity of conjugative plasmids of sequenced proteobacteria (Figure 6) (Garcillán-Barcia, *et al.*, 2009, Smillie, *et al.*, 2010).

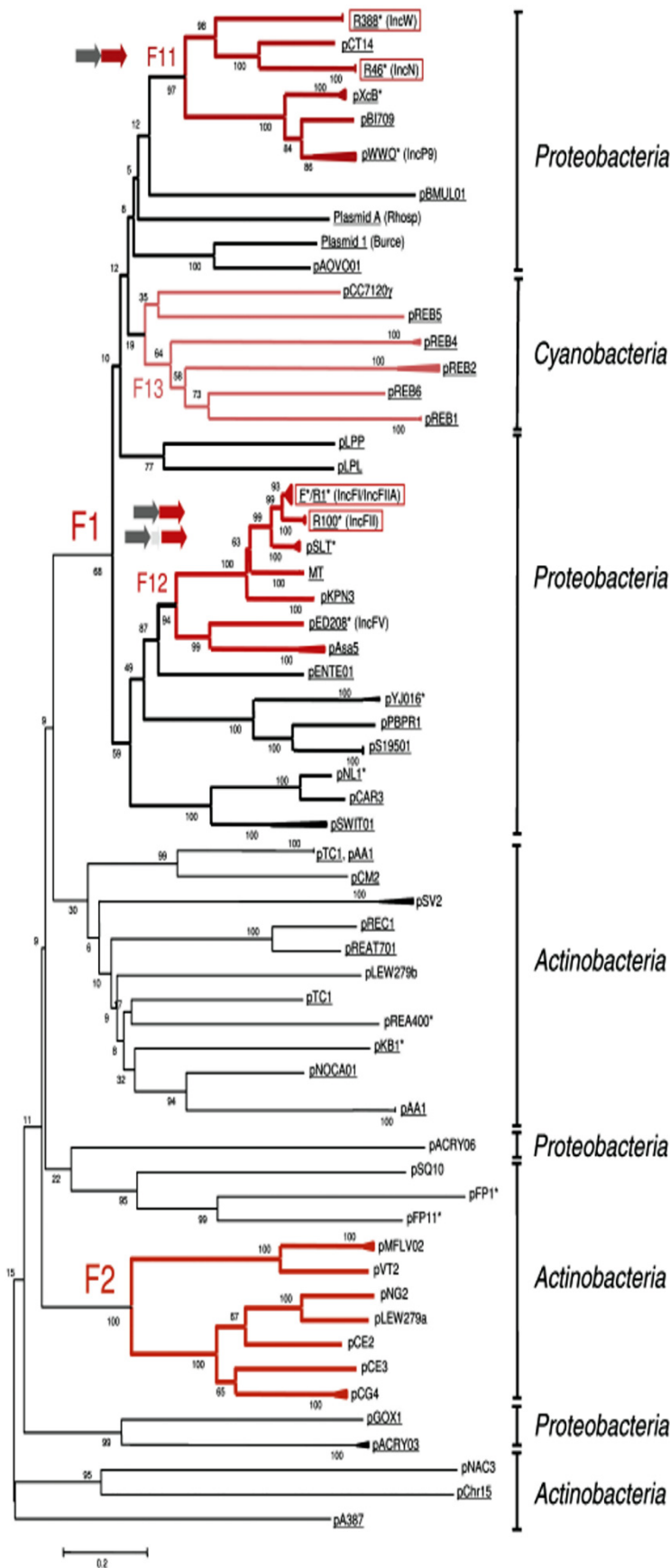


Figure 6: Phylogeny tree of MOB_F relaxases (Dtr_F). The dendrogram was constructed using a NJ algorithm. Bootstrap values for 1000 replicates are indicated. The tree was rooted with TrwC_R388 most distantly related homologues (pA387, pChr15 and pNAC3) that still belong to MOB_F. The distribution of plasmid origins according to the bacterial phyla is shown by vertical bars. Plasmids containing a T4CP gene in the vicinity of the relaxase gene (and thus presumed to be conjugative) are underlined. In the clades where the synteny relaxase-T4CP was conserved, this is shown in the figure by thick horizontal arrows where the T4CP is coloured dark grey, the relaxase gene is coloured red and any intervening gene(s), light grey or a thin black line. Plasmids whose relaxases have been analysed biochemically are boxed. Plasmids for which conjugation has been experimentally demonstrated are labelled with an asterisk. Rhosp, *Rhodobacter sphaeroides*; Burce, *Burkholderia cenocepacia*. Reprinted from Garcillán-Barcia *et al.*, 2009.

Other main conclusions of this work demonstrated how plasmids do not shuffle *Dtr* and *T4SSs* modules freely (see section 2.2.2) but they tend to cluster within given clades, and this preference would somehow be related to specific features of a given plasmid design and with the host physiology. Therefore, the authors remarked that what makes prokaryotic classification useful and meaningful appears to behave equally well in plasmid classification, respecting mobility systems most likely because of the adaptive coevolution of the different elements of the mobility machinery with the host. In addition, the author estimated the frequencies of conjugative (15%), mobilizable (24%), and nontransmissible (61%) plasmids in prokaryotes and in proteobacteria (28%, 23%, and 49%, respectively) (Figure 3, A). These findings will involve the revision of many evolutionary models of plasmid evolution assuming high rates of horizontal gene transfer for plasmid survival.

2.2.4 Plasmid conjugative transfer regulatory mechanisms

Mechanisms for the regulation of transfer gene expression are designed as the conjugative system they are modulating. Therefore, we can roughly distinguish two main groups: IncF-like and IncP-like transfer regulatory circuits. In the first case, the mechanism involved is relatively simple and transfer depends almost entirely on the activity of the host while the P system is highly independent of the host cell, encoding a highly complex regulatory network that allows it to be operative in a wide variety of cellular environments.

All plasmid transfer regulatory systems share in common the urgency to express plasmid genes in the newly formed transconjugant cells to promote a rapid plasmid establishment (Lawley, *et al.*, 2004). However, common needs may be satisfied in different ways: in narrow-host-range systems such as F-like systems, transfer genes are constitutively repressed in a process termed fertility inhibition, which is intimately linked to the host cellular machinery. A strong promoter called P_{traM} allows overriding the fertility inhibition to a low level (only 0.1% to 1% of cells become competent donors). In the presence of recipient cells, these fertile donors transfer immediately, originating transiently derepressed transconjugant cells for a period of time that may cover several generations. The high rates of transfer shown by this derepressed transconjugants leads to rapid epidemic spreading of the plasmid through the recipient cells population, even in the absence of selective pressure (Lundquist & Levin, 1986, Simonsen, 1990).

In contrast to F-like plasmids, there is no self-imposed fertility inhibition mechanism or host factor control involved in IncP system regulation. Instead, those are broad-host-range systems where transfer operons appear to be expressed at all times in an autogenously regulated way through negative feedback loops (Figure 7). One well-studied example which illustrates perfectly the degree of complexity attained by IncP transfer regulatory mechanisms is the

TOL plasmid pWW0 (White & Dunn, 1978) described in the previous section. In this plasmid, conjugation is regulated through feedback repression by open reading frames (ORFs) encoded in each of its main transfer genes, *traA* and *mpfR*, which control the *Dtr* complex or relaxosome and the *T4SS* or transferosome operons respectively (Figure 6) (Lambertsen, *et al.*, 2004).

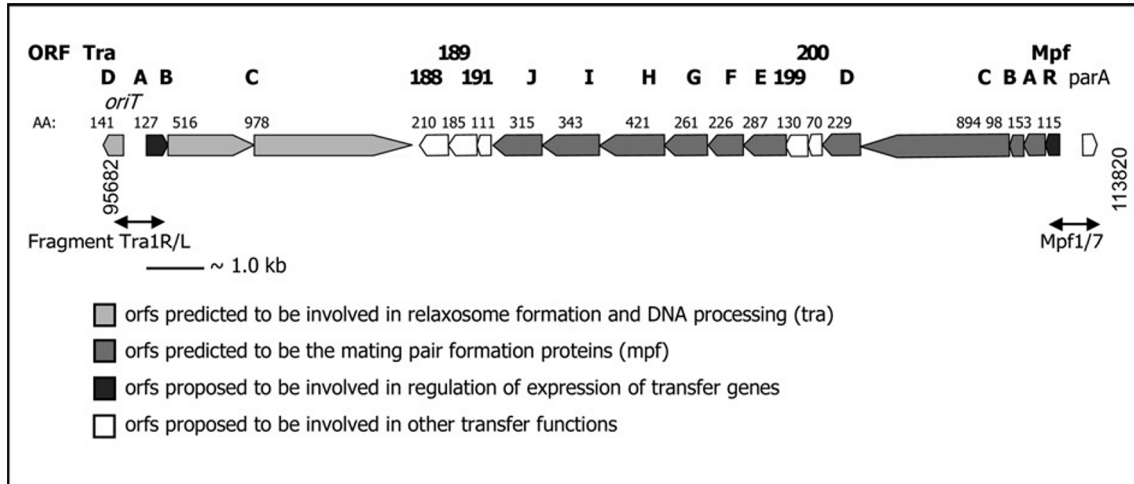


Figure 7 : Map of the putative transfer region of pWW0. The encoded ORFs are shown as box arrows, with the pointed end indicating the direction of transcription. The sizes of the predicted polypeptide products are shown above each ORF, and the name of the product is indicated above that. Reprinted from Lambertsen *et al.*, 2004.

Prior to this work, the observed continuous expression of the conjugative genes led researchers to think that this was the unregulated level of activity rather than the autogenously repressed level (Park, *et al.*, 2003). This conclusion was also consistent with the findings of Bradley and Williams (1982) who considered that the constant expression of pili on the bacterial surface was evidence that the genes were continuously derepressed. Although Lambertsen and co-workers (2004) also obtained similar results for conjugational genes expression in pWW0, they demonstrated the existence of an autogenously regulatory mechanism involved in pWW0 transfer by the inactivation of the *Dtr* genes. Those appeared to be particularly relevant since their expression products seemed to exert a negative control on the promoters *mpfRp1* and *mpfRp2*, which were also the strongest promoter regions detected in the system. In this way, the authors proposed that the presence of *mpfR* would shut down expression of the transfer genes to maintenance levels once the plasmid is established. This mechanism would aim presumably to reduce the metabolic burden and the risks of male-type specific phage infection that could be caused by plasmid genes expression (Thomas, 2006).

2.2.5 Plasmid metabolic burden

Plasmids contain a huge variety of different genes encoding for phenotypic traits that confers adaptative advantages to their host cells such as antibiotic resistance or new biodegradation pathways (Smets & Barkay, 2005, Sorensen, *et al.*, 2005). However, plasmid carriage is only beneficial for the host when environmental conditions provide positive selection for plasmid-bearing cells phenotypes. This is because plasmid carriage usually involves a small but measurable fitness cost. This discovery, together with the existence of plasmids that do not confer benefits to their host, has led plasmids to be considered as parasitic elements. pWW0 provides an illustrative example of plasmid presence negative effects. For example, Duetz and co-workers (Duetz & Van Andel, 1991) have shown that under succinate limitation and low dilution rates, the plasmid-free growth advantage increases up to 50%. In addition, when growing *P. putida* strains carrying pWW0 on benzoate (Williams, *et al.*, 1988), spontaneously “cured” derivative strains took over the culture after 60-80 generations. The negative effects of plasmid presence on bacterial growth have also been shown for other broad-host plasmids (De Bernardez & Dhurjati, 1987). It is important to remark that this fitness cost is not permanent, and plasmid-host co-evolution rapidly reduce metabolic burden after a few hundred generations in both F-like and P-like plasmids (Helling, *et al.*, 1981, Bouma & Lenski, 1988, Dionisio, *et al.*, 2005).

