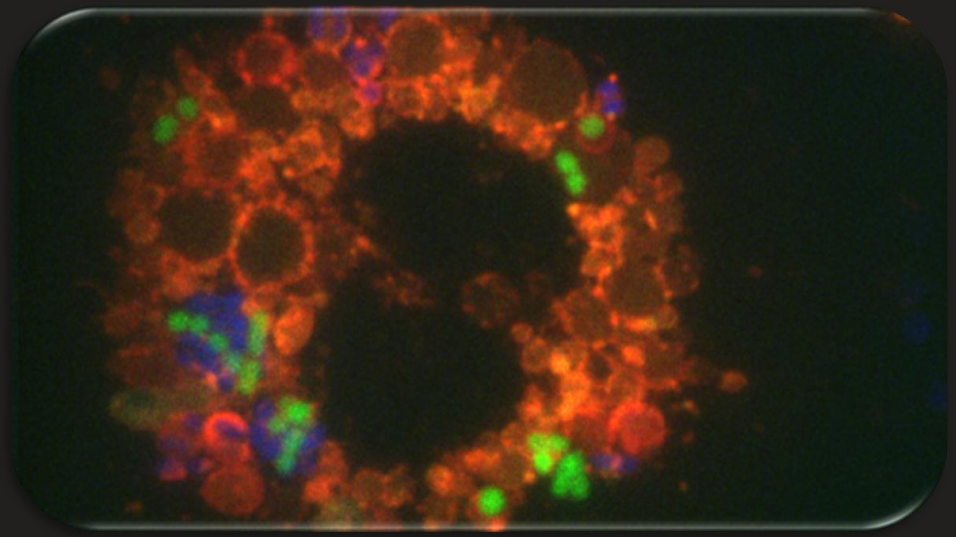
Role of biotic interactions in generating and maintaining biodiversity

Migla Miskinyte



Dissertation presented to obtain the Ph.D degree in Biology
(Host – microbe interactions)

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
May, 2013

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA /UNL
Knowledge Creation

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Dedikuoju savo mamai

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Summary

Microbial populations constantly face pressures imposed by exposure to host immune systems and antibiotics. Thus it is important to have a better understanding of how they can adapt to such pressures. In this context, studying the process of adaptation of commensal bacteria towards becoming a pathogen and how resistance to antibiotics affects bacterial fitness are pressing questions.

In this thesis I explored the adaptive strategies that commensal *Escherichia coli* can evolve to escape the first cellular line of defence of the innate immune system – macrophages. I described that in only a few hundred generations, bacteria can evolve and acquire specific adaptations such as increased intracellular survival and increased escape of engulfment by macrophages. These are strategies commonly found in clinical isolates, such as in small colony variant or mucoid clones isolated in many infections caused by different bacteria species. I also show the process of emergence of mucoid clones, in an *in vitro* experimental evolution setup with *E. coli*, and characterize them. Importantly, it is found that they exhibit increased pathogenesis by *in vivo* colonization and intraperitoneal infection assays. Furthermore, I determined the genetic basis of *E. coli* pathoadaptation and showed that transposable element insertions into both coding and regulatory regions drive the evolutionary process. This process is characterized by clonal interference, as indicated using both experimental evidence and a mathematical model. I also show that mucoid clones bear a selective advantage when interacting with other predators that *E. coli* can encounter in nature, such as protozoa and phages. I also show that small colony variant clones can have a competitive advantage in colonizing the mouse gut.

Finally, I estimated fitness effects of different antibiotic resistant mutations in the context of an interaction with macrophages. I showed that 67% of the mutants survive better than the sensitive bacteria in the intracellular niche of the macrophages. In particular, all streptomycin resistant mutants exhibit an intracellular advantage, due to specific environmental stresses that macrophages impose on bacteria, such as nutrient limitation and oxidative stress.

This thesis provides a test of the basic evolutionary hypothesis that biotic interactions increase rates of adaptation and a demonstration of the power of sentinels of the innate immunity as a selective pressure to help us understand the diversity of microorganisms in terms of evolution of antibiotic resistance and virulence.

Sumário

As populações microbianas enfrentam constantes pressões impostas pelo sistema imunitário do hospedeiro e pela utilização de antibióticos. Assim, à luz da notável capacidade de adaptação dos micróbios a novos nichos ecológicos é crítico que se entenda o processo de adaptação das bactérias comensais no seu percurso até se tornarem patogénicas e os efeitos da resistência a antibióticos.

Nesta tese, eu exploro diferentes tipos de estratégias de adaptação que a *Escherichia coli* comensal consegue evoluir, de forma a escapar à primeira linha de defesa das células do sistema imunitário inato – os macrófagos. Descrevo como, em apenas algumas centenas de gerações, estas bactérias conseguem evoluir e adquirir adaptações específicas. Uma das adaptações observadas foi o aumento de sobrevivência intracelular e outra o aumento da capacidade de escapar à fagocitose pelos macrófagos. Estas estratégias são normalmente encontradas em isolados clínicos, tais como os clones "small colony variant" (SCV) ou os clones mucóides (MUC). Além disso, os clones MUC evoluídos apresentam características patogénicas, tal como mostro pela colonização in vivo e em ensaios de infecção intraperitoneal. Adicionalmente, determino a base genética da adaptação patogénica da *E. coli* e mostro que inserções de elementos transponíveis, tanto nas regiões codificantes como nas regulatórias, conduzem o processo evolutivo. Este processo é caracterizado por interferência clonal, indicado tanto pelas evidências experimentais como pelo modelo matemático. Também demonstro que os clones MUC possuem uma vantagem selectiva quando interagem com outros predadores que a *E. coli* encontra na natureza, tais como protozoários e fagos. Para além disso, os clones SCV possuem vantagem na colonização do intestino dos mamíferos.

Finalmente, estimo os efeitos da aptidão (fitness) de diferentes mutações de resistência a antibióticos no contexto da interacção com os macrófagos. Cerca de 67% dos mutantes sobrevivem melhor do que a bactéria sensível no nicho intracelular dos macrófagos. Em particular, todos os mutantes resistentes à Estreptomicina exibem vantagem intracelular devido ao stress ambiental específico que os macrófagos impõem à bactéria, tais como a limitação dos nutrientes e o stress oxidativo.

Esta tese constituiu um trabalho interdisciplinar que fornece um teste da hipótese evolutiva e a demonstração do poder das sentinelas da imunidade inata como pressão selectiva de forma a nos ajudar a compreender a diversidade dos microrganismos em termos da evolução da resistência antibiótica e à virulência na natureza.

Chapter 1

Introduction

Microorganisms as models to study evolutionary processes

A vast diversity of microorganisms exists in our planet, from microbes that inhabit soil, fresh waters or the depths of our oceans, to those that colonize different animal species, including humans. With advances in molecular biology and increased accessibility of high-throughput sequencing techniques, it became evident that microbial cells outnumber human host cells by up to one order of magnitude. A tremendous diversity of microbes prevails across individuals and their body habitats (Li et al. 2012). One of the most intriguing questions in the field of evolutionary biology, emerging from those observations, is how such microorganisms adapted to particular niches, some living in mutual or symbiotic relationship with the human body, while others acquired means to harm their hosts.

For decades, evolutionary biologists tried to describe phenotypic variation of different traits in natural populations and to understand the evolutionary processes that shape them. This was mainly done using paleontology and comparative studies that are useful in order to understand evolution of a particular trait in a given phylogenetic context. However, by using such approaches it is difficult to unravel which type of selective pressures were responsible for acquisition of such traits and which evolutionary forces generated them. Experimental evolution has proven to be a powerful approach in evolutionary and experimental biology, because it allows to study evolutionary processes, such as diversification,

adaptation, natural selection, genetic drift, recombination, amongst others, under controlled conditions and in real time (see reviews (Mitchell-Olds et al. 2007, Bennett and Hughes 2009, Kawecki et al. 2012). Additionally, another highly useful experimental feature of this methodology is the ability to preserve evolved populations for a long time, making it relatively easy to compare evolved populations to its ancestors (for more details see Elena and Lenski 2003).

Using microorganism in experimental evolution became widely adopted due to their fast generation times, relatively simple handling, accessible genetic manipulations and the possibility of having several replicate populations (for more details see (Elena and Lenski 2003)). Additionally, genomic science brought the possibility of sequencing microbial genomes in little time and relatively low costs. This allows the prompt identification of arising *de novo* beneficial mutations in a population as well as estimating their effect on fitness and the tracking of their fate over thousands of generations. Once a beneficial mutation escapes genetic drift and gets established, it will get fixed in a population, provided that another mutation with larger fitness effect will not interfere, in a process called clonal interference (Gerrish and Lenski 1998). In accordance to these theoretical predictions, are the results on recent studies demonstrating, that clonal interference is one of the most likely forces for generating diversity in microbial populations adapting to novel environments (Hughes et al. 2012, Herron and Doebeli 2013, Traverse et al. 2013).

E. coli in experimental evolution studies

Escherichia coli has been frequently used as a model organism to study many important questions regarding evolution, such as how

organisms adapt to different selective pressures (Cullum et al. 2001, Riehle et al. 2001, Alcantara-Diaz et al. 2004, Lee and Palsson 2010), the extent to which evolutionary change is reproducible (Ibarra et al. 2002, Wick et al. 2002, Fong et al. 2005, Oxman et al. 2008), the relationship between phenotypic and genotypic changes during evolution, the distribution of fitness effects of beneficial mutations (Papadopoulos et al. 1999, Cooper et al. 2001, Perfeito et al. 2007, Herron and Doebeli 2013) among many others. Common to all these evolutionary processes is the result that environmental complexity affects the evolutionary outcome.

Numerous evolutionary studies have been carried in the simplest abiotic conditions with a constant environment (Lenski et al. 1998, Maharjan et al. 2006, Barrick et al. 2009). One of the most important and insightful set of experiments was initiated by Lenski *et al.*, by tracking adaptation (and genetic changes) in 12 initially isogenic populations of *E. coli,* since 1988, to a constant minimal glucose medium. The study of this long-term evolution experiment (*E. coli* populations passed over 57,000 generations as of March 2013) has demonstrated that the rate of adaptation decreases as populations adapt (Lenski 1991), that similar phenotypic traits emerge in parallel populations (Philippe et al. 2009) and that an entirely novel trait can be acquired (Blount et al. 2008). Lenski's group also tackled several other questions, such as the emergence of mutators (Sniegowski et al. 1997), changes in gene expression and DNA sequence evolution (Cooper et al. 2003) and the genetic basis of evolved phenotypic changes (Blount et al. 2012).

An additional level of complexity in evolutionary studies was added when abiotic environmental conditions changed over time and space (Reboud and Bell 1997, Habets et al. 2006, Beaumont et al. 2009, Cooper and Lenski 2010, Quan et al. 2012). In one of those studies, by Cooper

and Lenski (2010), 42 populations of *E. coli* were propagated for 2,000 generations either on single sugars or in combination of two sugars presented together or fluctuating daily. Interestingly, they showed that populations adapting to fluctuating environments showed greater fitness variation among populations than in constant environments.

Even though, abiotic factors are very important components of the environment in which microbes occur, they are not the only important aspect. In the real environments, many abiotic as well as biotic factors shape the environment to which microbes adapt. Biotic interactions, such as competition and predation, are not only overwhelmingly present in the real world, they also represent some of the strongest selection pressures to which organisms have to adapt. Therefore, increased selective pressures become important. Despite the importance of such interactions , only recently have they been incorporated into experimental evolution studies (Buckling and Rainey 2002, Mizoguchi et al. 2003, Yoshida et al. 2003, Yamada et al. 2008, Hillesland et al. 2009, Vos et al. 2009, Paterson et al. 2010). These studies demonstrated that bacteria can adapt to different biotic environments and antagonistic co-evolution between species is an important driver of fast diversification of bacterial populations.

In the real world, many abiotic as well as biotic factors shape the environment to which microbes adapt. Given *E. coli*'s potential to adapt to simple abiotic and complex biotic environments, seen above, it is important to understand which type of selective pressures *E. coli* encounters in its natural habitats.

E. coli in its natural ecosystems

Free-living bacteria are often exposed to environmental fluctuations in nature. While most enteric bacteria are obligate anaerobes and can survive only inside specific niches in the host, *E. coli* during its life-cycle can experience a transition between its primary host environment and its secondary external environment, which differ in the biotic and abiotic conditions and resources (Savageau 1983). Consistent with this observation, *E. coli* can be isolated from several environments including water, soil and sediments; however its primary habitat is the gastrointestinal tract (Gordon et al. 2002) of mammals, birds and reptiles. It is found in 56% of mammalian hosts and 23% of birds, and to a lesser extent in fish (10%), frogs (12%), lizards (10%), turtles (4%) or snakes (2%). In humans, *E. coli* can be detected in 95 to 100% of individuals. More recent studies observed that some strains of *E. coli* are primarily adapted in the human intestine, while others are better adapted to the external environments (Sampson et al. 2006).

E. coli usually colonizes the gastrointestinal tract of newborns in the first 40 hours after the birth, and colonization mode is influenced by the mode of delivery, infant diet, hygiene levels and medication (Grönlund et al. 2000, Magne et al. 2005). Some strains remain present for months to years while others are more transient and remain only for few days (Gordon 2004). *E. coli* is mainly a facultative anaerobe that resides in the large intestine. It is accustomed to a pH of 7 to 8 and a body temperature of 37 ^{o}C. In the large intestine, bacteria grow on nutrients acquired from the mucus layer (Duggan et al. 2008) and, in turn, performs useful functions for the host (i.e. fermentation of undigested carbohydrates, synthesis of vitamin K2 and B12 and some amino acids, protection against invading pathogens, control the development and homeostasis of the immune

system, regulation of epithelial cells (IECs) proliferation and others (Oginsky 1952, Bragg 1964, Guarner and Malagelada 2003, Baez-Viveros et al. 2004, Macdonald and Monteleone 2005).

It is possible, that due to the complexity of environmental niches and various lifestyles of *E. coli*, a wide diversity of *E. coli* phenotypes emerged during adaptive diversification. Therefore, a better understanding and characterization of particular *E. coli* phenotypes is needed in order to understand how they adapted to a particular niche and acquired unique and important traits.

Diversity and pathogenic potential of *E. coli* strains

Currently, *E. coli* strains present in human and animal populations are divided into four major recognized phylogenetic subgroups: A, B1, B2 and D. This separation is based on the analysis of genetic, phenotypic and ecological data. The distributions of the four *E. coli* subgroups are similar in mammalian herbivores and omnivores and differ from carnivores. A and B1 strains are closely related and occur in all vertebrate hosts and in the water; B2 subgroup is found in warm-blooded vertebrates with hindgut fermentation; subgroup D is found in warm-blooded vertebrates (Gordon 2004). In humans, considerable variation has been observed in the proportions of *E. coli* subgroups during the past 20 years (Tenaillon et al. 2010). However, it has been shown that socioeconomic factors, such as dietary habits and the level of hygiene, are presumably one of the main conditioners for phylogenetic group distribution, rather than geographical, climatic or host genetic conditions (Tenaillon et al. 2010).