The mechanisms by which plasmid metabolic burden operates are unclear. The different hypotheses trying to explain this phenomenon can be classified in three groups (Slater, *et al.*, 2008): those regarding the cost as an effect of plasmid-encoded protein expression (Bentley, *et al.*, 1990, Rozkov, *et al.*, 2004); those that related the cost to an effect of the replication and transfer on the plasmid DNA (Björkman & Andersson, 2000); and those which look for disorders in the cellular regulatory mechanisms (Ricci & Hernandez, 2000). In this sense, during the current work we have developed a methodology based in respirometry which has allowed us to measure very accurately the subtle metabolic burden associate with pWW0 carriage in *P. putida* (Seoane, *et al.*, 2010). From our results, both plasmid presence and protein expression encoded from the plasmid produced measurable effects on both the yield and the growth rates of our strain. Therefore, we could expect plasmid size as well as copy number to have an effect on the fitness cost of plasmid carriage. To the best of our knowledge, at the moment of writing the current work only one study had investigated the effect of carriage of 101 different plasmids on the growth rate of *E. coli*, showing that those plasmid that extended host generation times by more than 15% tended to be larger (>80 kb) (Bouma & Lenski, 1988, Slater, *et al.*, 2008). Other recent works have focused more on the characterization of the effects of plasmid carriage on the host cell metabolism. When global transcriptional expression analysis was applied on *E. coli* carrying the 7.3 kb NS3 plasmid, results showed a general trend of downregulated biosynthetic/energy metabolism genes, differentially

expressed transport genes and upregulated heat shock proteins (Ow, *et al.*, 2006). In addition, metabolic disorders caused by the broad-host-range plasmids pBBR1MCS-2 and pUCPM18 derived pAB4 and pAB8 have also been demonstrated in *Pseudomonas fluorescens* (Buch, *et al.*, 2010). In our opinion, there is enough evidence suggesting that the three proposed mechanisms of plasmid metabolic burden may coexist instead of being mutually exclusive, and their relative contributions to the overall fitness cost will be greatly dependant on the specific plasmid-host system tested.

2.3 Studying the extent of conjugal plasmid transfer in microbial populations: experimental approaches

Quantitative measurements are critical to address the extent of conjugal plasmid transfer in microbial populations. Traditionally, the efficiency of a conjugation system has been described by its transfer frequency, which usually involved the need to quantify donors, recipients and transconjugants frequencies.

2.3.1 Ex situ experimental approaches

Many different strategies have been developed so far to distinguish between the different bacterial types involved in plasmid dynamics essays, although most of them are based in the same concept: plasmid confers selective phenotypic traits to their host. Selective plating was the first method used to monitor gene transfer. Although the main advantages of this technique stand in its simplicity and sensitivity (detection limit down to 1 cell pr. sample), it also showed very important limitations: methods relying on culturing could not distinguish between effective transfer events and post-transfer selection (clonal growth of newly formed transconjugant cells). This made very difficult to quantify the effects of those environmental factors which were known to affect both plasmid transfer rates and population growth rates simultaneously, such as temperature (Richaume, *et al.*, 1989) or nutrient availability (Smets, *et al.*, 1993, Smets, *et al.*, 1995). In addition, ex-situ methods are inherently limited to the culturable fraction of bacteria (which is often under 1% of the overall amount of bacteria determined by direct counts) (Amann, *et al.*, 1995). Furthermore, this approach produce population-averaged measures of gene transfer, which did not account for the effects of heterogeneity or spatial structure on conjugative plasmid transfer in a bacterial population.

2.3.2 In situ experimental approaches

The use of *in situ* quantitative approaches such as those based on hybridization assays (Fujimura, *et al.*, 1996) or QPCR (Lee, *et al.*, 2006, Franiczek, *et al.*, 2010), has become very common in works analyzing conjugative plasmid transfer. Particularly important in the current PhD thesis is the use of reporter-

gene technology, which allows non-disruptive direct visualization of HGT at the individual cell level and remove the need for cultivation. Different reporter genes systems are available and have been successfully applied before to the study of HGT, including the luciferase genes *luxAB* and *luc* (Hoffmann, *et al.*, 1998), the β -galactosidase gene *lacZ* and genes encoding fluorescent proteins such as the green fluorescent protein (GFP) (Jaenecke, *et al.*, 1996). More specifically, the reporter gene system designed by Christensen *et al.* (1996), allows plasmid transfer detection by zygotic induction of the *gfp* marker gene after transfer in the recipient cell, allowing the assessment of conjugative plasmid in mixed populations without the need of selective cultures.

Later improvements on the same system (Dahlberg, *et al.*, 1998) allowed to avoid *gfp* marker gene expression in the donor cells but not in the transconjugant cells. This effect was obtained by fusing the *gfp* marker gene to a LacZ promoter, which was inhibited by a LacI repressor inserted in the donor's chromosome, producing in this way a solid HGT detection system that has been widely used since then (Christensen, *et al.*, 1998, Hausner & Wuertz, 1999, Nancharaiah, *et al.*, 2003). One example is provided by the estimation of the IncP plasmid pKJK10 transfer efficiency (10^{-2} transconjugants/donor) from an *E. coli* donor to indigenous freshwater bacteria in batch essays (Sørensen, *et al.*, 2003). Pinedo and Smets (2005) used a similar approach to asses in situ the effects of restriction proficiency and exposure to toxicants on the conjugational transfer rate of the TOL plasmid between *Pseudomonas spp.* on filter matings. A *Pseudomonas putida* KT2442-derived strain carrying a *gfp*-tagged variant of the TOL plasmid was used as a donor, and both restriction-deficient (PAO1162N) and -proficient (PAO2002N) *Pseudomonas aeruginosa* strains were used as recipients. The *in situ* enumeration of conjugation events allowed the authors to estimate plasmid transfer frequencies that were unbiased by transconjugant growth or plasmid retransfer. At standard donor-to-recipient ratios (10^{-3} for PAO1162N and 2×10^{-1} for PAO2002N) and total cell densities (10^5 cells/mm² for PAO1162N and 10^6 cells/mm² for PAO2002N), plasmid transfer frequencies were approximately 10^{-7} and 10^{-11} events per mm² respectively.

Other works have combined the previous construct with a zygotic marker system based on the red fluorescent protein DsRed from *Dicosoma* (Tolker-Nielsen, *et al.*, 2000), making possible to distinguish donor, recipient and transconjugant cells using fluorescence microscopy and/or flow-cytometry. This approach was used to identify the main drivers of plasmid transfer in the rhizosphere of pea and barley (Molbak, *et al.*, 2007). The donor *Pseudomonas putida* KT2442, containing plasmid pKJK5::*gfp*, was coated onto the seeds, while the recipient *P. putida* LM24, having a chromosomal insertion of dsRed, was inoculated into the growth medium. Mean transconjugant-to-donor ratios in vermiculite were $4.0 \pm 0.8 \times 10^{-2}$ in the pea and $5.9 \pm 1.4 \times 10^{-3}$ in the barley rhizospheres. In soil, transfer ratios were about 10 times lower. As a result of a 2-times higher root

exudation rate in pea, donor densities in pea were about 10 times higher than in barley.

However, in despite of the great improvements achieved during the last decade, experimental approaches have failed when trying to explain the observed plasmid spreading patterns within microbial communities growing on solid surfaces. An illustrative example is provided by plasmid invasion in biofilms, the most common form of bacterial life in nature. These structures are presumed to be hot-spots of horizontal gene transfer since they sustain high bacterial cells densities and provide a physically stable environment which supports cell-to-cell contacts (Sorensen, *et al.*, 2005). However, although some plasmids such as pWW0 has been observed to attain transfer frequencies as high as one transconjugant per donor under optimal conditions (Ramos, *et al.*, 1997), other works have shown its inability to fully invade colonies (Christensen, *et al.*, 1996) and biofilms (Christensen, *et al.*, 1998). In both cases, transconjugant cells were preferentially formed on the top layers and plasmid invasion was not detected. These observations have been proposed to be related to the nutrient and oxygen gradients typically formed within biofilm structures, which would originate low metabolic activity and low transfer genes expression levels. However, it has also been demonstrated that even starved cells may perform plasmid transfer and that above a certain threshold activity the level of conjugation is independent of metabolic activity (Normander, *et al.*, 1998, Hausner & Wuertz, 1999). Alternatively, it has also been suggested that cell-to-cell contact could be prevented by the production of extracellular polymeric substances (EPS) in high amounts (Molin & Tolker-Nielsen, 2003). Therefore, the reason why a plasmid cannot fully invade a receptive bacterial population remains unclear.

In addition, the large variety of different metrics still used to report plasmid-transfer frequencies *in situ* strongly remarks the need of a standardized metric for plasmid-transfer efficiency. Ideally, it should be reported as the number of transfer events per donor–recipient encounters (Sorensen, *et al.*, 2005). Such a ratio would allow true comparisons of the transfer efficiencies of different plasmids or in different environmental settings. During the current work, we have addressed this need by developing an experimental approach specifically designed to estimate conjugational transfer frequencies and other conjugation-related parameters at the individual cell scale (Figure 8, Annex II).

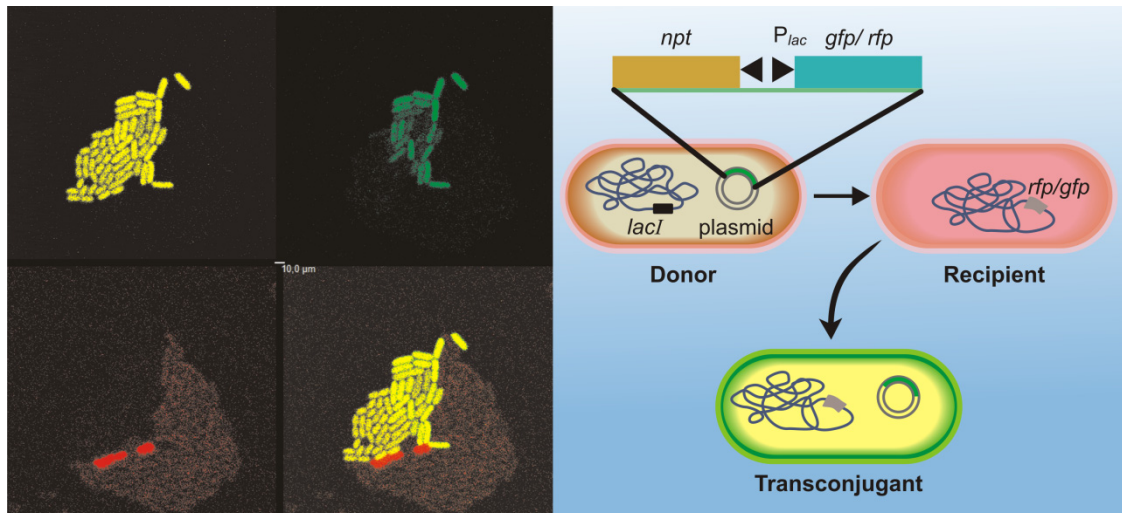


Figure 8: Direct visualization of conjugational plasmid transfer at the individual cell level. Right: In situ visualization of conjugation within a microcolony where donor *P. putida* KT2440 cells expressing DsRed LacI^q (bottom, left channel, red) transfer pWW0 TOL::GFP-LacI (top right channel, green) to recipient *P. putida* KT2440 cells expressing YFP (top left channel, yellow). The lower-left image displays the overlay of all these fluorescence images. Non-dividing inoculated donors present higher red intensities due to previous DsRed maturation. Left: An schematic of the reporter gene merged downstream from a synthetic LacZ promoter. GFP expression is repressed in the donor cell (red) by chromosomally tagged *LacI* gene inserted in the donor's chromosome. When the plasmid is transferred to a recipient cell (yellow), GFP expression is not repressed and transconjugants are detected as simultaneously yellow and green cells.

2.4 Studying the extent of conjugal plasmid transfer in microbial populations: theoretical approaches

2.4.1 Modeling plasmid population dynamics

During the last three decades, mathematical modeling has been a very useful complementary approach to explore plasmid fate within bacterial populations. In this sense, a steady plasmid copy-number in a bacterial population may be seen as an equilibrium between those processes that increase the number of plasmid copies (i.e. through horizontal gene transfer processes or replication promoted by selective advantage for plasmid encoded phenotypes) and those increasing the rates of plasmid loss (via vegetative segregation and/or fitness disadvantages).