E. coli is not just a commensal. According to the pathophysiology of a particular disease, *E. coli* strains are divided into several pathotypes, such as enterohemorragic (EHEC), enteropathogenic (EPEC), enterotoxigenic

(ETEC), enteroinvasive (EIEC) and extraintestinal (ExPEC) pathogenic *E.coli* (Denamur 2010). Pathogenic strains of *E.coli* can cause urinary tract infections, gastroenteritis, neonatal meningitis, hemorrhagic colitis, multifactorial hemolytic uremic syndrome (HUS), peritonitis, mastitis, septicemia and other diseases (Donnenberg and Whittam 2001). Within the different types of *E. coli*, commensal *E.coli* represents the most abundant bacterial flora in the colon where it normally does not cause disease (Denamur 2010). Nevertheless, even commensal *E. coli* can become an opportunistic pathogen if it breaks hosts physiological barriers, causing urinary tract infections or sepsis (MacFie et al. 1999, Russo and Johnson 2003).

Several studies have tried to address the genotypic and phenotypic differences between commensal and pathogenic *E. coli* strains (Dobrindt et al. 2003, Brzuszkiewicz et al. 2006, Zdziarski et al. 2010, Leimbach et al. 2013). DNA-DNA hybridization results have shown that genome contents of different *E. coli* isolates (22 pathogenic and commensal strains) differ distinctly from the laboratory strain K-12 MG1655 mainly in open reading frames (ORFs). Amongst these differences are virulence-related genes on mobile genetic elements, such as bacteriophages, plasmids and pathogenicity islands (PAIs). Also, pathogenic strains of *E. coli* differ from non-pathogenic ones in phenotypic traits such as antibiotic resistance, outer membrane permeability, growth rate, stress resistance and others (Levert et al. 2010, Lino et al. 2011). These results demonstrate that, in general, pathogenic *E. coli* strains exhibit a greater genotypic and phenotypic polymorphism than nonpathogenic *E. coli* strains. However, it is difficult to distinguish if this diversity in the pathogenic *E. coli* strains was generated due to a high selection pressure imposed by the hosts' immune system or due to other environmental factors inside and outside the host.

Over the past decades, global statistics on pathogenic *E. coli* strains reflect increasing recognition and surveillance to study emergence of disease outbreaks. Worldwide, ETEC strains are estimated to cause 600 million cases of diarrhea, 840 million gastrointestinal infections annually and 700,000 deaths in children younger than 5 years, predominantly in developing countries (Stamm and Norrby 2001, Dobrindt and Hacker 2008). According to the Centers for Disease Control and Prevention, several outbreaks of EHEC *E.coli* strain O157:H7, were observed during last decade. Though *E. coli* O157:H7 is a mutant form of EHEC pathotype, found mainly in the intestinal tract of cattle, it could be transmitted via many food vectors. As the population of EHEC O157 strains has increased in frequency and spread geographically, it has also genetically diversified. SNP (single nucleotide polymorphism) genotyping has shown that diverse lineages of O157 strains are associated with clinical infections and that some lineages have recently acquired novel factors that contribute to enhanced virulence (Zhang et al. 2007, Manning et al. 2008). Evolutionary changes in some of the *E.coli* strains could explain their emergence in several recent foodborne outbreaks. The most recent outbreak, in May 2011 in Germany, was caused by a new mutant EHEC *E.coli* strain O104:H4, resulting in 44 deaths and 3,792 infected people.

E. coli strains are extensively studied and can be viewed as rapidly evolving, capable of generating new pathogenic variants that have the ability to deceive host protective mechanisms. Thus it becomes crucial importance to understand which strategies can emerge when these microorganisms adapt in the presence of the immune system.

Immune system as selective pressure in bacterial populations

Infectious diseases caused by various microorganisms are a leading cause of morbidity and mortality worldwide, hence it is important to understand how the host immune system recognizes and responds to a microbe. After a microbe successfully overpasses the hosts' physiological barriers, it confronts the cells and mechanisms of the first line of defense against invading organisms - innate immune system. Among the variety of tissue cells involved in the innate immunity of the vertebrate hosts (e. g. neutrophils, eosinophils, basophils, dendritic cells) macrophages are key players in host defense by recognizing, engulfing and killing microorganisms (see Figure 1.1A).

Interaction between *E. coli* and macrophages

Recognition

Innate immune recognition is mediated by pattern-recognition receptors (PRRs), which are germline encoded. Each receptor recognizes specific molecular structures of different microbes. PRRs are unique to the host cells (e.g. epithelial cells or macrophages) and most extensively studied in macrophages. The main targets for PRR mediated recognition are called pathogen-associated molecular patterns (PAMPs). The best known PAMPs of bacteria are lipopolysaccharides, lipoteichoic acids, peptidoglycans, some lipoproteins and lipopeptides, CpG-DNA, carbohydrates, endotoxins and others (Medzhitov 2007). After many ligands and their receptors were discovered, it became obvious that the receptors do not distinguish "pathogens" *per se*, but rather invariant structures of microbial structure in general. The outcome of this foreign structures recognition, resulting either in activation of

proinflammatory/immunogenic responses or sometimes resulting in tolerance, remains to be explained.

All PRRs can be broadly categorized into three different functional groups (see summary in Table 1.1): signaling PRRs that initiate "pro-inflammatory" signaling pathways, phagocytic (or endocytic) PPRs that mediate microbe uptake to phagocytes and secreted PRRs which activate complement and facilitate phagocytosis (Miller et al. 2005, Medzhitov 2007).

Engulfment and phagosome maturation

After bacteria are recognized by phagocytic PRRs, different signaling cascades are activated depending on the nature of signaling receptors engaged in the activation. The following events result in physical and chemical changes in the macrophage cell that triggers the engulfment (Figure 1.1A).

Following the attachment, polymerization and then depolymerization of actin filaments take place, macrophages send pseudopods (also called pseudopodia or "false feet" – see Figure 1.1B) out to engulf the microbial particle and place it into endocytic vesicles called phagosomes (see Figure 1.1A and 1.1C).

Table 1.1. Different classes of PRRs and their best characterized PAMP ligands.

Functional groups	Class	Receptor	Characterized ligands	References
Signaling PRRs	TLR	TLR1 TLR2 TLR4 TLR5 TLR6 TLR9	LP LTA, PGN, lipoproteins, LPS LPS Flagellin diacyl lipoproteins CpG - DNA	(Miller et al. 2005, Medzhitov 2007, Ehlers and Rook 2011)
	NLR	NOD1 NOD2	meso-DAP MDP	(Medzhitov 2008)
Phagocytic (endocytic) PRRs	SR	SR-A MARCO	LDL, LPS, LTA, poly(IC) LPS, LTA	(Elshourbagy et al. 2000, Pluddemann et al. 2009)
		MR	carbohydrates	(Cotena et al. 2007)
		Dectin-1	glucans	(Cotena et al. 2007)
Secreted PRRs	collectins	SP-A SP-D	mannose, fucose and N-acetyl glucosamine	(Zamze et al. 2002)
	pentraxins	PTX3	OmpA	(James 2002)
		LPD	LPS	(James 2002)
		BPI	LPS and endotoxin	(Medzhitov 2008)
	PGRP		PGN	(Medzhitov 2008)

Upon formation of the early phagosomes, just after the microbe is ingested inside the macrophage, phagosomes undergo sequential fusion with early endosomes, late endosomes and lysosomes (Figure1.1A). The latter terminal step, when phagosomes fuse with lysosomes forming

phagolysosomes, is accompanied by acute changes in the composition of phagolysosomes lumen, which becomes a highly acidic, oxidative and degradative milieu. There are two major models that describe this endocytic pathway. The “vesicle shuttle model” stating that transport vesicles connect pre-existing endocytic organelles, and the “maturation model”, suggesting that the formation of endocytic vesicles occurs because of gradual maturation into the later stages vesicles (Tjelle et al. 2000).

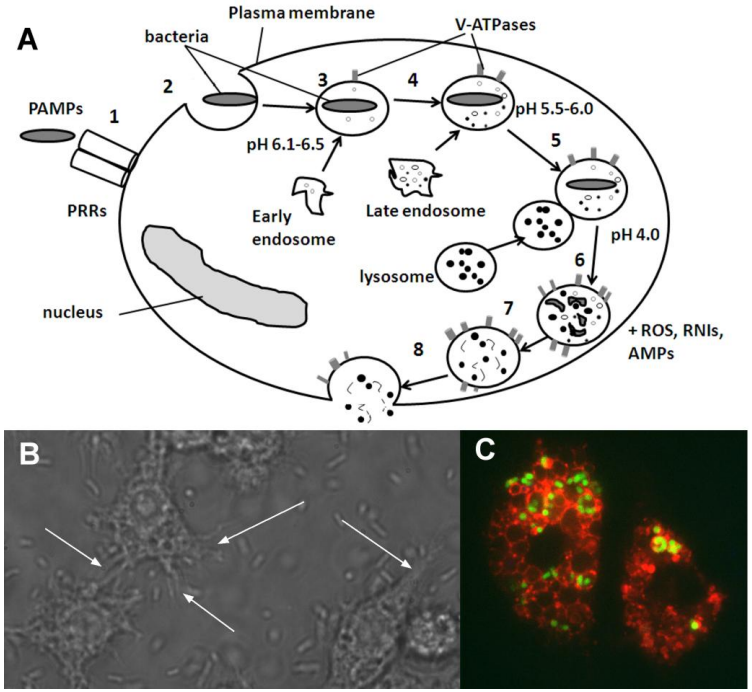

Figure 1.1. Interactions between macrophage and bacteria. (A) – recognition (1), engulfment (2), phagosome fusion with early endosomes (3) and late endosomes (4), phagolysosome formation (5), intracellular killing and digestion (6), formation of residual body containing indigestible material (7), discharge of waste

materials (8). **(**B) Pseudopod formation (indicated by arrows) during exposure of the macrophages to *E. coli*. DIC (interference contrast) picture was imaged with DeltaVision Core Olympus microscope equipped with EM-CCD camera with 60× oil immersion objective. (C) *E. coli* K-12 derivative which consitutively expresses yellow fluorescent protein (yfp, shown in green) inside endocytic vesicles of the macrophages. Macrophages (RAW 264.7) were labeled with LysoTracker Red (LTR) (in red) and imaged on a Nikon Eclipse Ti-E microscope with 100× oil immersion objective with Yokogowa CSU-x1 confocal scanner.

During the formation of early phagosomes, phagosomes fuse with early sorting and recycling endosomes making their lumen become mildly acidic (pH 6.1 - 6.5) and poor in hydrolytic activity. After several recycling processes, phagosomal lumen becomes more acidic (pH 5.5-6.0) with the help of proton- pumping V-ATPases. The final fusion of phagosome and late endosomes results in the maturation of phagolysosome, where intracellular killing of the microorganisms occurs. Insertion of additional V-ATPases increases pH dramatically, up to pH of 4.0. Phagolysosomal structure provide an enclosed space in which macrophages can direct high local concentrations of reactive oxygen species (ROS), reactive nitrogen intermediates and antimicrobial peptides to eliminate internalized microbes.

Oxygen-dependant and oxygen-independent killing mechanisms

After phagolysosome maturation, within few minutes of engulfment, bacterial physiology changes dramatically, resulting in the loss of viability due to microbicidal activities of the macrophages. The latter are divided into oxygen-independent and oxygen-dependent killing events.

The main mechanism by which phagocytes eliminate internalized bacteria is through ROS or through reactive nitrogen species (RNS). ROIs (reactive oxygen intermediates), ROS or ROMs (reactive oxygen metabolites) mainly contain oxygen (O_2), hydrogen (H_2) and halogen (Cl_2, Br_2). Some examples of ROIs include: free oxygen radicals (O_2^-), hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($RO_2^{\bullet}$), perhydroxyl radical ($HOO^{\bullet}$); some oxidants, such as hypochlorous acid (HOCl), ozone (O_3), peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Macrophages destroy pathogens in part through ROS generated directly or indirectly by the NOX2 NADPH oxidase. NOX2 transfers electrons from the NADPH to the molecular oxygen and generates NADP+, protons and superoxide anions:

$$2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+$$

This rapid reaction is called “respiratory burst” or “oxidative burst” which leads to the accumulation of large amounts of ROIs. Within the phagosome O_2^- can dismutate to H_2O_2, which can in turn react with O_2^- to generate hydroxyl radicals and singlet oxygen. H_2O_2 can also be converted by myeloperoxidase into hipochlorous acid and cloraminesin (Flannagan et al. 2009). NOX2 is part of the Phox multiprotein complex, which can be modulated by increasing concentrations of molecular oxygen, acidic pH and NADPH, Ca^{2+} depletion by store-operated calcium entry (SOCE) mechanism which in turn causes influx of extracellular Ca^{2+}. This process can be primed by LPS of gram-negative and gram-positive bacteria (Flannagan et al. 2009).

The second oxygen-dependent killing mechanism employs reactive nitrogen intermediates (RNIs), RNS and reactive nitrogen metabolites (RNM), which are composed mainly from nitrogen, oxygen and sometimes halogen or sulfur group. Some of the examples of RNS, RNI and RNM include: nitrite (NO_2^-), nitrate (NO_3^-), nitogen monoxide ($^{\bullet}NO$), nitrogen

dioxide ($^{\bullet}NO_2$), nitrosonium cation (NO^+), nitroxyl (NO^-) and peroxynitrite ($ONOO^-$) anions, dinitrogen trioxide (N_2O_3), nitryl chloride (NO_2Cl) and various S-nitrosothiols. The main enzymes in RNIs production are (NOS) nitric oxide synthases that generate $NO^{\bullet}$ from L-arginine. There are three isoforms of NOS: endothelial NOS , neuronal NOS (nNOS) and inducible nitric oxide synthase (iNOS or NOS2), which is induced only after exposure to pathogens, microbial ligans and/or cytokines (Bogdan 2010). iNOS functions as a dimer: one subunit transfers electrons from NADPH to FAD, then to flavin mononucleotide (FMN) and to the haem iron of the adjacent subunit, to produce $NO^{\bullet}$ and citrulline from L-arginine and oxygen (Bogdan 2010). $NO^{\bullet}$ is synthesized on the cytoplasmic side of phagolysosomes and freely diffuses across the membrane where it encounters ROS.

Collectively, highly reactive and toxic ROS and RNS effectively kill microorganisms as they interact with numerous microbial targets, such as thiols, metal centers, protein tyrosine residues, nucleic acids and lipids (Stuehr 1997). Moreover, the rate of spontaneous and intracellularly induced mutagenesis is at least 10-fold higher in the macrophage cells than in the absence of the macrophages (Schlosser-Silverman et al. 2000). Indeed, upon macrophage entry, the majority of *E. coli* cells are viable, but the mutagenic macrophage environment leads to an immediate arrest of gene expression and to DNA damage of *E.coli*. DNA damage generated is characterized by the DNA strand breaks and the modification 8-oxo-2*-deoxyguanosine, which are typical of oxidative damage.