This perception of plasmid transfer led Stewart and Levin (1977) to apply for the first time the quantitative mathematical approaches originally devised to study the population biology of higher organisms (Volterra, 1928) to the study of plasmid population dynamics. In their mass-action model for plasmid transfer, three cell types are recognized: donors (D), recipient (R), and transconjugants (T). D referred specifically to the original cells carrying the plasmid, while T referred to the newly formed plasmid carriers.

Plasmid dynamics between these three cells types are explicitly considered. The model assumes the feasibility of horizontal plasmid transfer from donor to recipients (to result in transconjugants) and from transconjugants to recipients (to result in more transconjugants). No plasmid transfer between plasmid harboring cell types is considered. Plasmid transfer is described through a mass-action model (1), meaning that the probability of transfer is jointly proportional to the density of the participating cell types (in the mating pair) multiplied by a probability coefficient. This probability coefficient is a measure of the plasmid ‘fertility’ and is typically referred to as the plasmid transfer rate coefficient γ (units: $\text{vol cell}^{-1} \text{ time}^{-1}$) (see section 2.3). In addition to horizontal transfer, plasmid loss from one of the cell types (τ) is implemented as a linear function of the cell density. In all of these processes, ‘r’ refers to volumetric reaction rates. Because plasmid transfer occurs between ‘individuals’, the concentration of the different cells types, X , is expressed in individuals-based concentrations (numbers or cells/volume) rather than in mass-based concentrations. The limiting solute concentration, S , remains in traditional units (mass/volume or moles/volume).

$$\begin{aligned}
 \text{D to R plasmid transfer rate} &\equiv r_{DR} \equiv \gamma_{DR} X_D X_R & (1) \\
 \text{T to R plasmid transfer rate} &\equiv r_{TR} \equiv \gamma_{TR} X_T X_R \\
 \text{plasmid loss rate from D} &\equiv r_{L_D} \equiv -\tau_D X_D \\
 \text{plasmid loss rate from T} &\equiv r_{L_T} \equiv -\tau_T X_T
 \end{aligned}$$

The growth dynamics of all cell types are typically described by Monod-like dependencies on the limiting substrate concentrations (2 & 3). In this case, μ are specific growth rates (h^{-1}), which are in their turn written as functions of the limiting substrate concentration, S and α refer to the fractional reduction in the specific growth rate due to plasmid carriage by the transconjugant cell type.

$$\begin{cases}
 \text{growth rate of D} = r_{X_D} = \mu_D X_D \\
 \text{growth rate of R} = r_{X_R} = \mu_R X_R & \mu_T = (1 - \alpha)\mu_R \\
 \text{growth rate of T} = r_{X_T} = \mu_T X_T
 \end{cases} \quad (2)$$

$$\mu_D = \mu_{\max,D} \frac{S}{S + K_{S,D}} \quad (3)$$

The substrate removal and cell growth rate expressions are coupled via the growth yields (4):

$$\text{substrate removal rate} = r_S = -\frac{r_{X_D}}{Y_{X_D S}} - \frac{r_{X_R}}{Y_{X_R S}} - \frac{r_{X_T}}{Y_{X_T S}} \quad (4)$$

Finally, these equations are combined in a mass balance to result in the governing equations for the different cell types in a chemostat environment (5). For the situation of a chemostat (a well mixed reactor, receiving a constant inflow and influent concentration of substrate, operated at a fixed dilution rate and having a constant volume), the equations become:

$$\begin{cases} \frac{dX_D}{dt} = r_{X_D} - DX_D \\ \frac{dX_R}{dt} = r_{X_R} - r_{DR} - r_{TR} + r_{L_T} - DX_R \\ \frac{dX_T}{dt} = r_{X_T} + r_{DR} + r_{TR} - r_{L_T} - DX_T \\ \frac{dS}{dt} = D(S_o - S) - \frac{r_{X_D}}{Y_{X_D S}} - \frac{r_{X_R}}{Y_{X_R S}} - \frac{r_{X_T}}{Y_{X_T S}} \end{cases} \quad (5)$$

with $X_{D,o}$, $X_{R,o}$, $X_{T,o}$ as initial conditions for X_D , X_R , X_T and S_o being the influent substrate concentration and D the dilution rate of the chemostat (flow rate through the chemostat/ volume of chemostat).

Solving the system described above leads to infer a simple ‘existence’ condition for a conjugal plasmid in a chemostat where an isogenic population community consisting of only a plasmid-free and a plasmid-carrying fraction is growing:

$$\gamma_{TR}(X_R + X_T) \geq \alpha D + \tau \quad (6)$$

The previous expression indicates that the rate of transconjugant generation must exceed the rate of transconjugant loss due to segregation. In other words, it indicates that a chemostat needs to support a minimal cell density to ensure transconjugant persistence given a certain set of plasmid dynamic parameters (γ and τ) as well as a plasmid growth effect (α).

Mass-action transfer models are the most extended and simple modeling method applied to the study of HGT in silico. Knudsen et al. applied this approach to

study the dynamics of survival and conjugation of *Pseudomonas cepacia* (carrying the transmissible recombinant plasmid R388:Tnl721) with a non-recombinant recipient strain in simple rhizosphere and phyllosphere microcosms, obtaining similar transfer rates in both environments (5×10^{-14} and 5.6×10^{-14} ml x cell⁻¹ x min⁻¹ respectively) (Knudsen, *et al.*, 1988). Simonsen and co-workers combined theoretical and experimental approaches to estimate the effects of growth rate, segregation and transitory derepression on plasmid R1 transfer rates in *E. coli* K12 growing in chemostats, obtaining intrinsic (i.e. independent of the experimental conditions) transfer frequencies values ranging between 1×10^{-12} and 9×10^{-13} ml x cell⁻¹ x min⁻¹ (Simonsen, 1990). Using a similar approach, conjugal transfer kinetics of pWW0 TOL plasmid in *P. aeruginosa* were estimated to be around 1.81×10^{-14} ml x cell⁻¹ x min⁻¹ (Smets, *et al.*, 1994). Other works have used mass-actions transfer models to address different questions: plasmids persistence over evolutionary time (Bergstrom, *et al.*, 2000), spreading of antibiotic resistance on hospitals (Webb, *et al.*, 2005), characterization of plasmid stability in different strains within the *Alpha*-, *Beta*- or *Gammaproteobacteria* carrying the IncP-1 β plasmid pB10 (De Gelder, *et al.*, 2007), plasmid population dynamics under non-selective conditions (Ponciano, *et al.*, 2007) or attachment and detachment dynamics (Zhong, *et al.*, 2010).

However, although this approach is able to capture some of the mechanistic elements of the biology of plasmid transfer, it involves that contacts occurs 'randomly' between the different cells types of a mating pair. This entails some major assumptions: every cell has equal probability of making contact with another cell and an equal fraction of these encounters results in plasmid transfer. Hence, the use of mass action models for HGT studies is only appropriated for homogeneous well-mixed environments (Turner, 2004). In addition, mass-action models cannot to capture the effects of the intra-population variability within a bacterial population, which maybe very high even in single species communities (Ponciano, *et al.*, 2009).

It is now widely recognized that the majority of bacteria found in natural, clinical, and industrial settings persist in association with surfaces were perfect mixing cannot be assumed (Davey & O'toole, 2000). Therefore, there is a direct need for the development of new mechanistically valid plasmid dynamic models that can address the spatial and biological heterogeneity typically associated with surface-attached bacterial communities. In this sense, Individual-based Modeling (IbM) (Kreft, 2004, Picioreanu, *et al.*, 2004) has been proposed as the most appropriate framework for modeling genetic transfer by conjugation, as this is a discrete event between two individual cells that contact forming a mating pair (Gilmour, *et al.*, 2001, Sorensen, *et al.*, 2005, Hellweger & Bucci, 2009). During the current work, we have addressed this suggestion by developing an individual-based model conceived for the study of plasmid conjugational transfer in colonies and biofilms (Annex III & Annex IV).

2.4.2 Modeling plasmid population dynamics in spatially structured environments: the Individual-based Modeling approach (IbM)

In order to capture the effects of spatial structure and the intra-population variability within a bacterial population on plasmid transfer, several colony scale HGT modeling approaches based in cellular automata (CA) have been proposed recently. These are discrete models consisting of a regular grid of *cells*, each one of which has a finite number of *states*. Lagido *et al.* (2003) proposed a CA model for horizontal transfer of plasmids on surfaces where independent donor and recipient colonies grew exponentially until complete nutrient depletion was reached and plasmid transfer occurred instantaneously if different colony types met. Although this model was able to describe trends observed in living colonies it tended to overestimate conjugation frequencies. The fact that some of the model assumptions (such as conjugation occurring every time a donor and recipient met) were biologically unrealistic could explain part of the inaccuracy observed (Lagido, *et al.*, 2003). More recently, Krone *et al.* (2007) produced a spatially explicit, stochastic CA of plasmid persistence on surfaces that incorporated HGT as well as plasmid loss (see section 2.2.4). In a later work, the same model was upgraded to describe three-dimensional structures (Fox, *et al.*, 2008). Although these models were able to describe some aspects of plasmid pB10 dynamics in *E. coli* colonies grown on filters, they failed when applied to the results obtained for plasmid R1 in *E. coli* K12 presented by Simonsen (1990). The authors explained this issue as a possible effect of not implementing the specific R1 plasmid regulatory system in the model. In addition, the CA modeling approach is not exempt of limitations, since biomass can only move in a finite number of lattice directions; as a result, the structures produced are the result of growth constrained to pre-defined directions and are extremely dependent on the coordinate system chosen (Laspidou, *et al.*, 2010). Therefore, although this approach was suitable for the spatial scale of interest in these studies (clusters and colonies of cells rather than individual microbes), it does not allow to capture the effects of the intra-population variability within a bacterial population, which maybe very high even in single species communities (Ponciano, *et al.*, 2009).

In this sense, Individual-based modeling or agent-based models (Kreft, 2004, Picioreanu, *et al.*, 2004) aim to describe the different characteristics defining a bacterial population as emergent properties arising from the biology and interactions occurring between individual bacterium and the single cell level. This approach is based on the same principles than CA models (substrate uptake, metabolism, maintenance, cell division and death), but there are still some major differences: each bacterium is individually simulated as a sphere of variable size in a continuous, three-dimensional space. The concentrations of substrates and products (i.e. oxygen, carbon sources, ammonia, nitrite, or nitrate) are obtained from diffusion and reaction, as described (Picioreanu, *et al.*, 2004). Using a 3D space, the movements of the simulated bacteria can have the same degree of

freedom as in reality, without using a predefined grid and no global laws such as exponential population growth are applied. In addition, movement of agents due to cellular growth and division is addressed through two complementary mechanisms: a biomass growth pressure (Klapper, *et al.*, 2002, Alpkvist, *et al.*, 2006) and a local particle shoving algorithm (Kreft, *et al.*, 1998, Xavier, *et al.*, 2005). Modeling details on the different processes involved in colony and biofilm growth simulation using IbMs are explained below.

Solute Dynamics

Two processes govern the solutes concentration fields within the computational domain: mass transport by diffusion and bacterial-mediated reactions. These two processes lead to the following partial differential equation (PDE) for solutes:

$$\vec{\nabla} \cdot (D_s \vec{\nabla} S) + r_s = 0 \quad (7)$$

In this equation, D_s is the solute diffusion coefficient and r_s is the local solute reaction rate. In using this equation it is assumed that the solute fields are in pseudo-steady-state with respect to biomass growth because the solute dynamics occur much faster than growth dynamics (Picioreanu, *et al.*, 1998). This equation is solved using the multi-grid method (Brandt, 1977), as has been used in previous models for biofilm growth (Picioreanu, *et al.*, 2004, Xavier, *et al.*, 2005, Lardon, *et al.*, 2009).

The solute fields are affected by the imposed conditions at the domain boundaries. In the current work, we have used a no-flux boundary for the substratum at the domain bottom, involving that the solute gradient normal to the boundary is set to zero. For the domain top, the solute concentration is fixed based on the imposed bulk compartment concentration. The domain sides are assumed to be periodic, and hence have constant solute concentration across the boundary.

For simulations utilizing a time-dependent bulk compartment, the solute concentrations in the bulk compartment are affected by a dilution process and by reactions occurring within the microbial population (Lardon, *et al.*, 2009). Thus, in the bulk compartment we have an equation of the form:

$$\frac{dS_B}{dt} = D \cdot (S_{In} - S_B) + \sigma_R \cdot \frac{1}{A_{BF}} \sum r_s \delta V \quad (8)$$

where S_B is the solute concentration in the bulk compartment, D is the dilution

rate of the bulk compartment, S_{In} the influent solute concentration, σ_R the specific surface area of the reactor (total area of carrier surface in the reactor divided by reactor volume), A_{BF} the surface of carrier potentially covered by biofilm, r_S the local reaction term within each grid element, and δV the volume of a grid element. The summation in (8) is over all grid elements in the computation domain. Note though that in all cases we assume a constant oxygen concentration in the bulk compartment, though the other solutes may vary.

Agent Dynamics

The space-occupying agents (bacteria and EPS particles) are represented by incompressible cylinders in two or three dimensions (spheres). The bacterial agents, in addition, possess an interior compositional structure consisting of active biomass, inert biomass, and capsular EPS; the active and inert biomass comprise an inner ‘‘cell’’ that represents the organism. The mass m_j and volume v_j of the entire agent may be found by considering the mass $m_{j,i}$ and density ρ_j of each component:

$$m_j = m_{j,active} + m_{j,inert} + m_{j,EPS}$$

$$v_j = V_j^{active} + V_j^{inert} + V_j^{EPS} = \frac{m_{j,active}}{\rho_{active}} + \frac{m_{j,inert}}{\rho_{inert}} + \frac{m_{j,EPS}}{\rho_{EPS}} . \quad (9)$$

Similarly, the radii of the inner cell and the entire agent may be estimated via:

$$\varphi_{j,cell} = \sqrt{\frac{1}{dZ} \frac{V_j^{cell}}{\pi}} \quad \text{and} \quad \varphi_{j,Total} = \sqrt{\frac{1}{dZ} \frac{V_j^{cell} + V_j^{capsule}}{\pi}} , \quad (10)$$

where $\varphi_{j,cell}$ is the radius of the cell including all intracellular compartments, $\varphi_{j,Total}$ is the radius of the entire agent (including capsular components), and the cylinder height is given by dZ . The radii are used in determining the time of cellular division.

Cellular Growth and Division

Reactions occurring in the model are described via stoichiometric matrices. The suite of reactions in Table III are a basic set of growth and maintenance equations meant to capture the basic essentials of aerobic bacterial growth on citrate. We have also included the effects of decay of active to inert biomass, as well as EPS production and hydrolysis.

Table III: Stoichiometric Matrix For Default Reactions

Reaction	Solutes		Biomass			Reaction Rate
	S_I	S_{O_2}	X_{Active}	X_{Inert}	X_{EPS}	
Growth on Substrate S_1	$-\frac{1}{Y_{S_1}}$	$-\frac{1-Y_{S_1}}{Y_{S_1}}$	$1-Y_E$		Y_E	$\mu_{max}^{S_1} \cdot \frac{S_1}{K_{S_1} + S_1} \cdot \frac{S_{O_2}}{K_{O_2} + S_{O_2}} \cdot X_{Active}$
Cell Maintenance		-1	-1			$b_m \cdot \frac{S_{O_2}}{K_{O_2} + S_{O_2}} \cdot X_{Active}$
Cell Decay			-1	1		$b_d \cdot X_{Active}$
Hydrolysis	1				-1	$k_{hyd} \cdot X_{EPS}$

Each reaction has an overall rate expression r_i that is, along with a yield coefficient Y_i , used to describe how a reaction affects solute and particulate components. The net reaction rate for a component j is found by summing all the reactions by which it is affected:

$$r_{j,Net} = \sum_{i \in \substack{\text{involved} \\ \text{reactions}}} Y_i \cdot r_i . \quad (11)$$

Reactions respect mass-conservation principles, and negative stoichiometric coefficients indicate consumption while positive coefficients indicate production. Several reaction rates are defined by the product of Monod kinetic factors representing saturation of a compound. Most reaction rates are also proportional to the mass of the compartment catalyzing the reaction; this means that within an individual agent several reactions may occur, each catalyzed by a different compartment.

Cellular division occurs instantly when cells reach a maximal size, with the size corresponding to a given amount of accumulated biomass. To avoid artificial synchronization of agents, the division decision is tested against a Gaussian distribution of the division radius using a 10% standard deviation of the division

radius, with the distribution cut off outside two standard deviations. Moreover, the division is not symmetric, with the daughter cells each receiving a slightly different amount of mass (chosen via a Gaussian distribution with 10% standard deviation that is centered around 50% and restricted to the 40-60% range) and being located in a randomly-chosen direction. Daughter cells are positioned such that they touch but without overlap at the end of the division. Cellular division may also affect plasmid presence, but this will be discussed later.

EPS excretion process can be introduced as described before (Xavier & Foster, 2007, Nadell, *et al.*, 2009), whereby bacterial agents release capsular EPS continuously rather than discretely. This approach to EPS release better captures the altruistic behavior of bacteria growing in biofilm communities.

Mechanical Interactions

Movement of agents due to cellular growth and division is addressed through two complementary mechanisms: a biomass growth pressure (Klapper, *et al.*, 2002, Alpkvist, *et al.*, 2006) and a local particle shoving algorithm (Kreft, *et al.*, 1998, Xavier, *et al.*, 2005).

For the biomass growth pressure, a mass balance is applied on each grid element, taking account of biomass growth and decay as well as advective motion. These growth and decay processes contribute to a biomass pressure P , and this pressure is alleviated through the advective movement of biomass. Advection within the bacterial population is described by Darcy's law, where the advective velocity \vec{u} is given by: $\vec{u} = -\lambda \vec{\nabla}(P)$, with λ called the Darcy parameter. Following Alpkvist *et al.* (2006), application of mass conservation for each grid element in the population yields the following elliptic equation for the pressure P :

$$\vec{\nabla} \cdot (-\lambda \vec{\nabla} P) + \sum_{j \in \text{zone}} \frac{r_j}{\rho_j} = 0 \quad (12)$$

where r_j and ρ_j are the mass production rate and density of agent j , respectively. The boundary conditions for calculating the pressure field P include a no-flux boundary at the substratum and periodicity in the lateral directions following the solutes, along with an imposed pressure $P=0$ outside the biofilm. These boundary conditions allow for calculation of the pressure via (12), and the local advective velocity may be computed by application of Darcy's law. Note that the value of λ may be chosen arbitrarily without altering the resulting velocity \vec{u} , so long as λ is kept constant throughout the biofilm. The resulting advective velocity is applied to each agent, and the global advection term is added to other movement terms caused by cell division and EPS particle excretion.

In addition to moving by advective motion due to the biomass pressure terms, agents are also affected by growth in the local neighborhood: growth may sometimes result in an overlap of agents, and the mechanism for resolving this overlap is local shoving. Any overlap of agents is estimated not on the basis of the cell radius $\varphi_{j,Total}$ but rather on the basis of a shoving radius, with $\varphi_{j,Shove} = k_{Shove} \cdot \varphi_{j,Total}$; the shoving factor k_{Shove} allows for adaptations of the shoving mechanics. Then the overlap δ between two adjacent agents is defined by:

$$\delta = d_{1,2} - k_{Shove} \cdot (\varphi_{1,Total} + \varphi_{2,Total}), \quad (13)$$

where $d_{1,2}$ is the distance between the centers of the two agents, as illustrated in Figure 9. During simulations of biofilm growth, it is more interesting to track the steady-state location of the agents, so therefore we use a relaxation algorithm (Lardon, *et al.*, 2009) to minimize the number of overlaps. Any overlap between agents is resolved by moving each in a direction opposite to the other by half the overlap distance. For a single agent, the movement vectors resulting from interactions with each of its neighbors are summed and the resulting movement is applied at the end of the step. This cycle is continued for all agents, and the shoving is stopped when the number of agents still moving is negligible (it is less than 5% of the total number of agents).

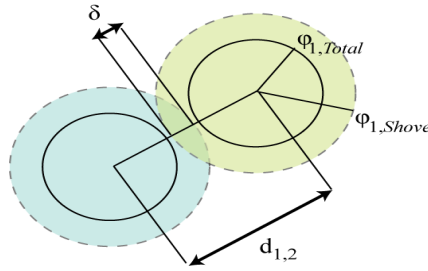


Figure 9: Agent overlap and shoving radius

Model outputs

An example of biofilm growth simulation using an individual based modeling approach is shown in Figure 10. Cells are represented as spheres, and every color address a different cell type. The biofilm grows upwards from the substratum (bar at the bottom) towards the bulk liquid (200 μm high).

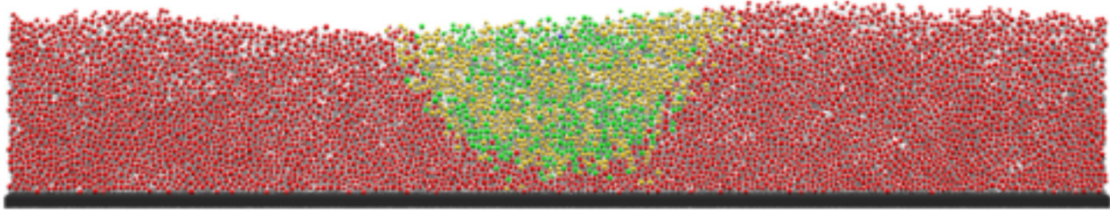


Figure 10: Example of IbM outputs for 2-D biofilm growth.

Figure 11 shows another example of IbM, this time used to simulate microcolony growth on an agar surface. Nutrients diffuse through the bottom to the top and there is not bulk liquid.

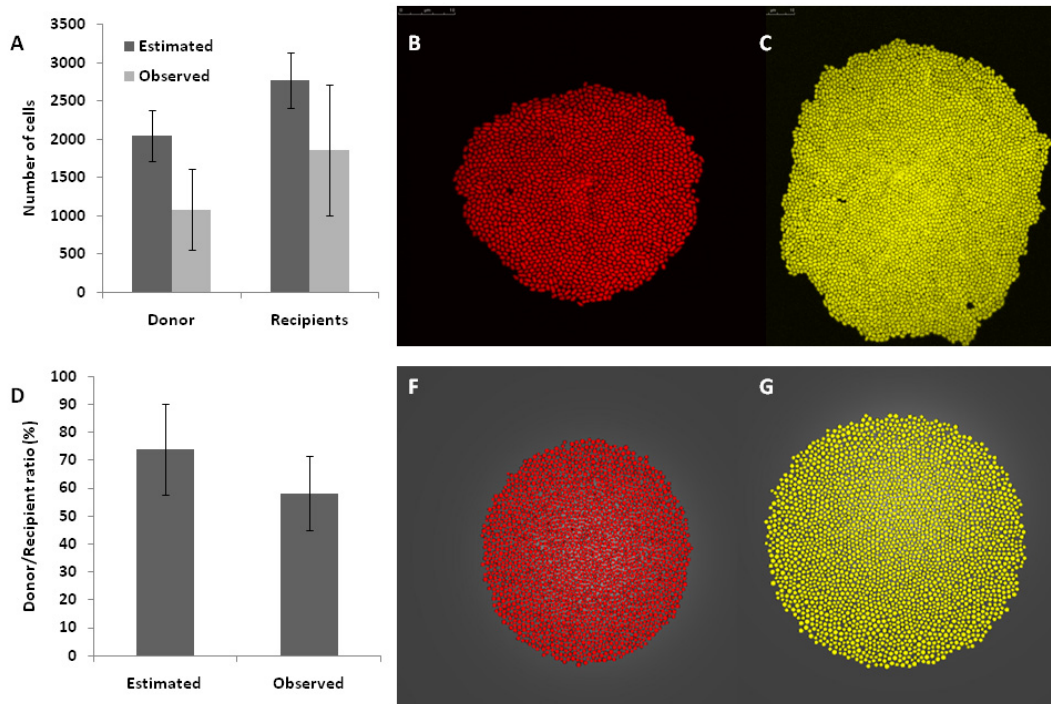


Figure 11: IbM validation for two cell types growth respectively. A: Comparison of the total number of cell observed and estimated using the model. B & C: Direct visualization of donor DsRed LacIq (red) and recipient KT2440::YFP (yellow) microcolony growth. Replicates = 45. D: Comparison of observed versus estimated donor-recipient ratio after 24 growth on ABC 0.5 mM. F & G: Examples of model output for donor (red) and recipient (yellow) microcolony growth after 24 hours on ABC 0.5 mM. n=5.