The oxygen-independent killing process involves many antimicrobial proteins and peptides secreted by the phagocytes (Ganz 2003). Cationic host defense peptides (HDPs) include two major classes of antimicrobial peptides - defensins and cathelicidins. HDPs recognize LPS or LTA in the bacterial membrane, but exact mechanisms of their

action are not known (Jenssen et al. 2006). Several models propose different targets for antimicrobial peptides action, which include disruption of membrane integrity, interference with essential processes dependent on membrane-bound enzymes (cell division, cell wall synthesis), or translocation across the membrane to access cytosolic targets (Yang et al. 2001).

Antimicrobial proteins are larger molecules than antimicrobial peptides (more than 100 amino acids) with a well defined tertiary structure. Like HDPs, they exhibit antimicrobial activities and immunomodulatory properties. Only three antimicrobial proteins are found in the mature macrophages: lysozyme, lactoferrin (Lf) and natural resistance-associated macrophage protein 1 (NRAMP1). Lysozyme can act only on very restricted range of bacteria as it kills bacteria by increasing osmotic stress that leads to the disruption of the peptidoglycan that composes bacterial cell wall. Gram-negative bacteria are generally more resistant to lysozyme activity due to stabilizing effect in the outer membrane and only higher levels of this protein can be antibacterial (Madera et al. 2010). Lf acts as bacteriostatic agent by sequestrating iron required for growth of many bacteria and as bactericidal agent by damaging the outer bacterial membrane and increasing membrane permeability (Madera et al. 2010). Another antimicrobial protein, NRAMP 1, an integral membrane protein expressed in late endosomes and lysosomes, exerts a bacteriostatic effect by extruding divalent cations, such as Fe^{2+}, Zn^{2+} and Mn^{2+}. Since those cations are cofactors for many microbial enzymes (e.g. Mn^{2+} is required for superoxide dismutase) it inhibits bacterial metabolism (Puddu et al. 2009).

In general, oxygen-independent (phagolysosome acidification, action of antimicrobial proteins and peptides) and oxygen-dependent killing mechanisms (ROS and RNS) are always interdependent. For example,

acidification of phagolysosomes by V-ATPases enhances the activity of many proteases that converts H_2O_2 into highly toxic halides and participates in ROI production.

Possible adaptive strategies that bacteria can evolve in the presence of macrophages

Just as the body has evolved mechanisms to defend itself from intruders, bacteria have evolved their own strategies to circumvent immune recognition at its initial steps. If bacteria breach the host's physical defenses, it must then overcome the host's phagocytic response to succeed in the following infection. Many bacterial pathogens have evolved to escape, resist or even kill phagocytic cells upon their encounter (Rosenberger and Finlay 2003). Altogether, several intracellular and extracellular strategies exist to fool innate immune system cells, in particular macrophages. Extracellular strategies include avoidance of detection and engulfment inhibition mechanisms. Intracellular strategies comprise of survival inside macrophages and escape from phagosomes mechanisms. The ultimate strategy that pathogens evolved can be classified either as intracellular or as extracellular, because pathogens can kill host cells by secretion of toxins either before or after engulfment of macrophages.

Avoidance of contact with macrophages

For some bacteria that do not have sophisticated genetic tools to interfere with macrophage functions, it is advantageous to remain extracellular in order to avoid being internalized and killed. To avoid detection by macrophages, some bacteria modify their surface by camouflaging or directly modifying the molecules that trigger TLR signaling. For instance, some extracellular pathogens (*Streptococcus pneumonia*, *E.*

coli K1, *Neisseria meningitides* and many others) mask bacterial surfaces by expressing a carbohydrate capsule, hereby avoiding opsonization and phagocytic clearance (Finlay and McFadden 2006). Many bacterial pathogens (*Salmonella enterica* serovar *Typhimurium*, *Brucella abortus*, *Yersinia pestis*), instead of hiding their bacterial surface, simply modify their LPS in such a way that they are less well recognized by macrophage receptors (Diacovich and Gorvel 2010, Pastelin-Palacios et al. 2011). For example, *Salmonella* modifies its lipid A structure by including 3-O-deacylase (PagL) and a lipid A palmitoyltransferase (PagP). As a result, modified lipid A is up to 100-fold less actively recognized by TLR4 receptor (Kawasaki et al. 2004).

Although several observations on filament forming bacteria suggested that bacteria undergo such morphological changes in order to avoid internalization (Garcia-del Portillo et al. 1993), this might not be true. For instance, several authors observed filamentous UPEC during bladder infections, a phenomena that is now described as being transient in the different stages of infection and insufficient to confer protection from phagocytosis *in vitro* (Justice et al. 2006). However, filamentous bacteria may show an advantage in the face of extracellularly excreted antimicrobial peptides, because such trait contributes to survival in different environmental stresses (Chen et al. 2005, Justice et al. 2006).

Inhibition of phagocytic engulfment

Several reviews discuss how bacterial pathogens, such as *Y. pestis*, *Pseudomonas aeruginosa* and EPEC or EHEC deliver specific proteins to interfere with bacterial uptake into host cells (Rosenberger and Finlay 2003, Bhavsar et al. 2007, Schmid-Hempel 2008). For example, Yops proteins by *Y. pestis* are deployed to macrophages to interfere with cytoskeleton to block proper phagocytosis (Hornef et al. 2002). Some

extracellular pathogens (for example, EPEC and EHEC) manipulate the actin cytoskeleton to generate actin-rich pedestal structures on the host-cell surface, where pathogen resides without being internalized (Bhavsar et al. 2007).

Escape from phagolysosomes

One way to avoid the harsh environment of phagolysosome is to rapidly escape maturing phagosomes or to arrest/reprogram phagosomal maturation. Various "professional" intracellular pathogens, such as *Mycobaterium tuberculosis*, *S. typhimurium*, *Coxiella burnetti*, *Legionella pneumophila, Listeria monocytogenes E. coli*, and others, survive intracellularly in different recycling early endosomes, late endosomes, lysosomes or endoplasmic reticulum (Underhill and Ozinsky 2002, Flynn and Chan 2003). For example, some strains of *E. coli* adhere to host cells with FimH type 1 pilus, actively enter macrophages through specific lipid rafts and persist inside membrane-bound compartments that are protected from oxidative and acidic stresses (Baorto et al. 1997). Bacteria such as *Shigella* spp, or *L. monocytogenes* produce proteins that allows bacteria to escape from vacuolar compartments into the cytoplasm (Portnoy et al. 2002). Recently, it was demonstrated that *L. monocytogenes* not only can enter cytoplasm and replicate rapidly (every 30 min) following fast dissemination, but it can also reside inside *Listeria*-containing vacuoles, where bacteria can replicate slowly (every 8 hours), without destroying the infected cell (Flannagan et al. 2009). Such switch between mechanisms could explain different outcomes of *L. monocytogenes* active and chronic infections. Other examples to escape from mature phagolysosomes are represented by *M. tuberculosis* and *Mycobacterium avium*. *M. tuberculosis* uses an array of effector molecules to survive inside macrophages by arresting phagosomal maturation (Flannagan et al. 2009). And *M. avium*

inhibits acidification of *Mycobacterium*-containing vacuoles thus enabling activation of lysosomal degradative enzymes.

Increased survival in the phagolysosome

If a pathogen is captured and reaches a phagolysosome, it can still survive by withstanding the microbicidal properties of the phagolysosome. Several studies have focused on the interactions that occur between macrophages and bacteria in this degradative milieu, where pH can decrease up to 4.0 and multiple antimicrobial peptides, ROS and RNS are present. Many intracellular pathogens (*M. tuberculosis*, *C. burnetti*, *Rhodococcus equi*, *Staphylococcus aureus*, *Bacillus anthracis* and others) survive this stress and some of them can even replicate inside the macrophages (Howe and Mallavia 2000, Pieters and Gatfield 2002, Toyooka et al. 2005). For example, *M. tuberculosis* survives and even replicates inside phagolysosomes, probably by excluding proton-pumping V-ATPases, however the exact mechanism is not known (Smith 2003). Moreover, *M. tuberculosis* cell wall contains mycolic acids and other lipids that protect bacteria from lysosomes. Other pathogens (for example *S. aureus* and *B. abortus*) produce enzymes, such as catalase and superoxide dismutase (SOD) that partially neutralizes reactive oxygen and nitrogen species (Kim et al. 2000, Das and Bishayi 2010).

Another astonishing obligate intracellular pathogen, *C. burnetti*, the causative agent of Q fever, shows a biphasic developmental cycle of infection (Flannagan et al. 2009). In the first phase of infection, *C. burnetti* displays dormant small-cell variant (SCV) morphology and delays phagosome fusion with lysosome. In the second phase of infection, small-cell variant transits to replication-competent large-cell variant (LCV), that behaves similarly to acidophile. It is believed that acidic environment of phagolysosome is required for many *C. burnetti* metabolic functions and

unknown virulence factors encoded in T4SS can protect from antimicrobial effects.

Killing of macrophages

Many most dreadful diseases, such as diphtheria, tetanus, botulism or cholera are caused by pathogens that are able to secrete toxins and kill macrophages (Lahiri 2000). Bacterial toxins can be divided into three groups according to their site of action: toxins that act at the plasma membrane and interfere with transmembrane signaling pathways (*E. coli* enterotoxin ST); that alter membrane permeability (*E. coli* hemolysin); or that modify cytosolic targets inside the macrophages (*E. coli* CNF) (Finlay and Cossart 1997). Toxins can also be divided in several groups according with the effect that impose on macrophage cells, from blockage of exocytosis or protein synthesis, actin depolymerization and cell shape changes to increase in cAMP and even cell death.

Traits selected in *E. coli* clinical isolates

Overall, many bacterial species evolved diverse mechanisms for interacting with, and manipulating, host cells and for evading host innate immune responses. Presumably, bacterial pathogens have evolved such strategies because they provide a selective advantage in the environments where pathogens can be found.

Several *E. coli* strains were isolated in clinics from patients, suffering from urinary infections, chronic chest infections, catheter-related infections, pyelonephritis, breast cancer, alcoholic cirrhosis, inflammatory bowel disease, diabetes, Crohn`s disease and other diseases (Taylor 1976, Borderon and Horodniceanu 1978, Vejborg et al. 2011). It is possible that many of those diseases resulted from evolution of *E. coli* to escape

immune surveillance in the host. Vidal *et. al* (1998) have shown that two clinical strains isolated from patients with catheter-related infections were able to form a thick biofilm and this phenotype could be explained by a single point mutation, resulting in the replacement of leucine by an arginine amino acid at position 43 in the regulatory protein OmpR (Vidal et al. 1998). While selection pressure responsible for the appearance of this phenotype could not be identified, it is striking that only one point mutation can be sufficient to acquire a pathogenic trait, such as adherence and biofilm formation. Other frequently sampled *E. coli* clinical isolates exhibit decreased growth ability, because of defections in the respiratory chain. Those metabolically deficient bacterial strains, previously called dwarfs (Borderon and Horodniceanu 1978) and later SCVs (for small colony variants), are more resistant to certain antibiotics and are responsible for various persistent infections (Proctor et al. 2006).

In order to combat pathogens that are able to overrun crucial host-cell mechanisms, it is important to disentangle the causes of the underlying disease. Despite progress towards understanding the molecular mechanisms that underlie microbial pathogenesis, further steps towards understanding how such traits evolve in the presence of selective pressures imposed by the host, are indispensable.

Bacterial adaptation to other environmental niches can result in increased survival in the presence of the macrophages

Many bacterial species are not solely obligatory human pathogens but rather versatile lodgers that can transit to other environmental niches. In such ecosystems as soil, freshwater and sea, microbes are confronted with a range of environmental predators and parasites (Finlay et al. 1998, Ronn et al. 2002, Chibani-Chennoufi et al. 2004, Fayer et al. 2004), and

thus, have to learn how to avoid, tolerate or defend against them. Because traits that bacteria evolve in the outside-host environment sometimes correlate with an increased survival to resist host's immune system (Wildschutte et al. 2004, Friman et al. 2009, Adiba et al. 2010, Chrisman et al. 2010), it is not clear which type of selective pressures are responsible for the acquisition of such traits. The coincidental evolution hypothesis suggests that some bacterial strategies, which are advantageous in the presence of immune system does not necessary result from directional selection in the host, but can be a by-product of adaptation to other ecological niches.

Such relationship between bacterial strategies to escape hosts immune system and increased survival in the presence of some simple eukaryotes and prokaryotes is very intriguing. It is believed that some traits that enhance the survival of bacterial pathogens in the presence of immune system by providing protection against environmental predators and parasites, such as bactevorous protozoa, bacteriophages, nematodes or even other bacterial species.

Protozoa

It is believed that some of the adaptations that evolved as defense mechanisms against protozoa may be important in the adaptation of bacteria to the immune system (for example, in *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Cryptococcus neofarmans*, *Salmonella enterica*, *E. coli* O157:H7 and others) and one of their primary environmental predators – protozoa (Matz et al. 2004, Wildschutte et al. 2004, Steinberg and Levin 2007, Chrisman et al. 2010, Messi et al. 2013). Indeed, pathogen detection mechanisms involved in innate immunity and prey detection by free-living protozoa are very similar (Jousset 2012). Moreover, protozoa and macrophages share similar mechanism by which

bacteria are uptaken and killed (Horwitz and Maxfield 1984, Rechnitzer and Blom 1989, Gao et al. 1997). In fact, many studies have shown that protozoa are able to select for bacterial virulence traits that help to survive in the presence of macrophages (Harb et al. 2000).

It is thought that adaptation of *Legionella pneumophila* to survive within environmental amoebae may have primed this pathogen to survive within macrophages (Cirillo et al. 1994, Brieland et al. 1997). Indeed, *L. pneumophila* grown within amoebae are several fold more invasive both for epithelial and macrophage cells and are 1000-fold more resistant to antimicrobial compounds (Barker et al. 1995). Moreover, several genes that enhance survival within macrophages are also required for survival within amoeba (Gao et al. 1997, Liles et al. 1999, Segal and Shuman 1999, Clark et al. 2013). Recently, however, it was also demonstrated that *L. pneumophila* grown within macrophages can become less invasive in protozoa (Ensminger et al. 2012). Examples from other bacterial species, such as production of toxins and biofilms in *P. aeruginosa* (Matz et al. 2004), O-antigen variation among *Salmonella* serovars (Tezcan-Merdol et al. 2004, Wildschutte et al. 2004) and advantage of *E. coli* Shiga toxin (Stx) (Steinberg and Levin 2007), were also observed during protozoan grazing. Altogether, there are, in fact, a variety of mechanisms that bacteria use to either evade or survive protozoan predation (Matz and Kjelleberg 2005). Such mechanisms resemble several adaptive strategies that bacterial pathogens evolved to circumvent recognition, engulfment or killing by macrophages (see previous chapter).

Nematodes

Little is known about bacterial evolution in nematodes, despite the vast body of studies using the nematode *Caenorhabditis elegans* as a model organism (Kurz and Ewbank 2003). This is disappointing given the fact that *C. elegans* and other nematode species are found in the same ecosystems as bacterial species, and form the second main group of bacterivorous predators (Abada et al. 2009). Only recently, however, *C. elegans* was recognized as a valuable model to study bacterial pathogenesis and even evolution of innate immunity (Schulenburg et al. 2004). It is known that when *C. elegans* encounters pathogenic bacteria, innate immune responses are mounted, such as production and release of different antimicrobial peptides (Irazoqui et al. 2010), very similarly to those observed in many eukaryotic cells in the human body, including macrophages. Moreover, there is an ever growing list of bacterial pathogens that are able to infect *C. elegans* and invade innate immune responses of the human host, such as *P. aeuruginosa*, *Vibrio cholera*, S. *typhimurium*, *Streptococcus pneumonia*, *Burkholderia pseudomallei* and many others (Darby et al. 1999, Aballay et al. 2000, Aballay and Ausubel 2002, Garsin et al. 2003, Vaitkevicius et al. 2006, Diard et al. 2007, Bhargava et al. 2009). For instance, toxins of *P. aeuruginosa* involved in the killing of *C. elegans* are also required for pathogenesis in mammals (Tan et al. 1999). And *E. coli* causing urinary tract infections in humans can cause digestive tract infections in *C. elegans* (Diard et al. 2007).

Bacteriophages and other bacterial species

Several other traits that are advantageous in the presence of the macrophages can be selected when bacteria are subjected to bacteriophages or even other bacterial species. It is known that some bacteriophages encode virulence factors that can be integrated into

bacterial genome (for explicit reviews see(Casas et al. 2010)). However, such phages provide direct advantage and thus cannot be applied to coincidental evolution theory. Indirectly, however, phages-mediated selection can shape several bacterial traits, such as motility and growth (Koskella et al. 2011) and even indirectly modulate bacterial pathogenesis (Friman et al. 2011, Laanto et al. 2012). Interestingly, Friman *et al.* (2011) demonstrated that after exposure of bacteria *Serratia marcescens* to predation by lytic bacteriophage PPV (*Padoviridae*), bacterial pathogens were less virulent in the *in vivo* insect model.

Curiously, other bacterial species, as *Myxobacteria* or *Bdellovibrio bacteriovorus*, can also display predatory behavior. Soil living bacteria, *Myxobacteria*, aggregate in motile swarms to concentrate enzymes that digest other bacterial species (Shimkets 1990). Or *B. bacteriovorus* kill other bacterial cells by entering in the prey's periplasm between its inner and outer membranes and utilizing the prey's macromolecules for replication (Nunez et al. 2003). Though, it is not known if such predatory mechanisms can select for traits that enhance survival of bacteria in the human host.

Overall, traits that affect ability to resist to environmental predators and parasites should be under strong selection in many bacterial pathogens. Hence, a number of virulence factors likely evolved in response to selective pressure within environment filled with predators and parasites. Therefore, it is important to ascertain which bacterial traits selected in the presence of phagocytes, could be advantageous when facing other ecological interactions and vice versa, in order to understand and predict evolution and maintenance of virulence in the complex inside-host and outside-host ecosystem.

Antibiotics as tools to eradicate bacterial populations

In terms of evolution and ecology, bacterial survival and adaptation to the innate immune system and to antibiotics is always interconnected. In the context of infection, bacteria are frequently exposed to multiple drugs and to hosts' immune system, sequentially or even simultaneously. Hence, it is important to understand how such interaction between immune system and antibiotics affects evolution of major public threats – antibiotic resistance and virulence.

In the course of evolutionary arms-race between hosts immune system and microbes, many different pathogens have emerged, such as *M. tuberculosis*, *S. aureus*, *Klebsiella pneumonia*, *Yersinia spp.*, *Clostridia spp*. and others, that can cause serious illnesses and even death to the host. The discovery of antibiotics decreased morbidity and mortality of human population worldwide; however the use of drugs was compromised by the development of tolerance or resistance to those compounds. There are many many excellent reviews on the genetics, evolution and mechanism of antibiotic resistance (Lenski 1998, Martinez 2008, Allen et al. 2010, Andersson and Hughes 2010, Kohanski et al. 2010b, MacLean et al. 2010, D'Costa et al. 2011, Andersson and Hughes 2012), however in my thesis I will focus on the cost of antibiotic resistance.

Generally, several studies have shown that antibiotic resistant strains have a lower fitness in antibiotic-free environment although resistance to some antibiotics can be neutral or even beneficial (reviewed in. (Andersson and Hughes 2010)) On first consideration it may seem that the most reasonable approach in clinical practice would be to prescribe antibiotics that carry the largest biological fitness cost for bacteria. However, two major difficulties exist here. Firstly, many *in vivo* and *in vitro* observations have shown that bacteria evolve very rapidly to compensate

such fitness costs (Bjorkholm et al. 2001, Sousa et al. 2012). Secondly, several studies have demonstrated that fitness effects of antibiotic resistant mutations vary when measured in different genetic backgrounds (Gagneux et al. 2006) or in different environmental conditions (Giraud et al. 2003, Hall and MacLean 2011, Trindade et al. 2012). While many of those studies determined the effects of resistance in diverse environments, only very few of those were conducted in the conditions relevant for the host, namely during the infection process in the host macrophages. This poses a challenge to both evolutionary biologists and microbiologists, as antibiotics are still fundamental tool to combat pathogens inside the host environment.

Similarities between ROS generated by macrophages and action of antibiotics

During entry into innate immune system cells – macrophages – bacteria experience a set of environmental stresses, such as host-induced nutrient limitation, acidification, toxic peptides, osmotic stress and reactive oxygen species (ROS), of which the latter is believed to be a major cause of bacterial killing (Schlosser-Silverman et al. 2000).

Several studies have tried to link oxidative stress and antibiotic-induced ROS formation (Kohanski et al. 2007, Dwyer et al. 2009). Kohanski *et al.* (2009) revealed that treatment of bacteria with different bactericidal antibiotics induces formation of hydroxyl radicals via common mechanism that affects tricarboxylic acid (TCA) cycle metabolism. Other studies reported that single point mutations in *soxR* gene, that protect *E. coli* from cytotoxicity of nitric oxide-generating macrophages (Nunoshiba et al. 1993), contributed to multiple-antibiotic resistance phenotypes (Koutsolioutsou et al. 2005). Moreover, Wang and Zhao showed that the combined activity of superoxide dismutases SodA and SodB were

important to killing by fluorquinolones and peroxidase AhpC was critical to killing by β-lactams and aminoglycosides antibiotics (Wang and Zhao 2009). A recent study has also shown that increase in mutation rate after addition of sublethal concentrations of various bactericidal antibiotics positively correlate with increase in ROS formation in bacterial cells (Kohanski et al. 2010a). However, it is also surprising, that the same phenomenon was not observed under anaerobic growth conditions, suggesting that antibiotic induced ROS-dependent killing is only important in aerobically respiring bacteria. Unfortunately, it may not be relevant inside human body, where oxygen may be limited in some micro-environments.

Taken together, these results suggest that bactericidal antibiotics, irrespective of the drug target of the applied antibiotic, may, in certain conditions, induce formation of ROS which in turn kills bacterial cells. In contrast, there is also very recent evidence to suggest that such unified mechanism of antibiotic killing may be incorrect (Keren et al. 2013). Keren *et al.* (2013) demonstrated that killing by antibiotics is unrelated to production of ROS at higher doses of antibiotics. Undoubtedly, there is a further need to investigate if antibiotics induce ROS formation in bacterial cells. Whichever is the correct answer for this dilemma; this does not change a fact that antibiotic resistance and virulence are commonly associated traits in many species of microbes and can influence evolution of each other.

In conclusion, the evolutionary and ecological mechanisms that select and maintain bacterial traits to resist hosts defenses or antibiotic pressure are fundamental to understand the emergence of diseases, as well as the causality and possible outcomes. Hence, this thesis tries to add some knowledge on the understanding the effect of immune system on the

evolution of bacteria in the context of pathogenesis and antibiotic resistance.

Thesis objectives

The main purpose of this thesis is to understand the process of adaptation to the complex biotic interaction between commensal strain of *Escherichia coli* and presence of innate immune system, namely macrophages.

In Chapter 2, I described different strategies that *E. coli* can evolve in the presence of innate imunity and revealed the nature of possible pathoadaptive mutations by which *E. coli* adapt to better resist phagocytosis by macrophages.

In Chapter 3, I tested if the rate of adaptation in the presence of this biotic environment is higher than in the abiotic environment and determined if clones adapted to macrophages also bear an advantage in other ecosystems.

Finally, in Chapter 4 I estimated fitness effects of antibiotic resistant mutations to address whether antibiotic resistant *E. coli* can have an advantage or disadvantage in the context of an interaction with macrophages.

Internet Resources

Centers for Disease Control and Prevention (CDC)
http://www.cdc.gov/nczved/dfbmd/disease_listing/stec_gi.html

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Chapter 2

The genetic basis of *Escherichia coli* pathoadaptation to macrophages

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Summary

Antagonistic interactions are likely to be important determinants of the diversity observed in bacteria and the complexity of their genomes. An important trait, relevant for bacterial pathogenesis, is the ability to escape innate immunity. Here, we use a combination of experiments, genome sequencing and mathematical modeling to address the question of how fast and through how many adaptive steps a commensal strain of *Escherichia coli* acquires such a trait. Using a novel setup of experimental evolution, we show that less than 500 generations are sufficient for *E. coli* to evolve clones able to better escape macrophage phagocytosis and acquire *in vivo* pathogenicity. We show that transposable elements drive this pathoadaptive process. Particularly, we find that a single insertion into the promoter region of *yrfF* emerges in all lines, which potentiates three main adaptive walks. Strikingly, in one-third of the evolved lines evidence was found for increased mutagenesis driven by an insertion of IS186 into the promoter of the Lon protease, which likely accelerated the pathoadaptive process. Finally we show that clonal interference dominates the dynamics of new genotypes during evolution, using both direct experimental evidence, and a mathematical model, which is able to reproduce the dynamics observed in the experiment.

Introduction

Bacteria are currently used to study evolution in real time, in controlled environments through experimental evolution (Elena and Lenski 2003). Different studies have demonstrated that bacterial populations have an enormous potential to adapt to relatively simple and abiotic challenges in laboratory environments (Perfeito et al. 2007, Sniegowski and Gerrish 2010). On the other hand our knowledge on how biotic interactions shape adaptive evolution is still in its infancy. Antagonistic interactions (predation, parasitism) are likely to be important determinants of the rate of adaptive change observed in bacteria, their trait diversity and the complexity of their genomes (Matz and Kjelleberg 2005, Young 2007, Stern and Sorek 2011). The most studied antagonistic interaction in an evolutionary laboratory setting is that between bacteria and their phages. This has been shown to increase rates of bacterial adaptation and diversification (Buckling and Rainey 2002, Meyer and Kassen 2007, Paterson et al. 2010), demonstrating that biotic interactions can have an important role in bacterial evolution. Another common antagonistic interactions bacteria face is with the cells of the immune system. Yet, the short-term adaptive

process in this ecological context has not been studied. Here, we determine the strategies *E. coli* can evolve to overcome the hosts' first defense line – the macrophages. *E. coli* is both a commensal and a versatile pathogen, killing millions of humans each year (Tenaillon et al. 2010). Moreover, there is evidence that some pathogenic strains evolved from commensal *E. coli* (Crossman et al. 2010, Denamur 2010). This makes *E. coli* an ideal organism to study the transition from commensalism to pathogenicity. *E. coli* colonizes the infant gastrointestinal tract within hours after birth, and typically builds a mutualistic relation. However non-pathogenic strains of *E. coli* can become pathologic, when the gastrointestinial barrier is disrupted as well as in immunosuppressed hosts (Sanz-Garcia et al. 2009, Sharma et al. 2011, Janny et al. 2012). Finally pathogenic strains of *E. coli* are able to cause disease in healthy.

Macrophages (MΦs) are a key component of host defense mechanisms against pathogens. They can cause a direct bactericidal response through generation of reactive oxygen species and phagocytosis, a process by which bacteria are engulfed into phagosomes. Yet many bacterial species are capable to escape, resist and even kill eukaryotic cells, such as macrophages (MΦs) (Amer and Swanson 2002, Schmid-Hempel 2008), suggesting that several defense strategies could evolve when bacteria encounter MΦs. Strategies to resist outside MΦs include surface masking and capsule formation (to avoid engulfment and phagocytosis), increased mobility, filamentation and biofilm formation. Strategies to resist inside MΦs include toxin release and even intracellular growth. Within the species of *E. coli* alone, there are examples of several different strategies (Hunstad and Justice 2010).

In this study we have established a system in which *E.coli* is allowed to evolve to the continuous selective pressure of MΦs. Using this

system, we ask which types of adaptive strategies emerge in a commensal strain of *E. coli* to avoid killing by one of the sentinels of the innate immune system-the MΦs.

Materials and Methods

Ethics statement

All experiments involving animals were approved by the Institutional Ethics Committee at the Instituto Gulbenkian de Ciência (project nr. A009/2010 with approval date 2010/10/15), following the Portuguese legislation (PORT 1005/92) which complies with the European Directive 86/609/EEC of the European Council.

Strains and media

The RAW 264.7 murine macrophage cell line was maintained in an atmosphere containing 5% CO_2 at 37^{o}C in RPMI 1640 (Gibco) supplemented with 2mM L-glutamine (Invitrogen), 1mM sodium pyruvate (Invitrogen), 10mM hepes (Invitrogen), 100U/ml penicillin/streptomycin (Gibco), 50µM 2-mercaptoethanol solution (Gibco), 50µg/ml gentamicin (Sigma), with 10% heat-inactivated FCS (standard RPMI complete medium). Before infection assays, MΦs were cultivated for 24h in the same medium as before except for the three antibiotics which were now replaced by 100µg/ml Streptomycin antibiotic (RPMI-Strep medium). All bacterial cultures were also done in RPMI-Strep medium, except if stated otherwise.

Escherichia coli strains were MC4100-YFP and MC4100-CFP (MC4100, galK::CFP/YFP, $Amp^{R}Strep^{R}$) which contain the yellow (*yfp*) and cyan (*cfp*) alleles of GFP integrated at the *galK* locus in MC4100 (*E.coli* Genetic Stock Center #6152) and differ only by YFP/CFP locus that is

constitutively expressed (Hegreness et al. 2006). MC4100-CFP strain was used for the evolution experiment and MC4100-YFP as a reference strain for the fitness assays.