The interest for the IbM approach is growing rapidly between microbiologists. IbMs have been already applied to a wide variety of bacterial systems such as wastewater plants (Picioreanu, *et al.*, 2004), medical settings (Murphy, *et al.*, 2009), microbial fuel cells (Picioreanu, *et al.*, 2010), porous media (Graf von der Schulenburg, *et al.*, 2008) or bacteria in food (Ginovart, *et al.*, 2002). In addition, IbMs have been proven to be extremely useful to study different processes when occurring within the biofilm environment such as EPS synthesis (Kreft & Wimpenny, 2001, Xavier & Foster, 2007), quorum sensing (Nadell, *et al.*, 2008) or cell aggregation (Johnson, 2008).

Between 46 IbMs articles recently reviewed (Hellweger & Bucci, 2009), the motivations for the use of the IbM approach were justified as follows: 46% of the authors looked for capturing the effects of heterogeneity within a bacterial population; 24% aimed to analyze the emergence of population level patterns; 5% wanted to represent individuals as discrete entities and the resting 24% justified this choice because of others reasons.

In despite of their capabilities, individual-based models have also important limitations (Laspidou, *et al.*, 2010): tracking every single particle produced and its interactions with the environment individually is a highly computationally demanding process. In addition, most experimental data are still derived from population-level studies. This is a major constraint since the use of population-averaged parameters cannot capture the effects of individual's heterogeneity within a bacterial population, producing similar outputs as those obtained from mathematical models describing higher levels of organization. Therefore, the acquisition of individual-based observations is a crucial step in order to fully exploit all the abilities inherent to individual-based models (Murphy, *et al.*, 2008, Hellweger & Bucci, 2009). Furthermore, some authors consider that this technique still misses a solid methodological framework for its development, implementation and validation.

However, many of the mentioned limitations have been overcome during the last years. Although IbM is a computationally demanding technique, the rapid advancements in computer science has reduced very significantly this technical constraint. One in particular, the flexible large-scale agent modeling environment FLAME-GPU (Richmond, *et al.*, 2010), is a template driven framework for IbM based on the use of the graphics processing unit (GPU) for calculations instead of the conventional central processing unit (CPU). This programming technique has reported a massive number of individuals explicitly represented and therefore a very significant improvement in performance over more traditional IbMs frameworks. Alternatively, different modeling techniques have been developed to reduce the number of individuals explicitly represented while minimizing the impacts on the final simulation outputs. First, the spatial extent of the simulated area can be reduced to a representative space (RS approach), and "edge effects"

can be solved by wrapping the boundaries (Kreft, *et al.*, 2001). Second, individual cells maybe gathered together in subgroups of super-individuals (SI approach), each representative of a larger number of bacteria (Scheffer, *et al.*, 1995). These two approaches may be combined simultaneously in the same model (Picioreanu, *et al.*, 2004). Moreover, guidelines for building and using IbMs have been developed (Grimm, *et al.*, 2006) and are already well established within the IbMs users community, as demonstrate the 125 IbMs constructed following these guidelines during the last three years.

Unfortunately, other limitations are inherent to the nature of IbMs as stochastic simulation models. IbMs applied to biological process often combine the randomness of individual-level interactions and evolve in a large state and parameter space that can only be sampled. Therefore, the complexity and limited generality are the main limitations of IbMs (Uchmanski & Grimm, 1996). In addition, the application of IbMs to the study of conjugative transfer mediated by plasmids still suffer of some of the classic constraints inherent to all IbM's in their early days (see section 2.3.2). The most significant one is probably the lack of individual-based observations for the main parameters describing conjugation at the individual-cell level. Available parameter estimates to date derive from population-averaged instead of individual-based observations, which makes it very difficult in practice to apply IbMs to the study of bacterial conjugation. As mentioned before, the obtaining of individual-based observations is a crucial step in order to fully exploit all the abilities inherent to individual-based models (Murphy, *et al.*, 2008, Hellweger & Bucci, 2009).

3 Individual-based analysis of plasmid population dynamics in spatially structured environments

The current PhD thesis has been carried out within the frame of a bigger EU project (RaMaDA) which included within its objectives the development and validation of an IBM platform (iDynoMiCs) able to describe and predict the dynamics of growth and conjugative plasmid transfer in surface-associated bacterial populations. One of my main objectives has been to identify and estimate the growth and conjugal plasmid transfer parameters to be included in iDynoMiCs. In this sense, we can distinguish two functional modules within the model structure (Figure 12): one submodel for microbial growth and one submodel for conjugative plasmid transfer. In this chapter, I will introduce the development of experimental methods for the identification and estimation of the main parameters describing bacterial growth together with those designed to quantify plasmid transfer parameters at the individual cell level. Finally, I will present the validation of our model by comparing the outputs obtained using iDynoMiCs with experimental results on plasmid invasion in microcolonies.

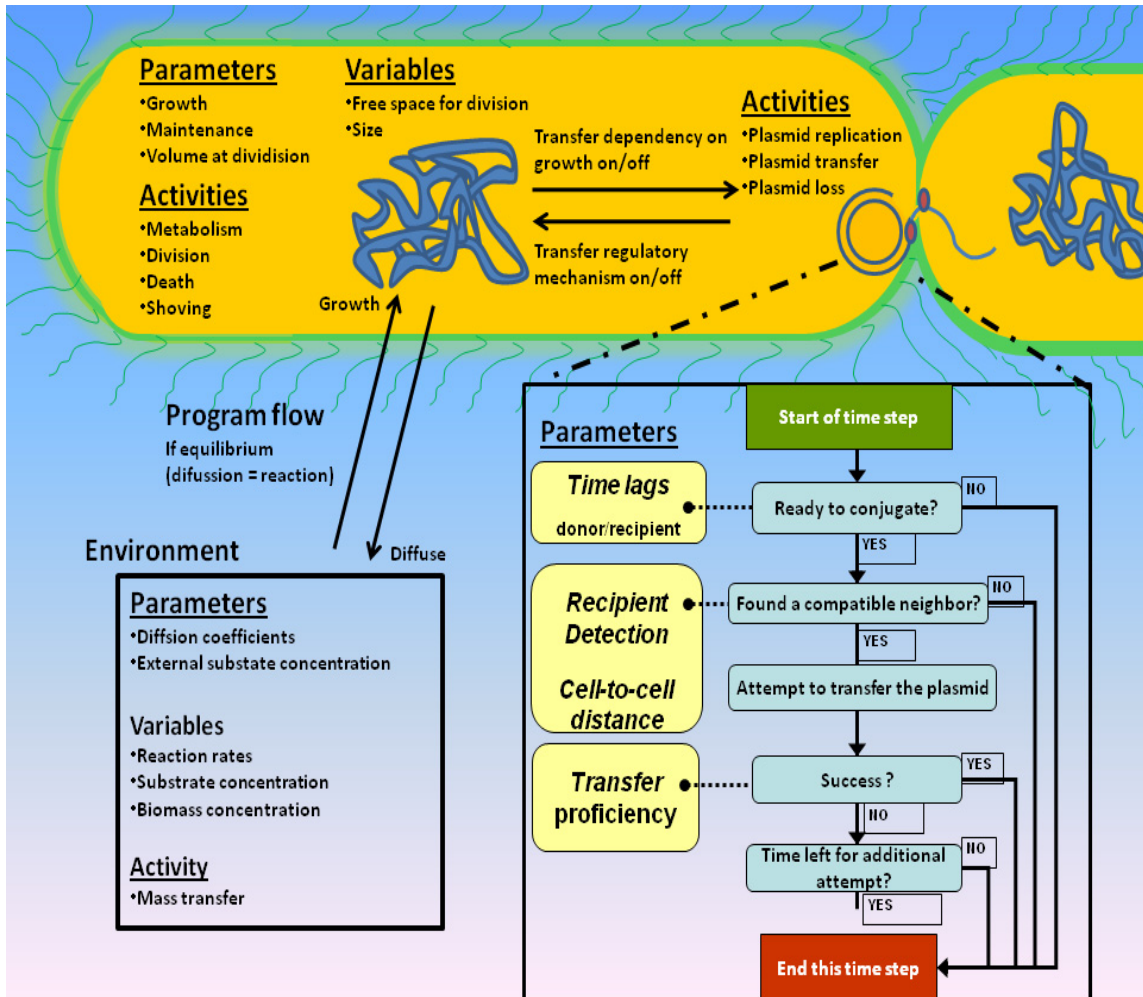


Figure 12: IDynoMiCs model structure overview. Two main modules may be distinguished: cell interactions with the environment (left half) and plasmid replication and transfer (right). In addition, several chromosomal DNA-plasmid interactions have been implemented.

3.1 Parameter estimation for Individual-based models (Annex I & II)

The IbM platform IDynoMiCs has been designed to accommodate the possibility of describing different plasmid conjugative transfer regulatory mechanisms (see section 2.2.4). Although the methods I present here may be applied to any specific conjugative plasmid, I will focus in the analysis of P-like systems. More specifically, I have chosen the model organism *Pseudomonas putida* KT2440 and the natural TOL plasmid pWW0. This plasmid is highly suitable for the study of conjugal HGT because of its high transfer frequencies (Ramos-Gonzalez, *et al.*, 1991), well known regulatory transfer mechanisms (see section 2.2.4), low copy-number, high stability and big size (see section 2.2.1). Conjugative plasmid transfer was detected by using the reporter gene system originally employed by Christensen *et al.* (1996) and described in section 2.3.2.

3.1.1 Estimation of plasmid metabolic burden and growth parameters (Annex I)

Plasmid metabolic burden (see section 2.2.5) has been usually quantified using pair-wise growth competition assays, which provide a fast, simple and sensitive method for the estimation of the relative fitness of two different strains which are grown together within the same experimental system (De Gelder, *et al.*, 2007, Fox, *et al.*, 2008). However, this methodology also presents many limitations such as very limited kinetic parameter outputs and the need of multiple controls to remove the effects of cell-cell and plasmid-cell interactions (i.e. horizontal gene transfer, plasmid loss or commensalism), which may affect the final value of the fitness coefficient. It does not help that very often these interactions are not well known and they cannot be controlled, as for example the use of pWW0 metabolism debris by plasmid-free cells (Duetz & Van Anandel, 1991).

Alternatively, isogenic strains with and without the plasmid can be grown separately and, in this way, growth parameters obtained from the biomass growth kinetics (Haft, *et al.*, 2009) or the substrate depletion (Joshi, *et al.*, 2009) can be compared to establish the metabolic burden associated with the plasmid carriage. However, this method may become highly time-consuming and tedious, especially when testing strains with very low growth rates.