Evolution experiment

Twelve populations were founded from a single MC4100-CFP clone and were therefore genetically uniform in the beginning of the experiments. All populations were evolved in RPMI, 6 populations in the presence of the MΦs (M1-M6) and the other 6 (C1-C6) in the absence of MΦs. Before each infection cycle, MΦs (0.7×10^6 to 1.3×10^6/ml) were centrifuged at 1200 rpm for 5 min, resuspended in RPMI-Strep medium and activated with 2 µg/ml CpG-ODN 1826 (5´TCCATGACGTTCCTGACGTT 3´ - Sigma) for 24 h (Utaisincharoen et al. 2002). Cells were then centrifuged (1000 rpm for 5 min), resuspended in 3 ml of fresh RPMI-Strep medium and seeded in 12-well microtiter plates (0.8×10^6 to 1.6×10^6/). Subsequently they were incubated at 37^{o}C for 2 h, washed in RPMI-Strep and infected with a MOI of 1:1 (10^6 bacteria to 10^6 MΦs). After 24 hours of infection, MΦs were detached with cell scraper and the whole culture was centrifuged at 4000 rpm for 10 min to pellet cells. This procedure lyses MΦs releasing intracellular bacteria. Then these were washed twice with phosphate-buffered saline (PBS) and counted by flow cytometry using a FACscan cytometer (Becton Dickinson). Approximately 10^6 of recovered bacteria were used to infect new activated MΦs in the same manner as before. The same procedure was applied to control populations, except that 10^4 bacteria were transferred daily. This is so, because after 4 hours of infection with the MΦs bacteria drop to 10^4. This adjustment allows the same number of generations in both environments. In both treatments (with and without MΦs), bacteria were allowed to propagate for approximately 15 generations per day. Generation time is estimated as: $G=\log_2(Nf/Ni)$,

where Ni is the initial number of bacteria and Nf is the final number of bacteria. Nf was approximately 6×10^8 in both treatments. Evolution occurred during approximately 450 generations in both environments.

Dynamics of infection at 3 h post infection

Bacterial uptake was measured by the gentamicin protection assay as previously described (Glasser et al. 2001), with modifications, as follows. MΦs were infected at MOI 1:1 as described above to determine the number of intracellular and extracellular bacteria after 3 h of incubation. The number of extracellular bacteria at 3h of incubation was estimated by taking a sample of the culture medium (without detaching the MΦs), centrifuging (4000 rpm for 10 min) to pellet the cells and finally washing these in PBS prior to plating on LB agar plates. The number of intracellular bacteria was estimated by washing infected MΦs twice with PBS and adding fresh medium containing 100 µg of gentamicin/ml to kill extracellular bacteria. After incubation for an additional hour, the medium was removed, the monolayer of macrophages was washed 3 times with PBS, detached using a cell scraper and centrifuged (4000 rpm for 10 min) to pellet the cells. These were further resuspended in PBS and the appropriate dilution was plated on LB agar plates to determine the number of intracellular bacteria. Relative abundance (Rr) of evolved clones to that of the ancestral in intracellular or extracellular environment of MΦs was estimated as:

$$Rr = (N3h_e/Ni_e)/(N3h_a/Ni_a),$$

where $N3h_e$ and $N3h_a$ are the numbers of evolved (*e*) and ancestral (*a*) bacteria at 3 hours post infection (in the intracellular or extracellular niche of MΦs) and Ni_a and Ni_e are the initial numbers of evolved (*e*) and ancestral (*a*) bacteria used for inoculation.

Colanic acid purification and quantification

The method used to extract colanic acid was based on a procedure described previously (Obadia et al. 2007). Briefly, 50 ml of a bacterial cell culture was heated for 15 min at 100°C to denature EPS-degrading enzymes, cooled down and centrifuged at 13,200 rpm at 4°C for 30 min. Then 40 ml of the supernatant was precipitated by addition of three volumes of ethanol. The mixture was maintained at 4°C overnight and centrifuged again at 13,200 rpm at 4°C for 30 min. The resulting pellet was dissolved in 5 ml of distilled water, dialyzed for 48 h against distilled water (membrane MWCO, 3500 Da) and dried in SpeedVac. Residual polypeptides were removed by precipitation with 5 ml of 10% (v/v) trichloroacetic acid and centrifuged at 13,200 rpm at 4°C for 30 min. The supernatant was dialyzed for five days against distilled water and dried. The resulting preparation was resuspended in 1 ml of distilled water. Quantification of colanic acid was carried out by measuring non-dialyzable methylpentose (6-deoxy-hexose), namely fucose, which is a specific component of this exopolysaccharide. 10 to 100 µl of the colanic acid preparation were diluted to 1 ml with distilled water, and mixed with 4.5 ml of H_2SO_4/H_2O (6:1; v/v). The mixture was prepared at room temperature, then heated at 100°C for 20 min, and finally cooled down to room temperature. For each sample, absorbance at 396 nm and 427 nm was measured either directly (control sample ($A_{\text{-co}}$)) or after addition of 100 µl of 0.3% freshly prepared cysteine hydrochloride (cysteine sample ($A_{\text{-cy}}$)). The absorption due to the unspecific reaction with H_2SO_4 was subtracted from the total absorption of the sample: $A_{396\text{-co}}$ and $A_{427\text{-co}}$ were subtracted from $A_{396\text{-cy}}$ and $A_{427\text{-cy,}}$ respectively, to obtain ΔA_{396} and ΔA_{427}. Values of (ΔA_{396}– ΔA_{427}) were directly correlated to methylpentose concentration by using a standard curve obtained with a fucose concentration ranging from 2 µg/ml to 100 µg/ml.

SCV reversion rate and auxotrophy to hemin

To determine the reversion frequency of SCV to the ancestral phenotype, single colonies grown on LB agar plates were resuspended in PBS the appropriate dilution was plated on LB agar plates and incubated at 37^{o}C. After 48 h small and large colonies were counted (Roggenkamp et al. 1998). To test for the auxotrophy to hemin, individual SCV colonies were isolated, resuspended in PBS and plated on M9 minimal medium agar plates containing 2% glucose with and without hemin 50 µg/ml (Sigma-Aldrich). After incubation at 37^{o}C for 48 h, CFUs were counted to estimate the ratio between the number of cells able to grow in presence and in absence of hemin.

MIC determination

Minimal inhibitory concentrations for aminoglycosides (gentamicin-10 µg/ml, kanamycin – 30 µg/ml, amikacine –30 µg/ml, tobramycin – 10 µg/ml, netilmycin – 10 µg/ml) and other type of antibiotics (nalidixic acid – 30 µg/ml, tetracycline - 30 µg/ml) were determined for the small and large colonies using antibiotic disc tests (LIOFILCHEM s.r.l., Italy) following manufacturer`s instructions.

Whole Genome re-sequencing and mutation prediction

Both the ancestral and 7 isolated MUC clones (M3s_d19 sampled from population M3 after 19 days of evolution with macrophages and M1s to M6s_d30 sampled from M1 to M6 pops after 30 days of evolution) were grown overnight in 10 ml of RPMI-Strep at 37ºC. DNA isolation from these cultures was done following a previously described protocol (Wilson 2001). The DNA library construction as well as the sequencing procedure was carried out by BGI. Each sample was pair-end sequenced on an Illumina

HiSeq 2000. Standard procedures produced data sets of Illumina paired-end 90bp read pairs with insert size (including read length) of ~470bp.

Mutations in the two genomes were identified using the BRESEQ pipeline (Barrick et al. 2009). To detect potential duplication events we used SSAHA2 (Ning et al. 2001) and the paired-end information to map reads only to their best-match on the genome. Sequence coverage along the genome was assessed with a 250 bp window and corrected for GC% composition by normalizing by the mean coverage of regions with the same GC%. We then looked for regions with high differences (>1.4) in coverage. We did not find any such difference between the ancestral and evolved clones. See Table 2.2 for the identity and precise location of mutations identified in the sequenced clones. All mutations were confirmed by direct target sequencing.

Mutation frequency estimation

The clone M3_D19 was tested for increased mutagenesis, due to increased transposition, in comparison with the ancestral strain. For that, fluctuation assays were performed and the number of bacteria resistant to D-cycloserine (resistance can occur due to mutations or transpositions into the *cycA* gene) and to rifampicin (to which resistant mutants typically carry point mutation in *rpoB*, without involving transposition) were assayed. D-cycloserine resistance assays were performed as previously described (Feher et al. 2006), with slight modifications. Briefly 50 tubes with 1 ml RPMI medium were inoculated with $5x10^4$ each which grew for 24 hours. 100 µl of different dilutions from each tube were then spread on M9 minimal medium (MM) agar plates supplemented with 0.2 % glucose and D-cycloserine at the final concentration of 0.04 mM. The frequency of mutagenesis was estimated as the number of mutants observed on D-cycloserine plates, over the total number of cells (measured by Flow

cytometry). Clones were tested for increase in spontaneous single point mutation rate by plating 100 µl of an overnight culture of both ancestral and MUC_M3_D19 in LB supplemented with rifampicin at the end-concentration of 100 µg/mL.

EPS quantification

To quantify the total amount of exopolysaccharides (EPS), bacterial cultures were grown for 24 hours in 1 ml RPMI media, heat-inactivated at 100 ^{o}C for 15 min, centrifuged at 14000 rpm for 15 min and the supernatant collected for colorimetric assay to measure the binding of Congo red (adapted from Black and Yang (Black and Yang 2004)). A total of 450 µl supernatant was mixed with 50 µl of a dye (stock solution 150 µg/ml in deionized distilled water). All samples were vortexed briefly and incubated in the dark at room temperature for 30 min. The absorbances of the supernatants were measured at 490 nm in triplicate. Relative amount of EPS was estimated per bacterial cell by calculating CFUs by Flow Cytometry.

Detection of mutations

In order to determine the frequency of the mutations in clones sampled along the experiment, DNA was amplified by PCR (to identify IS insertions) and sequencing PCR was performed (to identify SNPs). DNA was amplified by PCR in a total volume of 50 µl containing 1 µl bacterial culture, 10 µM of each primer, 200 µM dNTPs, 0.5 U *Taq* polymerase and 1× *Taq* polymerase buffer. The amplification profile was 15 min at 95°C, followed by 35 cycles at 94°C for 30 s, 60°C for 90 s, 72°C for 2 min with a final extension at 72°C for 10 min. All gene fragments were amplified using these conditions and oligonucleotide primers (Appendix A, Table A2). The same primers were used for sequencing straight from the PCR product.

LD50 – test for increase of pathogenesis

We maintained male C57/BL6 mice, aged 8-10 weeks (in house supplier, Instituto Gulbenkian de Ciência), on *ad libitum* food (RM3A(P); Special Diet Services, UK) and water, with a 12 hour light cycle, at 21 ºC. We initiated infections by intra-peritoneal inoculation of bacteria in 100 μl saline. Four groups of 5 mice per bacterial strain were injected with doses ranging from $7x10^5$ to $7x10^7$. The inoculum for ancestral bacteria consisted of a single clone and the inoculum for the mucoids consisted of a mixture of equal numbers of the 6 sequenced clones from day 30 (M1s-M6s; see Figure 2.3). Furthermore, as a control, we injected a group of 3 mice with only 100 μl of saline (these did not display any signs of disease). We monitored mice for a period of 10 days (twice a day for the first two days and daily for the remainder 8 days). Upon death (or at the end of the experiment for survivors), we obtained blood from each animal by hearth-puncture, performed peritoneal lavages and plated bacteria from both samples in LB media with streptomycin (100μg/ml). We analyzed the survival data by fitting a binomial generalized linear model for each morphotype, using survival as a response variable and $\log_{10}$ bacterial dose as explanatory variable and estimated LD_{50} following KERR AND MEADOR 1996 (Kerr and Meador 1996).

Growth parameters of Ancestral-looking bacteria

We tested for changes in growth of 36 clones exhibiting an ancestral morphotype (4 replicates/clone) compared with the original ancestral clone (20 replicates). Growth rate measurements were obtained by following OD_{600nm} using a Bioscreen C system (Oy Growth Curves Ab).

Cultures were done by seeding 150 μl RPMI MOI media with 10^4 bacteria, covered with oil, and low speed agitation (5s every 30min).

Mathematical model of clonal interference

We assume a simple model for the interaction between bacteria and macrophages: $\frac{dB}{dt} = B\left(r - \frac{B}{K} - a_m M_0 e^{-\delta t}\right)$, where B represents the number of bacteria along time, r their growth rate, K their carrying capacity and δ the death rate of macrophages. For the experimental conditions used $M_0=10^6$ and $B(0)=10^6$. During a 24 hour period the infection dynamics expected under this model are similar to those obtained experimentally, with r=2.3 (per hour), $K=10^8$ and δ=0.1 (per hour) (Appendix A, Figure A2). As in the experiments, every 24 hours bacterial population numbers are reduced to $B(0)$ and macrophage numbers increased to M_0. Having these initial conditions, we then assume that new mutants arise and are not stochastically lost at a given rate. More specifically, the ancestral clone can mutate to two new types of adapted clones: one by transposition (upstream of *yrfF*) and another by point mutation. These mutations can cause changes in both r and a_m, i.e., the growth rate of mutant bacteria (r_m) can change and their ability to interact with macrophages (a_{mm}) can also change. Given the experimental evidence in Figure 2.2 and in Appendix A, Figure A5, we assume that mucoid bacteria will exhibit an increased ability to escape macrophages $a_{mm} < a_m$ but also a decreased growth rate $r_m < r$, due to the cost of producing exopolysaccarides. The following equations determine the evolution of a population where 3 new haplotypes can successfully emerge:

$$\frac{dB}{dt} = B\left(r - \frac{(B+M+B'+M')}{K} - a_m M_0 e^{-\delta t}\right) - UB - U_{is}B$$

$$\frac{dM}{dt} = M\left(r_m - \frac{(B+M+B'+M')}{K} - a_{mm} M_0 e^{-\delta t}\right) - U_{is'}M + U_{is}B$$

$$\frac{dB'}{dt} = B\left(r' - \frac{(B+M+B'+M')}{K} - a_{mb} M_0 e^{-\delta t}\right) + UB$$

$$\frac{dM'}{dt} = M'\left(r'_m - \frac{(B+M+B'+M')}{K} - a_{mm'} M_0 e^{-\delta t}\right) + U_{is'}M$$

where U and U_{is} are the rates of occurrence of successful beneficial SNPs and transpositions, respectively. Note that U is the spontaneous rate of mutation times the probability that such mutation is not lost by drift. B represents the number of ancestral genotype, M of the first mucoid genotype that emerges, B' of a derived genotype exhibiting a non-mucoid phenotype and M' of a derived mucoid genotype. The solutions of these equations for a given set of parameters were obtained using Mathematica 8.0 and are given in Figure 2.5.

Statistical analysis

The statistical analysis was performed using the R software: http://www.r-project.org/.