In order to overcome these limitations, I implemented a new experimental design based on respirometry (Figure 13). This was achieved through the development of a two-step respirometric assay: first, the initial conditions were set to a S_0/X_0 ratio higher than one hundred, and hence the respirometric profile obtained (Figure 13, region B) was representative of the ultimate growth capabilities of the tested strain (the intrinsic biokinetics). OD_{600} was used to check the initial

biomass concentration at the beginning of each experiment. From 50 to 350 mg COD/L, a linear correlation ($Y = 461.57 X + 11.045$; $R^2 = 0.97$) was obtained between dry weight and optical density. Second, I performed an extant biokinetic test over the population obtained previously by adding small substrate pulses to maintain a very low S_0/X_0 ratio (Figure 13, region C). This assay results in minimal physiological changes in the microbial population and results in kinetic parameters which describe the respirometric profile obtained previously.

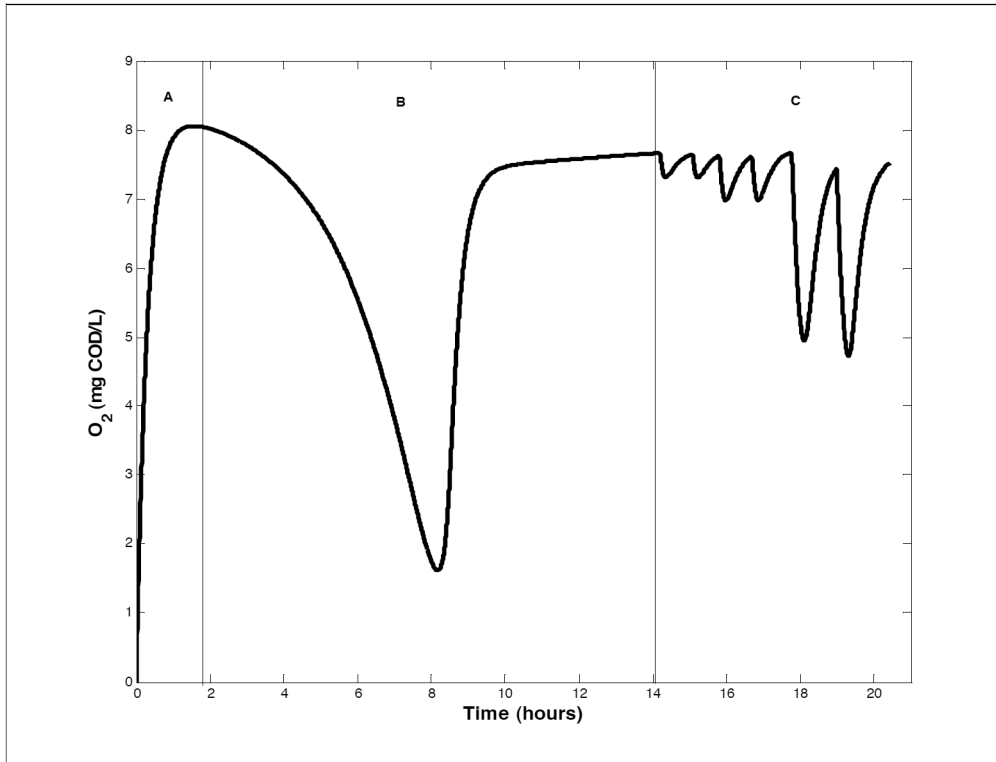


Figure 13: Modeled respirometric profile representative of the experimental approach with three well-differentiated regions: A: re-aeration; B: initial growth; C: extant assays.

In addition, the proposed experimental design maximizes the accuracy and reliability of the parameter estimates in two ways. First, high quality data are obtained by repeating substrate pulses at increasing, but relatively low, concentrations. Second, identifiability analyses complemented with parameter estimation uncertainty (95% confidence interval) are applied to provide statistically reliable and accurate parameter estimates from the experimental data.

In order to prove the high sensitivity of my experimental design, I quantified the subtle differences existing between the metabolic burden caused by just pWW0 presence (replication and maintenance) and the effects produced by both pWW0 presence and plasmid-encoded protein expression in *P. putida* KT2440 (Table IV). Results showed that plasmid presence was able to reduce significantly the bacterial fitness, as reflected by the observed reduction in the yield (11%) and the specific growth rate (17%) compared to its plasmid-free counterpart. When the

effect of expression of additional recombinant proteins from pWW0 was tested, the strain KT2440 (pWW0::*gfp*) had a specific growth rate μ_{\max} decreased by 31% and 14% compared to KT2440::*gfp* and KT2440::*gfp* (pWW0), respectively.

Table IV: Parameter estimates and confidence interval (c. i.) for the four data sets per strain with parameter estimation for all assays simultaneously (n = 4)

Strain	Y_H (mg COD/mg COD)	μ_{\max} (1/h)	K_s (mg COD/L)	b (1/h)
KT2440	$0.68 \pm 5.7E-03$	$0.58 \pm 2.3E-02$	$5.08 \pm 3.8E-01$	$0.001 \pm 7.0E-04$
KT2440:: <i>gfp</i>	$0.66 \pm 4.9E-03$	$0.51 \pm 3.3E-02$	$5.08 \pm 5.6E-01$	$0.001 \pm 6.0E-04$
KT2440:: <i>gfp</i> (pWW0)	$0.61 \pm 4.3E-03$	$0.42 \pm 7.8E-03$	$4.82 \pm 1.2E-01$	$0.03 \pm 6.0E-04$
KT2440 (pWW0:: <i>gfp</i>)	$0.71 \pm 2.5E-03$	$0.35 \pm 1.1E-03$	$5.16 \pm 3.5E-02$	$0.03 \pm 2.0E-04$

These results are consistent with previous works suggesting that plasmid metabolic burden is due to the cost associated with the plasmid replication and maintenance (Björkman & Andersson, 2000) but also with other hypothesis pointing towards the additional energetic cost required for plasmid-encoded protein expression (Rozkov, *et al.*, 2004). During the work I present here, I have uncoupled and quantified the relative contribution of both processes showing that they are equally important in the overall metabolic burden caused by pWW0 in his host. These results together with those presented in previous works (see section 2.2.5), suggest that the three proposed mechanisms of plasmid metabolic burden may coexist and should not be considered mutually exclusive.

At this point, my approach may result surprising because I have estimated growth parameters in a planktonic population with the aim to describe growth in a solid surface. This is based in the principle that when a bacterium from the chemostat starts growing in a solid surface, the environmental conditions change but the genome potential to respond to these changes (signaling/gene regulation) is still the same. In other words, bacterial growth dynamics on solid surfaces can be predicted from chemostat data as long as we are able to model the effects of the spatial structure, the individuality and the stochasticity involved (Kreft, *et al.*, 2001, Picioreanu, *et al.*, 2007).

3.1.2 Estimation of TOL plasmid conjugation parameters (Annex II).

One of the main constraints for the application of IbMs to the study of conjugative plasmid transfer is the lack of observations for the main parameters describing conjugation at the individual-cell level (see section 2.4.2). Fortunately, the fast development of individual-based observation technology (Brehm-Stecher & Johnson, 2004) now gives the opportunity of studying the main bacterial processes at the individual cell scale. In this sense, I have designed and successfully implemented an individual-based experimental framework to identify and estimate the main parameters governing bacterial conjugation at the single cell level. More specifically, the initially targeted parameters (Table V) were (i) the conjugation rate (Stewart & Levin, 1977, Simonsen, 1990, Smets, *et al.*, 1994), (ii) the donor-recipient distance (Gregory, *et al.*, 2008) and (iii) the lag times between plasmid receipt and plasmid transfer (Massoudieh, *et al.*, 2007).

In order to quantify conjugation rate and donor-recipient distance, matings were performed on filters and donor-recipient cell growth and retransfer was automatically removed from the image analysis, allowing me to obtain unbiased measurements on the conjugation frequency of the originally inoculated donors. However, due to the smaller time scale (minutes), this methodology was not suitable for the acquisition of conjugational lag times.

Table V: pWW0 conjugation parameters values

Parameter	Description	Estimated Value	Units	Source
γ	Conjugation rate	6×10^{-1} to 2.1	h^{-1}	This study
d_p	Pilus reach distance	0-1	μm	This study
$T_{regulated}$	Transfer lag during transfer regulated periods	1.4	h	This study
$T_{unregulated}$	Transfer lag during transfer unregulated periods	0.33	h	This study
p_{loss}	Probability of segregative loss during cell division	1×10^{-9}	%	1
b_p	Plasmid maintenance rate	0.025	h^{-1}	2

Sources: 1, (De Gelder, *et al.*, 2007); 2, (Seoane, *et al.*, 2010)

With this aim, additional conjugation experiments were carried out using perfusion chambers (Reinhard & Van der Meer, 2009) containing solid media, which allowed to monitor on-line plasmid spreading within the bacterial populations without disrupting their spatial structure (Figure 14).

Results have shown that conjugation could happen even in the absence of an added carbon source, but at a very low frequency (only 10% of the donors in contact with recipients have transferred the plasmid after 24 hours). In 91.1 % of the cases, successful mating pairs occurred through direct cell-to-cell contact (0-1

µm range). Those contacts arose randomly from microcolony morphogenesis and no pulling action (by conjugal pili) was observed. An elongation of 60-70% compared to the maximal cell length (length attained immediately prior to septum appearance and division) was required for conjugation to occur and, in 75% of cases, the cells exceeded 80% elongation. No successful mating pair was detected that comprised recipient cells that did not divide shortly after transfer, suggesting that recipients are more susceptible to receive the plasmid at advanced stages of cell growth cycle. In addition, the lag times needed for newly formed transconjugant cells (unregulated) to transfer pWW0 (Table V) were significantly shorter than in the case of initial donor cells (regulated).

Table VI: Comparison pWW0 conjugation parameters values

Donor	Recipient	Environment	γ (h ⁻¹)	References
<i>P. putida</i> PAW1	<i>P. areuginosa</i> PAO 11612	Liquid	2.14*10 ⁻¹⁵	1
<i>P. putida</i> AC37	<i>P. putida</i> mt-2	Liquid	1.3*10 ⁻²	2
<i>P. putida</i> AC37	<i>P. putida</i> mt-2	Solid	2.5*10 ⁻¹	2
<i>P. putida</i> KT2440	<i>P. putida</i> KT2440	Solid	6*10 ⁻¹ to 2.1	This study

Sources: 1, (Smets, *et al.*, 1993); 2, (Bradley & Williams, 1982)

In order to assess the adequacy of this methodology, the obtained conjugation rate estimates were compared with those presented in previous works (Table VI). Results were within the range expected, following Bradley & Williams (1982). In addition, a rough comparison to the pWW0 conjugation rates obtained in liquid media for intraspecies (Bradley & Williams, 1982) and interspecies (Smets, *et al.*, 1993) transfer essays showed much higher transfer rates when pWW0 was transferred to its natural host *P. putida* than to a different (although highly related) specie such as *P. areuginosa*. These differences suggest that pWW0 transfer mechanisms are highly adapted to their natural host, and therefore we have to be very cautious when extrapolating the results presented here to other systems or other plasmids.

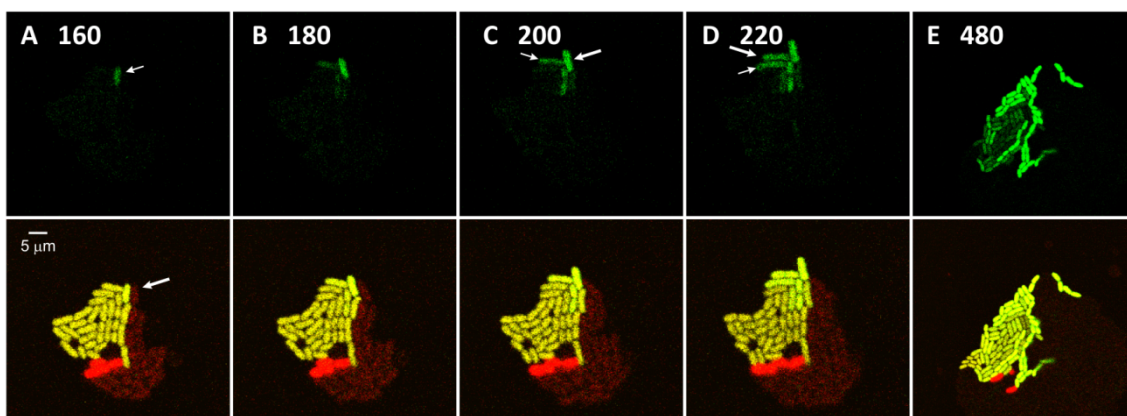






Figure 14: Time-lapse CLSM images of a microcolony where donor *P. putida* KT2440 cells expressing Dsred and LacIq are transferring pWW0 TOL::GFP to recipient *P. putida* KT2440 cells expressing YFP. Transconjugants are simultaneously expressing GFP and YFP. GFP signal (transconjugant cells) and the corresponding overlay of all fluorescence signals (all cell types) are displayed on the top and on the bottom channels respectively. Non-dividing inoculated donors have higher red intensities due to previous DsRed maturation. Thick arrows mark the individual cell transferring the plasmid while thin arrows indicate the resulting new transconjugant cell. The cells were inoculated on a nutrient agar slab, and images were taken every 20 minutes. After 160 minutes of donor-recipient contact, conjugative transfer was detected (A). It then took less than 40 minutes for this transconjugant to retransfer twice (C) and less than 20 minutes for the new transconjugants to retransfer again (D). After 480 minutes (approx. 5 division cycles) most of the recipients in the microcolony contained the plasmid. See also supplementary information (Annex II, SI movie_1).

During the current work, the possible effects of the relative orientation of donor cells vis-a-vis recipient cells on successful plasmid transfer were analyzed (Table VII). Results suggested that pWW0 plasmid junctions may appear at any point of contact on the surface of donors and that DNA can be transferred to any available location along the recipient membrane. However, some orientations were more favorable than others: conjugation is more likely to occur through the lateral wall of the donor than through the poles, which is consistent with previous observations showing that plasmids are situated preferentially at the characteristic centre or quarter cell position in the cell and not in the poles (Lawley, *et al.*, 2002).

Table VII. Surface of contact and relative donor-recipient orientation frequencies observed at the moment of transfer

Case	Orientation*	Surface contact (μm) [†]	Successful pairs (%) [†]	Regular pairs (%) [†]
1		1.1± 0.27	24	13
2		1.7 ± 0.28	61.6	41
3		--	13.6	32
4		1.1± 0.12	0.79	13

* The relative orientation (n=380) of donor (black cell) and recipient (white cell) and the surface of contact (n=36) at the moment of transfer were analyzed. Regular non-conjugative pairs were also analyzed for relative orientation (n=107). †Means (\pm SE) and orientation frequencies (%) are given.

In this sense, two additional parameters were shown to have a significant effect in the probabilities of successful plasmid transfer to occur at the individual cell level: the donor-recipient relative orientation within the mating pair and the degree of elongation of the recipient cells. The obtained results indicated that (i) there is an almost complete absence of transfer observed under non-growing conditions (and therefore no cell elongation), (ii) there is a positive dependency of successful plasmid transfer on the donor-recipient surface of contact (which is dependent on cell elongation) (iii) plasmid transfer occurs preferentially at advanced stages of the cell cycle (when elongation is high). Therefore, I suggest that cell elongation during bacterial growth may be an important process influencing the probabilities of successful conjugation to occur. This is because this process increases the surface of contact between the mating pair, which favors the formation of conjugative junctions. A complementary explanation could be that during growth, the loosening of the peptidoglycan matrix that forms the cell wall would facilitate the mating pore formation and therefore the entry of plasmid DNA in the recipient cell. Unfortunately, the demonstration of these hypotheses was beyond the goal of the current PhD studies and further research will be needed in this sense.

3.2 Individual-based Modeling of plasmid spreading in spatially structured bacterial populations (Annex III & IV).

3.2.1 Individual-based Modeling of plasmid spreading in biofilms (Annex III)

Biofilms (the most common form of bacterial life in nature) are assumed to be hot-spots of conjugational plasmid transfer (Sorensen, *et al.*, 2005). This is probably because, within these bacterial communities, cells are highly packed enhancing cell-to-cell contact. However, plasmid spreading in biofilms have been shown to be surprisingly limited (Christensen, *et al.*, 1998). It was suggested that that this observed lack of plasmid spread into the deeper biofilm layers can be explained by a dependence of conjugation on growth rate. In order to further explore this hypothesis, a pre-existent individual-based model of microbial growth called IDynoMICs (Lardon, *et al.*, 2009) was extended to include the dynamics of plasmid carriage and transfer by individual cells (Figure 15 and Table VIII). Details on the model parameters and equations are provided in Annex III.

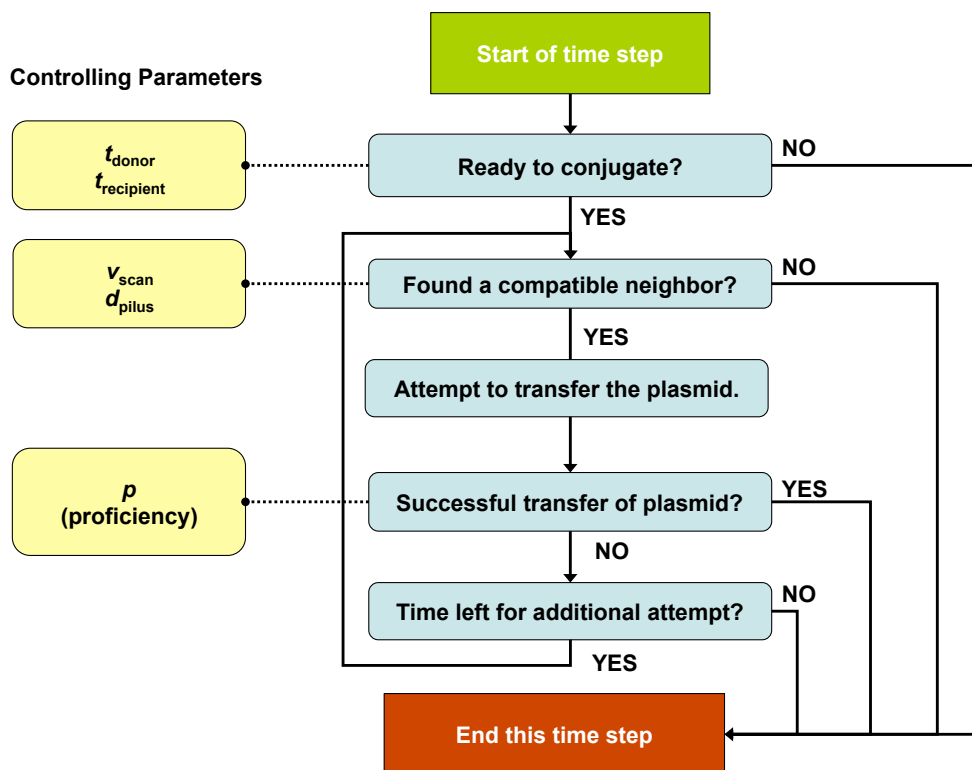


Figure 15: Algorithm ruling conjugational plasmid transfer in IDynoMICs

Table VIII: Default HGT parameter values

Parameter	Description	Value	Units	Source
v_s	Conjugal pilus scan speed	10	h^{-1}	1
d_p	Pilus reach distance	7	μm	1
p	Transfer proficiency (probability of success)	1	-	A
$t_{\text{recipient}}$	Maturation period following initial plasmid receipt	1.2	h	2
t_{donor}	Recovery period following plasmid transfer	0.3	h	A,2
p_{loss}	Probability of segregative loss during cell division	1×10^{-9}	-	3
b_p	Plasmid maintenance rate	0.025	h^{-1}	4,5

Sources: 1, (Clarke, *et al.*, 2008); 2, (Massoudieh, *et al.*, 2007) ; 3, (De Gelder, *et al.*, 2007); 4, (Seoane, *et al.*, 2010); 5, (Smets, *et al.*, 1993); A, assumed.

In a first step, the influence of model parameters on model output was ranked by conducting a sensitivity analysis following established methodology (Brun, *et al.*, 2001, Sin & Vanrolleghem, 2007). The recipient lag, scan speed, and transfer proficiency had the largest effect (Table IX). Model outputs were over twice as sensitive to the recipient lag as to other parameters, meaning that the time required for plasmid maturation and expression in a transconjugant cell has a strong impact on the ability of a plasmid to invade a biofilm. The donor lag was relatively unimportant, presumably because the longer recipient lag acts as a bottle-neck on permitting transfer events.

Table IX: Sensitivity of model output to model parameters

Parameter	Sensitivity	Rank
$t_{\text{recipient}}$	0.32	1
v_s	0.14	2
p	0.10	3
d_p	0.097	4
t_{donor}	0.023	5
b_p	0.006	6
p_{loss}	0.0072	7

As shown in Figure 16, simulations were run to assess the degree of dependency of plasmid transfer on growth required to reproduce the plasmid spreading patterns observed experimentally.

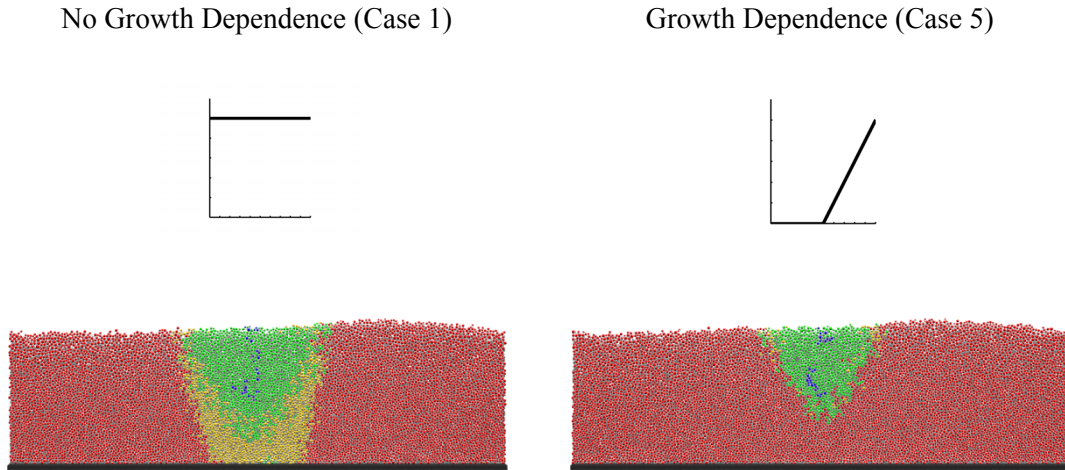


Figure 16: Plasmid spread for different growth dependencies. The left plot illustrates plasmid spread if there is no growth dependence, and the right a moderate level of dependence. Red represents recipient agents, blue donor agents, yellow new transconjugants, and green the daughters of transconjugants. Each image shows one representative outcome of the 5 replicate simulations. Note the inability of the plasmid to invade the previously-established biofilm in the growth-dependent case.

Results suggested that limiting transfer only to those cells growing at least at the 33% of their maximal growth rate can prevent deep invasion of a biofilm by a plasmid, whereas plasmid spread should not be limited if there were not such a dependency. In addition, timing parameters such as transfer lags had the strongest effect on plasmid invasion, while distance parameters are of lesser importance. In conclusion, the new individual-based model presented here has been shown to be a powerful tool to explore in silico the spread and role of plasmids in bacterial biofilms.

3.2.2 Model validation (Annex IV)

Although IbM is a very suitable approach for the study of plasmid conjugative transfer in silico, the lack of individual-based estimates available for the main parameters describing this process makes very difficult in practice to implement this approach. During this period of the current PhD studies, I overcame this limitation by using an experimental framework specifically designed to quantify the most relevant parameters describing conjugational plasmid transfer at the individual-cell level (see section 3.1.2 and Annex II). Using the parameter estimates obtained in this way, I validated the IbM IDynoMICs (see previous section and Annex III) by comparing its ability to predict plasmid population dynamics in bacterial microcolonies with observations obtained in living cells.