Results

Emergence of morphological diversity during the evolution experiment

We followed the evolution of six *E. coli* populations (all founded from the same ancestral clone), when adapting to the antagonistic interaction with MΦs. The bacterial populations (M1 to M6) evolved in RPMI medium with MΦs and were propagated at a multiplicity of infection (MOI) of 1:1 (10^6 *E. coli* to 10^6 MΦs). In parallel we also evolved *E. coli* in the absence of MΦs. All populations were evolved for a period of 30 days, which corresponds to approximately 450 generations, given the increase in CFUs observed during a 24 hour infection.

Adaptation of the bacterial lines to the presence of MΦs was characterized by the emergence of phenotypic variation within populations. After 4 days of evolution, corresponding to approximately 60 generations only, diversity started to be observed in the populations. This diversity was detected in all populations, by the appearance of distinct colony morphologies, when plating on LB plates (Figure 2.1A). Such morphological diversity was never observed in control populations that were evolved in the absence of MΦs for 30 days (n=6). Two distinct heritable morphs could be clearly identified and scored: small colony variants (SCV) and large translucid mucoid colonies (MUC).

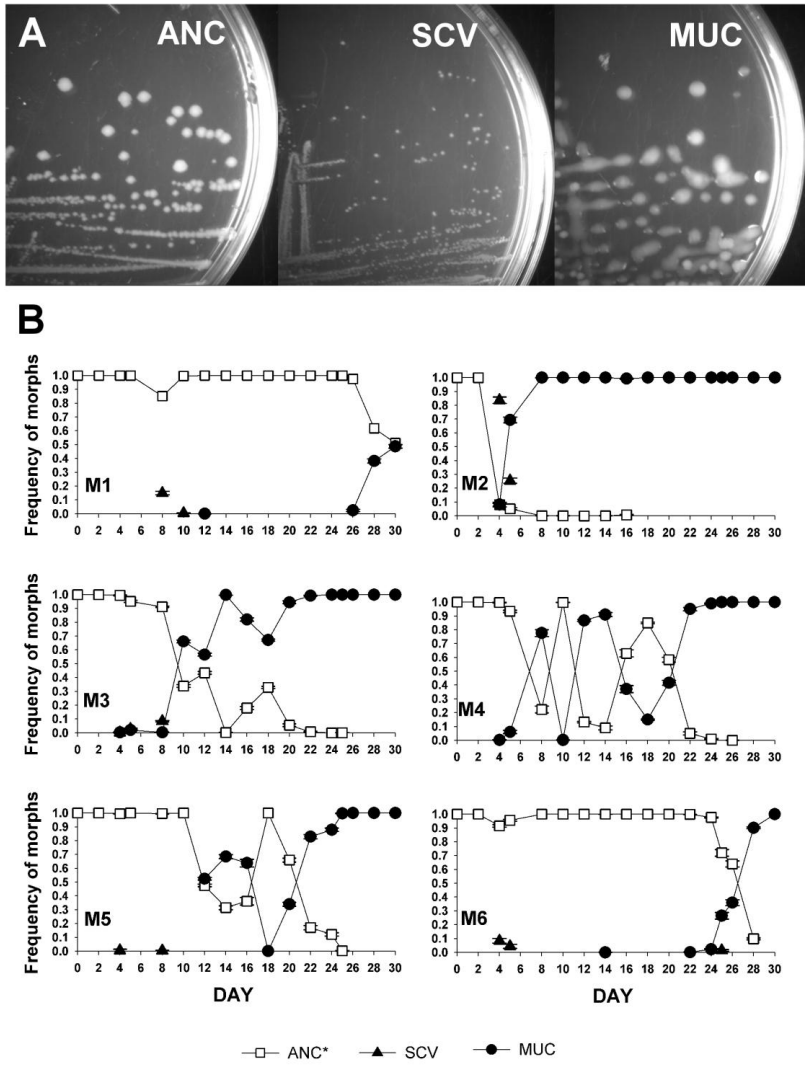

Figure 2.1. Emergency of morphological diversity in the bacterial populations adapting to MΦs. (A) Examples of the variability for colony morphology that emerged in *E. coli* populations adapting to MΦs, from the left to the right – ANC stands for morphology of ancestral, SCV for the small colony

variants morphology and MUC for mucoid colony morphologies. (B) Dynamics of frequency change of the evolved morphs in each replicate evolution experiment M1 to M6 populations: white squares indicate ANC, black triangles SCV, black circles MUC morphs.

We followed the frequency of these morphs over time (Figure 2.1B). While SCVs could be observed in five out of six populations, this morph remained at low frequency and was detected transiently. The observed parallelism for the emergence of SCVs, indicates that this phenotype constitutes a possible initial adaptive strategy in the interaction of *E. coli* with MΦs. On the other hand, the MUC rose in frequency in all populations, reaching fixation in 5/6 populations by day 30. These results show that morphological diversity in *E. coli* can emerge very rapidly as a result of their adaptation to MΦs.

Dynamics of phenotypes and phenotypic characterization of SCV and MUC morphs

The changes in frequency of each of the emerging morphs (SCVs and MUCs) showed a complex dynamics (Figure 2.1B). In the populations where both morphs were detected, whenever one morph (which gives rise to SCVs) decreased in frequency the other morph (MUC) tended to increase (see population M2 and M3 where the emergence of SCV is followed by the emergence of MUC). This suggests that MUC morphs are able to outcompete SCV morphs, thus having a larger fitness advantage.

SCVs emerged in at least 80% of the independent evolving populations, though their frequency was always low. This strongly suggests that these morphs have a selective advantage but can be outcompeted. To better understand the selective advantages of the SCVs we performed two assays: 1) an infection with SCVs to test for possible change in their abundance inside and outside MΦs, relative to that observed in the

ancestral strain; 2) a competitive fitness assay to test for SCV ability to outcompete the ancestral strain, in the presence of MΦs. While we did not observed any difference in SCV relative abundance, either intracellularly (Rr= 0.99+0.16 (2SE)) or extracellularly (Rr= 1.01+0.13 (2SE)), an advantage was found in the competitive fitness assay (Figure 2.2A). Interestingly, SCV clones SCV_M1_D8 and SCV_M3_D5, exhibited an advantage relative to the ancestral inside MΦs in the early phase of infection. However, after 2 hours of infection, the SCV tested showed a disadvantage in relation to the ancestral outside MΦs (Figure 2.2A). This may explain why these SCV clones increased in frequency in the populations but never reached fixation (see Figure 2.1B).

We tested SCVs for resistance to different antibiotics, catalase activity and their ability to use hemin. These are common properties of SCV clones sampled in natural populations (Proctor et al. 2006b). Indeed, the evolved *E. coli* SCV clones showed an increased resistance to aminoglycosides, but not to other antibiotics (see Table 2.1), and were catalase negative, just as found for clinical isolates of SCVs in several bacterial species (Proctor et al. 2006a). Furthermore, SCVs showed a remarkable plasticity because in rich medium they reverted to a phenotype of large colony forming bacteria at a frequency of $9x10^{-4}$ ($2SE=4x10^{-4}$), a property similar to that of clinical isolated SCVs (Roggenkamp et al. 1998). Supplementation with hemin enhanced growth of the SCVs tested when compared to the ANC clones (SCV_M1_D8: 2.9 ± 1 (2SE) and SCV_M3_D5: 2.5 ± 0.7(2SE)). In a clinical setting it is hard to define the selective pressures that lead to the acquisition of such pathogenic traits. But in our experimental system the selection pressure is readily identified since only in the presence of MΦs were these traits observed.

Table 2.1. Increased resistance of SCVs to aminoglycoside antibiotics.

	Mean zone diameter (mm)						
Antibiotic (µg)	**ANC**	**SCV1**	**SCV2**	**SCV3**	**MUC2**	**MUC3**	**MUC4**
Kanamycin (30)	24S	11R	12R	10R	23S	20S	21S
Gentamicin (10)	20S	10R	10R	10R	16S	16S	18S
Amikacine (30)	24S	12R	11R	11R	20S	17S	18S
Tobramycin (10)	18S	11R	10R	10R	17S	17S	17S
Netilmycin (10)	24S	16S	15S	15S	19S	17S	21S
Tetracycline (30)	29S	33S	31S	32S	30S	33S	32S
Nalidixic acid (30)	27S	34S	32S	33S	34S	34S	36S

Minimal inhibitory concentration (MIC) of each clone was measured in triplicate by a disc diffusion assay. S indicates sensitive clones and R resistant clones. SCV_M1_D8, SCV_M2_D4, SCV_M3_D5 and MUC_M2_D19, MUC_M3_D19, MUC_M4_D19 clones are shown.

To better understand the selective advantages of the MUCs, we tested if they exhibited an increased ability to escape MΦ engulfment. Figure 2.2B shows the abundance of MUC inside and outside MΦs, relative to that observed for the ancestral clones, after 3 hours of infection. The figure clearly shows that the abundance of all MUC clones inside MΦs is much smaller than that of the ancestral strain from which the MUCs evolved. Moreover, the abundance of 4/6 MUC clones is higher outside MΦs. To further complement this finding we performed 1:1 population mixture of evolved MUC (MUC_M3_D19 that is marked with CFP) and ancestral bacteria (marked with YFP) and checked for intracellular bacteria after infection using confocal microscopy. As shown in Figure 2.2C, only ANC clones and not MUC clones, are detected inside MΦs. Taken together, these results strongly suggest that MUC clones are better

adapted to escape MΦs. MUCs also have a visible overproduction of exopolysaccharides when plated on LB plates. Since colanic acid is present in most natural *E. coli* isolates (Majdalani and Gottesman 2005), and this capsule is made in mutants of *E. coli* that emerge under stress conditions (Gottesman and Stout 1991), we tested mucoid clones for overproduction of this exopolysaccharide. We determined that mucoid clones show overproduction of colanic acid (see Appendix A, Figure A1). Our results clearly show that rapid evolution to change this trait can occur under the specific selection pressure imposed by MΦs. Contrary to what was found for SCVs, mucoid clones did not show increased resistant to antibiotics (Table 2.1).

Genetic basis of the adaptation to macrophages

Given the phenotypes of the MUC clones and their dynamics, we sought to determine the beneficial mutations responsible for their success along the evolutionary process. Whole genome sequencing (WGS) of 7 clones was performed, six clones isolated from each population at the last time point (day 30) of the experiment (M1s, M2s, M3s, M4s, M5s, and M6s) and a clone sampled from M3 population at day 19 (MUC_M3_D19). WGS of this clone revealed that it carries two transposon insertions: an insertion of IS186 into the promoter region of *lon* and an IS1 insertion upstream of *yrfF* (see Table 2.2). Strikingly, this last transposition event occurred in all other sequenced clones (Table 2.2, Figure 2.3A), which shows parallelism at the genetic level across all independently evolved lines.

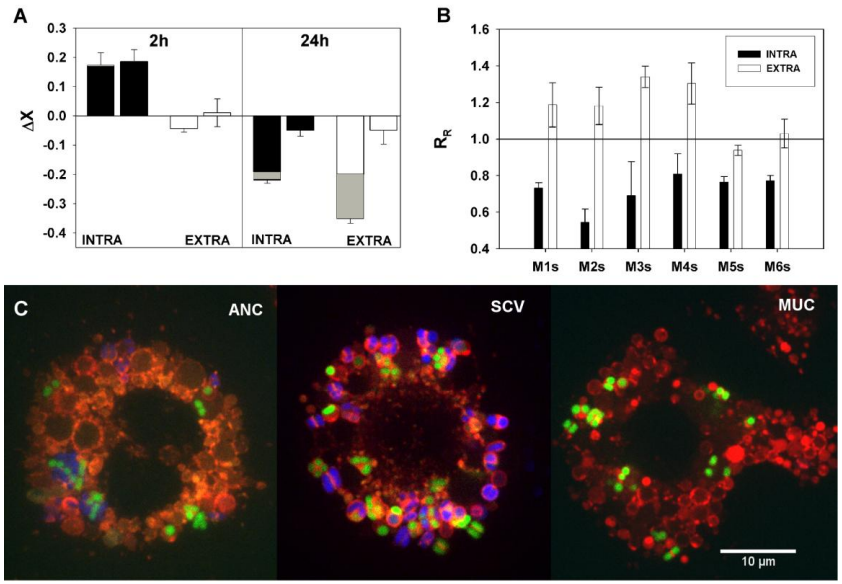

Figure 2.2. Phenotypic characterization of SCV and MUC clones. (A) Competitive fitness of SCV clones in presence of MΦs. The change in frequency (ΔX) of the evolved bacteria against the ancestral in the intracellular (black bars) and extracellular (white bars) niche of the MΦs at MOI (1:1). Clones are ranked in the following order: SCV_M1_D8 and SCV_M3_D5. Because SCV clones revert to ancestral looking colonies, frequencies of those phenotypic revertant SCV_REV colonies are shown in grey. (B) Evidence that clones ad
phagocytosis. Rr represents the relative abundance (R_r) of evolved clones to that of the ancestral. Clones M1s to M6s were sampled from each independent evolution. In black bars the relative abundance inside MΦs and in white bars outside MΦs. All evolved clones show a smaller abundance inside MΦs, suggesting that these are better adapted to escape MΦs phagocytosis. (C) Confocal microscopy images taken at 2 hours after infection of MΦs at MOI 10:1 (10 *E. coli* to 1MΦ) with a 1 to 1 mixture of *E. coli* expressing CFP (shown in blue) or YFP (shown in green). From left to right: ANC clones (CFP and YFP); SCV (CFP) and ancestral (YFP); MUC (CFP) and ancestral (YFP). MΦs are labeled with LysoTrackerRed (LTR).

The function of *yrfF* is unknown in *E. coli* but its homologue in Salmonella, *igaA,* is known to prevent over-activation of the Rcs regulatory system, which regulates colanic acid capsule synthesis (Dominguez-Bernal et al. 2004). It is therefore likely that the insertion upstream of *yrfF* alters *E. coli* ability to produce colanic acid (Appendix A, Figure A1).

Other important parallelisms were detected involving two transposition events: one in the coding region of *yiaW* and the other in the coding region of the *pot* operon. *potD* is one of the four genes of the potABCD operon, a spermidine-preferential uptake system (Igarashi and Kashiwagi 2010). All four genes are essential for spermidine uptake, so the insertions in *potD* detected in clones MUC_M4_D30 and MUC_M6_D30, or the insertion in *potA* observed in clone MUC_M3_D30, are likely to result in inability to uptake spermidine. Interestingly, during adaptation to MΦs, insertion in *yiaW* (whose function is unknown) was rapidly followed by insertion in *potA* or *potD* genes (see Figure 2.3B, M3, M4 and M6 populations), indicating a potential interaction between these genes. The parallelism observed suggests that insertions in *yiaW* are an important adaptation of *E. coli* to MΦs.

Table 2.2. Mutations acquired by evolved clones identified through whole genome re-sequencing (WGS).