Regarding cell growth, the model predicted an adequate donor/recipient ratio after simulation of 24 hours, although both donor and recipient cell number were overestimated by approximately one generation (Figure 17, A).

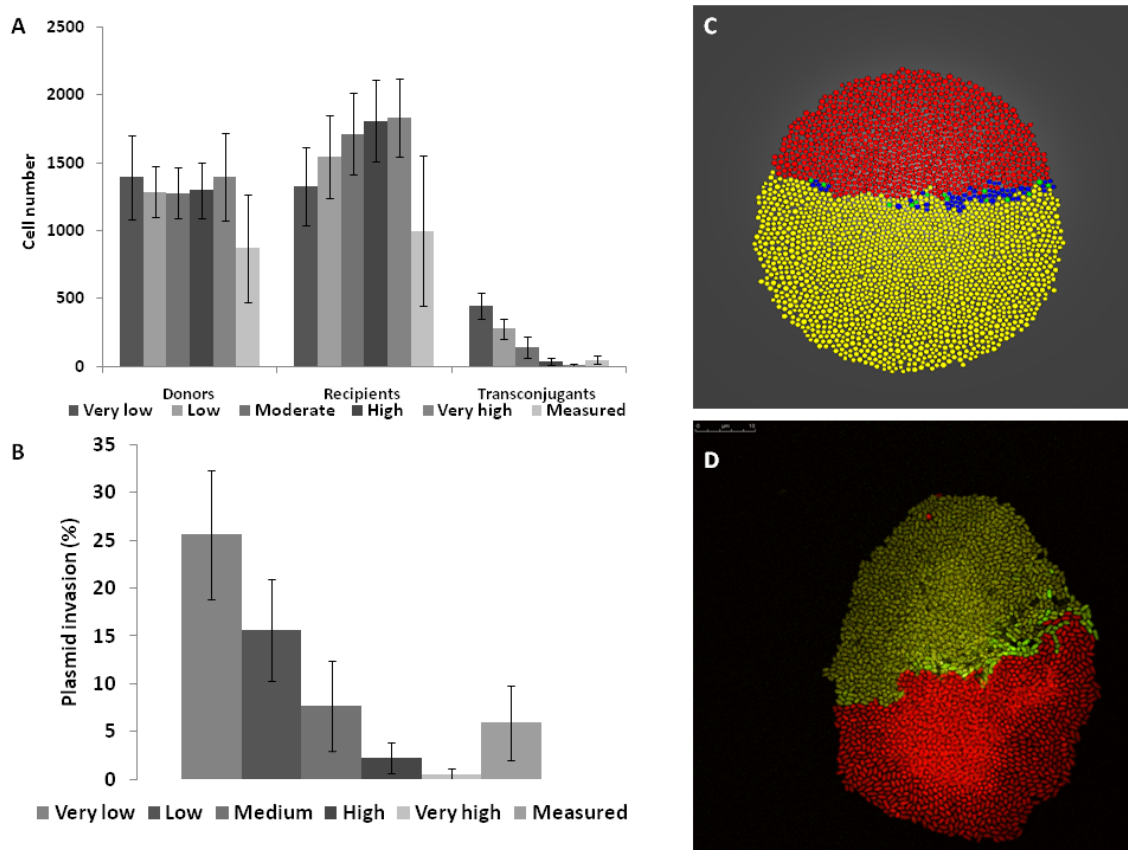


Figure 17: Estimation of plasmid transfer dependency on growth rate. A & B: Comparison of the total cell number and plasmid invasion (%) obtained in vivo (n=50) and in silico (n=5) for the different cellular types at differences grades of plasmid transfer dependency on growth. C: Model output for 24 hours

mating simulation. The system starts with a single donor cell and a single recipient cell placed in the center of the image. D: Direct visualization within a microcolony where donors DsRedLacIq (red) transfer pWW0::GFP-LacI (green) to recipients KT2440::YFP (yellow) n= 45.

The best agreement between model predictions on expected transconjugants frequencies and experimental results were obtained when transfer dependency on cell growth and division was set as medium ($6 \pm 4\%$ of all recipient cells carrying a plasmid copy after 24 hours simulation compared to $6 \pm 4\%$ measured) although high dependency values also produced acceptable results. In addition, my individual-based model allowed me to estimate the contribution of the conjugative plasmid transfer process to the overall transconjugant cell population, which was estimated to be $20 \pm 9\%$. Therefore, plasmid vertical spreading processes (due to cell growth and division) were behind the origin of the remaining $80 \pm 9\%$ transconjugant cells observed. Moreover, I used this model to explore the main hypothesis explaining the inability of pWW0 to fully invade bacterial microcolonies (see section 2.3.2), showing that a moderate dependence of plasmid invasion on bacterial growth was enough to explain the observed patterns of pWW0 invasion in *P. putida* KT2440 microcolonies, while no effect of EPS synthesis at biological levels could be observed.

In conclusion, it was shown that a population-scale parameter such as the plasmid frequency can be successfully predicted in a spatially structured bacterial population by inputting parameter estimates obtained at the individual cell level. These results also demonstrate the suitability of the experimental framework presented here to obtain accurate plasmid transfer parameter estimates from individual cells (Annex II).

4 Conclusions and perspectives

During the last two decades, much experimental effort has gone into obtaining better knowledge about the processes and factors that govern conjugational plasmid transfer in bacterial populations. In this sense, particular attention have been paid to biofilms because being highly crowded bacterial populations with high cell-to-cell contact, they could be expected to be hot-spots for conjugation plasmid transfer. Surprisingly, it has been shown that many promiscuous plasmids such as pWW0 cannot fully invade a receptive colony or biofilm and transconjugant cells remained in the top layers of the population. These observations have been proposed to be related to nutrient and oxygen depletion, low metabolic activity and low transfer gene expression levels in the deeper cell layers of the bacterial population (Christensen, *et al.*, 1996, Christensen, *et al.*, 1998, Haagensen, *et al.*, 2002). However, it has also been shown that even starved cells may perform plasmid transfer and that above a certain threshold activity the level of conjugation is independent of metabolic activity (Normander, *et al.*, 1998, Hausner & Wuertz, 1999). Therefore, the reasons why a conjugal plasmid cannot fully invade a spatially structured bacterial population remain unclear.

The development of new modeling tools such as Individual-based models (IbM), which can address the spatial and biological heterogeneity typically associated with spatially-structured bacterial communities, offers now the possibility to get new insights into this question. However, the lack of individual-based estimates available for the main parameters describing this process makes very difficult in practice to implement this approach. This thesis illustrates, through extensive experimental work combined with mathematical modeling, the design and development of an experimental framework specifically conceived to facilitate the analysis of conjugative plasmid transfer at the individual cell level. In addition, I have successfully applied the proposed methodology to the calibration and validation of a pre-existent individual-based modeling platform (iDynoMiCs) describing microbial growth and conjugative gene transfer dynamics in colonies and biofilms.

The resulting methods have been shown to be highly suitable for the obtention of accurate estimates of the five main parameters describing bacterial conjugation in solid surfaces: (i) the conjugation rate γ , (6×10^{-1} to $2,1 \text{ h}^{-1}$); (ii) the maximal pilus reach distance, d_p ($0 - 1 \text{ }\mu\text{m}$); (iii) the lag times between donor-recipient cells contact and transfer, t_{lag} ($0,33$ to $1,4 \text{ h}$); (iv) plasmid loss p_{loss} (1×10^{-8}) and (v) plasmid maintenance ($0,025 \text{ h}^{-1}$). In addition, it was possible to quantify at the individual cell level the effects of cell orientation within the mating pair and the phase of cell division at which transfer is more likely to occur. From this analysis, transient periods of unregulated transfer together with contact mechanics arising

from cellular growth and division were shown to be determinant in pWW0 invasiveness. Furthermore, pWW0 conjugation occurred mainly at advanced stages of the growth cycle and non-growing cells, even when exposed to high nutrient concentrations, did not display conjugal activity. However, transient periods of elevated plasmid transfer in newly formed transconjugant cells seemed to be offset by unfavorable cell-to-cell contact mechanics, which ultimately precluded the pWW0 TOL plasmid from fully invading tightly packed multicellular *P. putida* populations like microcolonies and biofilms.

Once implemented in the model, the parameter estimates obtained from the previous experimental work allowed the prediction of the degree of plasmid spreading in bacterial microcolonies, together with the spatial plasmid spreading patterns and other macroscopic aspects such as colony morphology. These results demonstrated that a population-scale parameter such as the frequency of plasmid-carrying strains in a spatially structured bacterial population can be successfully predicted by inputting parameter estimates obtained at the individual cell level. Although this concept is the main theoretical pillar of the individual-based modeling approach, the lack of high-quality individual-based observations makes it very difficult to find practical examples in the field of microbial ecology. In this sense, results also demonstrate the good performance of the experimental framework specifically designed to obtain HGT parameter estimates at the individual cell level. In addition, this model was used to test the main hypothesis explaining the inability of pWW0 plasmid to invade tightly packed bacterial structures, such as microcolonies or biofilms. The main conclusions obtained from these simulations were that a moderate dependence of plasmid invasion on bacterial growth (no EPS synthesis) was enough to explain the observed plasmid spreading patterns.

In conclusion, during the present PhD thesis I have successfully developed and applied an individual-based experimental framework specifically design for the calibration of IbMs aiming to study bacterial conjugation. With the adequate calibration, the IbM iDynoMiCs was able to predict, both qualitative and quantitatively, the spatial patterns of plasmid spreading observed in living microcolonies, demonstrating the suitability of the proposed methodology. Therefore, we have shown that when high quality individual-based parameter estimates are available, IbMs have the potential to make predictions on the effects of environmental fluctuations, virus infections or antibiotic treatments in plasmid dynamics on bacterial populations.

4.1 Perspectives

The individual-based modeling approach has become increasingly popular during the last few years. However, in despite of the suitability of this methodology for the study of bacterial conjugation, its application to the field of plasmid biology is still in its infancy. One of the main difficulties to extend this approach to plasmid conjugation studies lies in the lack of individual-based observations available, which makes very difficult to calibrate these models.

In this sense, I expect that the experimental methods provided here will facilitate the characterization of the population dynamics of other relevant plasmids and their hosts as well as to get deeper knowledge on the mechanisms controlling conjugation between two individual cells. Additionally, the proposed experimental set-up offers the possibility to test on-line how changes in different environmental conditions (such as temperature, heterogeneity in the substrate distribution, periodic antibiotic treatments or new sources of nutrients) affects plasmid spreading within a bacterial community. In this sense, it would be very interesting to determine experimentally the set of conditions that enhance or fully prevent conjugational plasmid transfer between individual cells.

However, the proposed experimental approach only allows the analysis of plasmid spreading in bacterial populations during short periods. I hope that this work brought some convincing evidences that, with the appropriate calibration, IbMs can acquire a very valuable predictive power in the long term. With the adequate support, IbMs have the potential to become extremely useful tools for the management of bacterial populations containing plasmids which confer beneficial traits to their hosts (i.e. pollutant biodegradation) or, conversely, those which are behind of undesirable traits such as antibiotic resistance.

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Appendix

- I. **Seoane, J.**, Sin G., Lardon L.A., Gernaey K.V. & Smets B.F. (2010) A new extant respirometric assay to estimate intrinsic growth parameters applied to study plasmid metabolic burden. *Biotechnology and Bioengineering* **105**: 141-149.

- II. **Seoane, J.**, Yankelovich, T., Dechesne, A., Merkey, B.V., Sternberg, C., & Smets, B.F. (2010). An individual-based approach to explain plasmid invasion in bacterial populations. *FEMS Microbiology Ecology*. *Accepted*.

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- IV. **Seoane, J.**, Merkey, B.V. & Smets, B.F. (2010). An Individual-based analysis of plasmid spreading in bacterial microcolonies. *Microbiology*. *Submitted*.

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ISBN 978-87-92654-15-1