Clone	Genome Position	Gene	Mutation	Annotation
MUC_M3_D19	360771	*clpX/lon*	intergenic (+88/-100)	IS186 +12 bp
	3411601	*nudE/yrfF*	intergenic (-273/-47)	IS1 +10
M1s	2029672	*yegH*	G→T	A422S GCC→TCC
	3356932	*fusA*	A→C	S588A TCC→GCC
	3411601	*nudE/yrfF*	intergenic (-273/-47)	IS1 +10
M2s	3356932	*fusA*	A→C	S588A TCC→GCC
	3411605	*nudE/yrfF*	intergenic (-277/-43)	IS1 +6
M3s	459734	*folD/sfmA*	G→T	intergenic (-10/-461)
	1088154	*potA*	coding (589/1137 nt)	IS1 +10
	3411601	*nudE/yrfF*	intergenic (-273/-47)	IS1 +10
	3640515	*yiaW*	coding (263/324 nt)	IS1 +9
	3922002	*trkH*	T→A	L389Q CTG→CAG
M4s	1084946	*potD*	coding (1032/1047 nt)	IS1 +9
	3411601	*nudE/yrfF*	intergenic (-273/-47)	IS1 +10
	3640515	*yiaW*	coding (263/324 nt)	IS1 +9
M5s	1480525	*ydeS/hipA*	intergenic (-1603/+205)	Δ208 bp
	2024227	*wzc*	G→T	P645T CCG→ACG
	3411601	*nudE/yrfF*	intergenic (-273/-47)	IS1 +10
M6s	1084946	*potD*	coding (1032/1047 nt)	IS1 +9
	3411601	*nudE/yrfF*	intergenic (-273/-47)	IS1 +10
	3640515	*yiaW*	coding (263/324 nt)	IS1 +9

Mutations in intergenic regions have the two flanking genes listed (e.g., *clpX*/*lon*). SNPs are represented by an arrow between the ancestral and the evolved nucleotide. Whenever a SNP gives rise to a non-synonymous mutation the amino acid replacement is also indicated. The symbol Δ means a deletion. For intergenic mutations, the numbers in the Mutation row represent nucleotides relative to each of the neighboring genes, here + indicates the distance downstream of the stop codon of a gene and - indicates the distance upstream of the gene, that is relative to the start codon. Insertions of IS elements are denoted by the specific IS element followed by the number of repeated bases caused by its insertion.

Besides IS insertions, parallelism at the level of point mutations was also observed. Two clones had the same SNP in *fusA*, a gene coding for elongation factor G (EFG). EFG catalyzes the elongation and recycling phases of translation (Savelsbergh et al. 2009). It is known that mutations in *fusA* reduce the rate of protein synthesis and thus may have pleiotropic effects on bacterial physiology (Andersson and Hughes 2010).

Dynamics of haplotypes along the adaptive walk and evidence for clonal interference

To understand the dynamics of adaptation in each independent evolved line, we sought to determine the frequency of the mutations found (see Appendix A, Table A1), in clones sampled at different time points. Figure 2.3B shows the frequencies of the haplotypes detected along the evolution experiment. Figure 2.3 clearly shows that adaptation involved the competition between distinct haplotypes and the successive accumulation of beneficial mutations, mainly caused by IS insertions. Such haplotype dynamics is characteristic of clonal interference (Sniegowski and Gerrish 2010), where clones carrying distinct beneficial mutations compete for fixation.

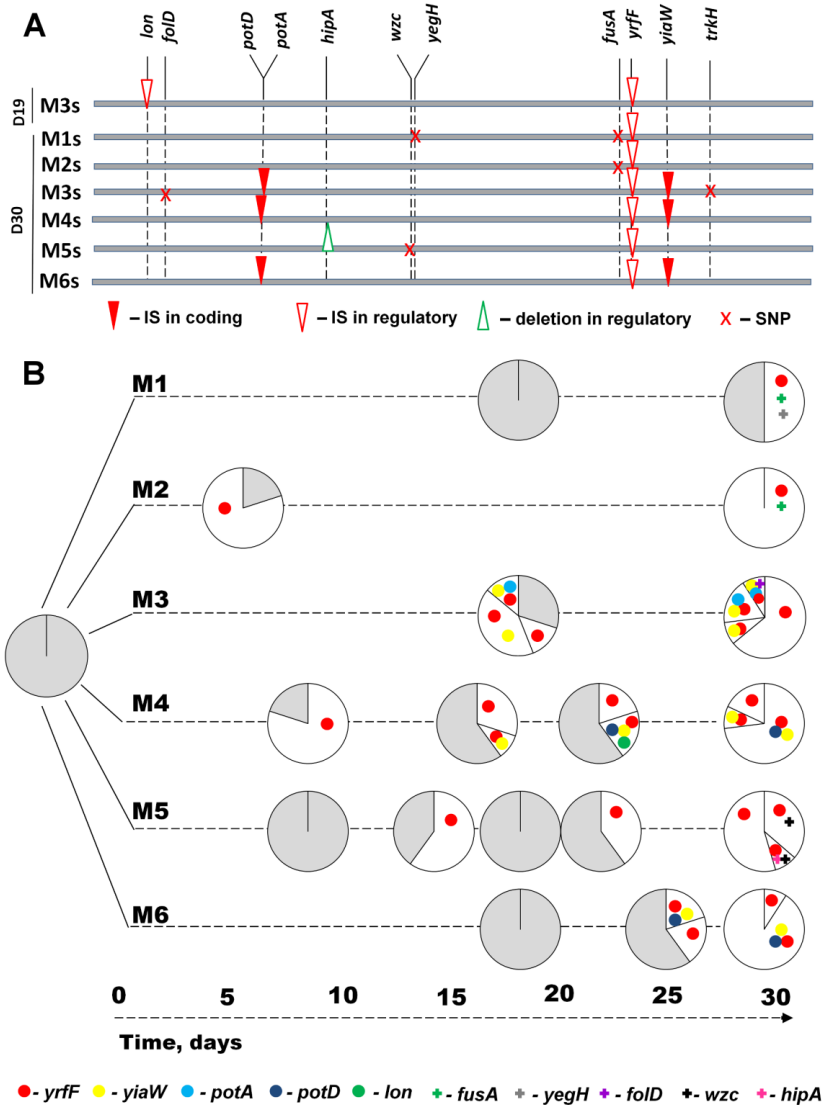

Figure 2.3. Genetic characterization of adaptive mutations and the dynamics of their appearance. (A) Mutations identified in M1s to M6s clones isolated from M1 to M6 populations (evolved for 450 generations), represented along *E. coli* chromosome. For simplicity, the genomes are represented linearly and horizontally drawn. The types of mutations are represented in the following way: SNPs are shown as crosses, IS insertions as inverted triangles and deletions as triangles. Filled symbols represent mutation in the coding region of the gene and empty symbols in the regulatory region. (B) Emergence and spread of adaptive mutations in M1 to M6 populations. Dynamics of haplotype frequencies in evolving populations at different days of evolution experiment are represented by circles. The color and symbol (IS insertions are represented as circles and other mutations as crosses) of each sector represents different haplotypes and the area of the circle their frequency in the population. Grey area represents the frequency of clones in the population that were typed for existing mutations in the population and did not differ from ancestral haplotype.

Indeed we can model this process, within the basic ecological scenario of our experiment, and ask if clonal interference alone is sufficient to reproduce the dynamics observed in Figure 2.1. In this model of clonal interference we make the simplifying assumption that two traits, *r* and *am*, the growth rate of bacteria in RPMI and their ability to escape MΦs, respectively, are the most important for bacterial fitness in this environment. Both these traits can evolve, as it is evident from the emergence of new morphologies (in particular mucoid morphs) and phenotypic tests of the evolved clones (see Methods, Figure 2.2, Figure 2.4 and 2.5).

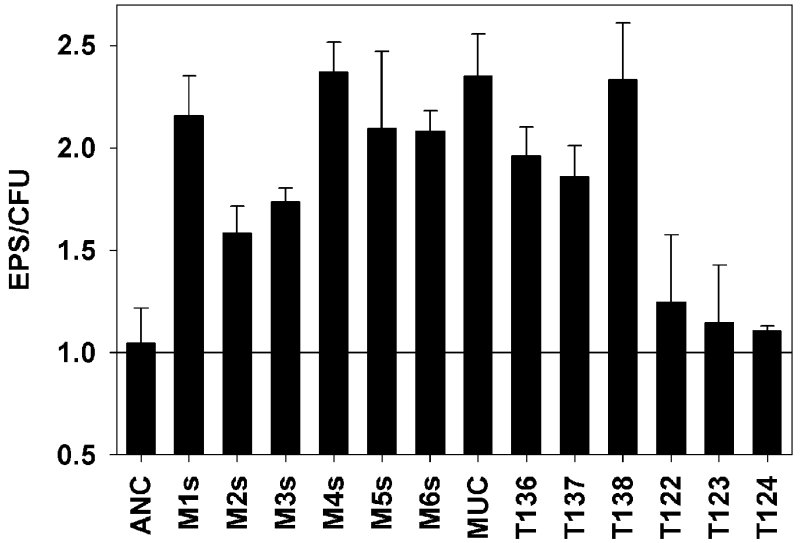

Figure 2.4. Variation in exopolysaccharide production among evolved clones. Amount of EPS per bacterial cell was measured for the ancestral strain (ANC) and six mucoid clones that evolved independently (M1s to M6s): we also measured the amount of EPS in MUC_M3_D19 (MUC) and in six other clones derived from this clone after a growth in RPMI (T136-T138, T122-T124). None of these derived clones have the IS186 insertion in *lon* promoter region and all have the IS1 insertion upstream of *yrfF*. T136-T138 are visibly mucoid and T122-T124 show a non-mucoid colony morphology. All measurements were done in triplicate.

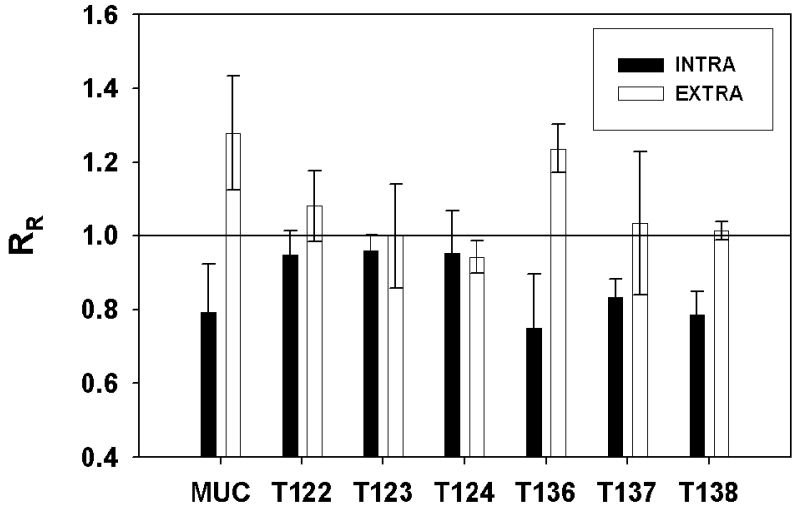

Figure 2.5. Relative abundance of evolved clones in the presence of the MΦs. Relative abundance (R_r) of MUC_M3_D19 (MUC) clone and six clones derived from this clone (T122-T124 non mucoid and T136-T138 mucoid clones) are represented either inside MΦs (black bars) or outside MΦs (white bars).

Figure 2.6 shows the outcome of simulated evolution experiments of the frequency of mucoid morphs under different parameter values. These parameters were chosen because they are able to reproduce the initial infection dynamics of the ancestral strain (Appendix A, Figure A2) and its relation with the derived evolved clones (see Methods). We are able to find conditions that can reproduce the observed dynamics of morphs under a scenario of intense clonal interference and accumulation of multiple beneficial mutations (see Appendix A, Figure A3). The model is able to reproduce the observed changes in frequency of the mucoid and non-mucoid phenotypes, if we assume that distinct beneficial mutations may

occur which will change each of the two fitness traits in both morphs. Specifically, we assume that a successful beneficial mutation occurs, which produces the first mucoid morph (the mutation upstream of *yrfF*). Such mutation increases the ability of bacteria to escape MΦs but also carries a cost in that it diminishes the growth rate (see Appendix A, Figure A4). This mucoid morph can acquire further beneficial mutations, which can alter those traits values, such that a new derived mucoid haplotype can have a reduced cost of producing colanic acid and/or an increased ability to escape MΦs. Importantly, we also assume that non-mucoid clones can acquire beneficial mutations which increase their growth rate. Experimental support for this assumption is provided in the supplement (Appendix A, Figure A5) Under these assumptions, and with the direct evidence that several distinct clones are segregating in the populations, complex dynamics are to be expected (Figure 2.3 and Appendix A, Figure A3).

It is important to note that we cannot exclude the possible occurrence of other forms of selection during the evolution experiment. Indeed it is known that even in simple abiotic environments adaptive diversification, involving frequency dependent selection can repeatedly evolve. It is generally difficult to distinguish this form of selection from simple clonal interference (Herron and Doebeli 2013). However if strong negative frequency dependent selection would have occurred in our lines, it would have led to maintenance of distinct lineages along the evolutionary process. Such expectation seems inconsistent with the fixation of the IS insertion upstream of *yrfF* and the fixation of the mucoid phenotype in majority of the lines that we have observed.

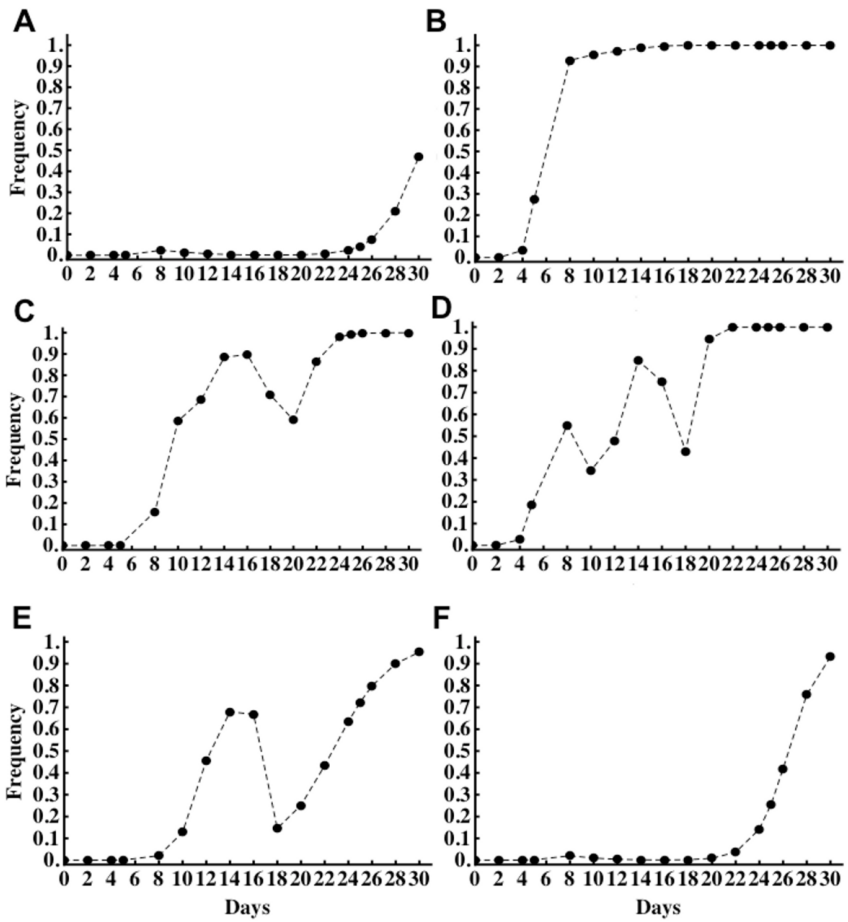

Figure 2.6. Model of clonal interference during the evolution process. Simulations of the adaptive dynamics over the period of the experiment (30 days). The frequencies of mucoid phenotypes are plotted and can be compared to those observed in the experiments (Figure 2.1). The dynamics were obtained by the model of clonal interference described in the methods. The values of parameters used and the dynamics of haplotypes that compete for fixation are shown in Appendix A, Figure A5.

Emergence of a transient mutator with increased transposition rate

An important parallelism was revealed when genotyping clones for determining polymorphism levels. This involved an IS186 insertion into the promoter region of *lon*. Lon (Long Form Filament) is a heat shock protease responsible for degradation of defective proteins in the cell. The promoter region of *lon* has been described as having a single site that is a natural hotspot for IS186 insertions (saiSree et al. 2001). The selection of independently evolved clones carrying an IS element at this site argues for it causing a beneficial effect and the locus being a insertion hotspot suggests that it could rapidly emerge. As *lon* mutants tend to overproduce colanic acid (Torres-Cabassa and Gottesman 1987), a trait that appears to be strongly selected for in our experiment, it is possible that this was the main beneficial effect caused by that insertion. However the IS186 insertion could only be detected in two populations (M3 and M4) at intermediate time points in the experiment. It was no longer detected at day 30 (see Figure 2.3B). Interestingly, *lon* has been reported to be a mutator gene in mutants that bear an IS186 insertion in its promoter, thus increasing the rate of IS transpositions 10- to 100-fold (Nicoloff et al. 2007). This happens because the stability of several transposases is dependent on the Lon protease (Derbyshire et al. 1990, Rouquette et al. 2004), which seems to regulate their transposition activity.

In MUC_M3_D19 an IS186 inserted in -10 promoter region and since mutations in this position were shown to significantly decrease level of *lon* transcription (Chin et al. 1988), it is likely that this clone is a mutator where a burst of transposition events could have occurred during the adaptation. To test for increased mutagenesis in this clone, a fluctuation test was performed to determine number of D-cycloserine resistant mutants, a phenotype caused by a knock out of *cycA*, which can be caused

by IS insertions (Trindade et al. 2012). Interestingly we found a significant increase in the frequency of *cycA* mutant clones in the MUC_M3_D19 clone relative to the ANC clone (median frequency $2.6x10^{-6}$ vs. $1x10^{-7}$, for the ancestral background, $P=5.5x10^{-13}$, W=203.5, Mann-Whitney U test, Figure 2.7).

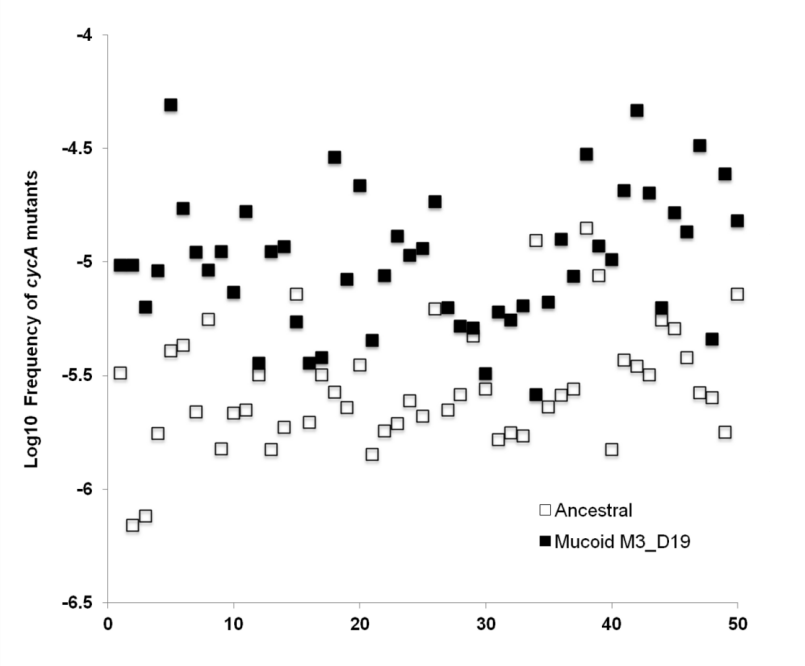

Figure 2.7. Fluctuation test of evolved MUC clone (MUC_M3_D19) and ANC clone to test for increase in mutagenesis. Black squares (for MUC clone) and white squares (for ANC clone) each represent 50 independent measurements of the frequency of spontaneous mutants resistant to d-cycloserine. Mutation rates were significantly different between MUC and ANC clones (median MUC=2.6x10-6 and ANC=1x10-7, P=5.5x10-13, W=203.5, Mann-Whitney U test).

Consistent with this increased mutagenesis being driven mainly by IS insertions we did not observe significant differences in the frequency of rifampicin resistant clones, which are caused by point mutations (median frequency $3.3x10^{-7}$ vs $6.9x10^{-7}$ for the ancestral background, P=0.1, W=21, Mann-Whitney U test). The presence of IS186 in the *lon* promoter region was also found to be highly unstable since a spontaneously derived non-mucoid clone from MUC_M3_D19 (MUC_M3_D19_REV) shows a precise excision of this element while maintaining the IS1 insertion in regulatory region of *yrfF* (see Table 2.2). These results indicate that this IS186 insertion visibly enhances mucoidy levels, increases mutagenesis and is also very unstable in this genetic background (see also Figure 2.4). The later may explain why it did not fix in the population. Recently, it has been shown that, in Salmonella, the transcription of *igaA* is regulated by *lon* (Garcia-Calderon et al. 2009). The dynamics of the IS186 insertion in populations M3 and M4 suggest that this mutation was beneficial in the background with an IS1 insertion upstream of *igaA* is present.

Clones evolved to escape MΦs have increased pathogenicity

Translocation of commensal *E. coli* from the gut can be associated with severe health complications (e.g. sepsis), particularly in immunosuppressed hosts or after surgery (MacFie et al. 1999, Russo and Johnson 2003). Bacteria which reach the mesenteric lymph nodes or the peritoneal cavity (extensively populated by MΦs) and that are able to escape destruction by MΦs should have a fitness advantage and potentially cause more severe disease. As the evolved MUC clones can better escape MΦs, we tested whether this adaptation leads to increased pathogenesis. To do so, we infected mice via the intra-peritoneal route (simulating peritonitis and sepsis) with different doses of ANC and MUC bacteria and measured survival. As can be seen in Table 2.3, the lethal

dose 50 (LD_{50}) is, on average, one log lower for the MUC than for the ANC bacteria. Moreover, we plated samples of blood and peritoneal contents for all animals and observed that animals surviving to the end of the experiment had no bacteria in their blood and 41% had low doses of bacteria in the peritoneal cavity (<250 CFUs/ml). On the contrary, animals failing to survive infection had high bacterial densities, both in the blood (range: $7x10^5$ – $9x10^8$/ml) and in the peritoneal cavity ($3.4x10^5$ – $2x10^8$/ml; Appendix A, Figure A6). Thus, our results show that the mucoidy due to overproduction of colanic acid, evolved during the interaction with MΦs, leads to increased pathogenicity.

Table 2.3. LD_{50} estimates for the ANC and the MUC (evolved) clones. Estimates were obtained following KERR (see methods).

Morphotype	log_{10} LD50 (± std. error)
Ancestral	7.51 (7.20 – 7.83)
Mucoid	6.69 (6.55 – 6.84)

Discussion

In principle bacterial evolution towards becoming more pathogenic may occur through the acquisition of new genes – a gain of function mechanism- or modification of their current genomes, including loss of genes - change-of-function mechanism (Sokurenko et al. 1999). The later constitutes a pathoadaptation. Pathoadaptive mutations are therefore mutations that enhance bacterial virulence without horizontal transfer of specific virulence genes. For example the knock out of *hemB* in *Staphylococcus aureus*, increases its ability to persist intracellularly (von

Eiff et al. 1997) and the loss of *mucA*, increases *Pseudomonas aeruginosa* ability to evade phagocytosis and resist to pulmonary clearance (Yu et al. 1998).

We have followed the evolution of a commensal strain of *E. coli* under the selective pressure imposed by MΦ phagocytosis, to determine the rate of adaptive evolution and to uncover the nature of possible pathoadaptive mutations in *E. coli*. From the infection dynamics (Figure 2.1 and 2.2) we conclude that at least two different strategies, detected by different colony morphology changes, emerged during adaptation to the selective pressure imposed by MΦs: i) an intracellular strategy evolved by SCV clones early in the process; ii) an extracellular strategy evolved by MUC clones emerging later in the evolution. The intracellular is characterized by increased bacterial resistance, plasticity and survival in the early phase of interaction with MΦs, and was accompanied by a reduced growth in the extracellular medium. The extracellular is characterized by overproduction of colanic acid and increased resistance to escape MΦ phagocytosis, dominated in all populations.

The adaptive strategies emerged from commensal bacteria adapting to MΦs, occurred as fast as a few hundred generations and were characterized by traits reminiscent of those found in pathogenic bacteria. Indeed some clinical isolates sampled from patients suffering from recurrent and persistent infections, for example in the blood (Funada et al. 1978) or urinary tract (Roggenkamp et al. 1998, Tappe et al. 2006), are SCVs. The distinctive traits of this phenotype are: ability to form small colonies, to revert to larger colony forming bacteria at high frequencies and increased resistance to certain antibiotics. Mucoidy is also a property that can be frequently observed in certain infections, for example in *Pseudomonas aeruginosa* or *E. coli* (Govan and Deretic 1996, Bottone

2010). Our results suggest that these traits can rapidly emerge due to the selective pressure imposed by MΦs solely, which has implications for the treatment of bacterial infections. Indeed we found that the increase ability to escape MΦs of *in vitro* evolved clones lead to increased pathogenesis *in vivo*, when these were tested in a mouse model. We also found that this rapid pathoadaptative process was characterized by three main adaptive paths. Although distinct in the number and type of mutations, these paths share an initial mutation: a IS insertion in the same exact position upstream of *yrfF*, a gene which shares 84% sequence similarity at the protein level to IgaA of *Salmonella enterica* serovar Typhimurium. In *S. Typhimurium* it was shown that the stability and responsiveness of the RcsCDB system depends on IgaA (Mariscotti and Garcia-Del Portillo 2008). The RcsCDB system controls the production of colanic acid, virulence in diverse pathogens (Page et al. 2001, Ferrieres and Clarke 2003, Majdalani and Gottesman 2005, Vianney et al. 2005, Huang et al. 2006, Majdalani and Gottesman 2007), modulates responses to environmental changes and is activated upon exposure to antimicrobial peptides (Conter et al. 2002, Sailer et al. 2003, Kaldalu et al. 2004, Erickson and Detweiler 2006). IgaA represses the RcsCDB system (Cano et al. 2002) and mutations causing instability of IgaA activate the RcsCDB system, leading to overproduction of colanic acid capsule (mucoid phenotype) (Cano et al. 2002). Given the repressive function of IgaA on the RcsCDB, which controls many traits likely to be important for bacterial fitness, it is likely that the observed IS insertion upstream of *yrfF* to be an adaptive mutation with pleiotropic effects. If so the adaptive path may proceed through the occurrence of new mutations, which may compensate for the pleiotropic effects of that first adaptive step. Interestingly the same amino-acid substitution in *fusA* occurred in two independent lines. *FusA* is an elongation factor and is part of the str operon of *E. coli*, which has 3 other genes: *rpsL*, *rpsG* and *tufA*.

Since the strain that we studied carries a mutation in *rpsL* that confers streptomycin resistance, which is costly in RPMI yet increases survival inside MΦs (Miskinyte and Gordo 2013), it is possible that the SNP in *fusA* could be compensatory to cost of the *rpsL* mutation in the milieu outside MΦs.

One of the adaptive paths taken by *E. coli* involved insertions into the coding regions of *yiaW* and *potA* or *potD*. While the function of *yiaW* is unknown the later genes are involved in spermidine transport, which may affect *E. coli* interaction with MΦs. Spermidines are polyamines, polycationic molecules, which interact with nucleic acids and have been described as important in escape from phagolysosomes, biofilm formation and protection from oxidative and acidic stress amongst other traits important in bacterial pathogenesis (Shah and Swiatlo 2008).

The adaptive process was also marked by the occurrence of an important IS186 insertion into the promoter region of the Lon protease. Such insertion was not only likely adaptive (it was observed in two independent lineages and it increases mucoidy), but also likely leads to increased rates of transposition. Given that many of the adaptive mutations observed under the stresses imposed by MΦs were caused by ISs, the IS186 insertion may constitute an example of Barbara McClintock proposal that TE movement under stress could aid organisms to adapt to new environments (McClintock 1984).

Overall our results show that *E. coli* can adapt to better resist to MΦs within a few hundreds of generations and that clones with different morphologies and traits similar to those of pathogenic bacteria rapidly emerge.

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M.M participated in planning the experiments, obtained and analyzed results for Figures 2.1, 2.2, 2.3, 2.4, 2.5, 2.7 and Table 2.1, and participated in writing the manuscript. A.S analyzed whole-genome sequencing results (Table 2.2), mathematical model and simulations were mainly performed by J.S and I.G (Figure 2.6). R.R performed and analyzed LD50 test (Table 2.3). I.C and S.M participated in planning the experiment.

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Chapter 3

Presence of innate immunity accelerates bacterial adaptation

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Summary

Microbial populations constantly face a pressure imposed by other species in different environments, thus it is important to understand the rate and the mode of adaptation when living interactions are present. In particular, the rate of fitness increase and diversity are expected to increase in ever changing environments compared to environmental conditions that are static. Although recent studies emphasized the importance of biotic interactions in microbial experimental evolution, we are lacking studies addressing the pressure of the immune system in microbial evolution, a factor important in many bacterial isolates sampled from clinical specimens. It is not known how much such pressure can accelerate microbial adaptation. Understanding the rate and the mode of adaptation to the pressure imposed by the hosts' immune system unravels the adaptive potential of bacteria to both protect and kill their hosts. We use experimental evolution to show that in the presence of innate immune system cells – macrophages – the rate of adaptation of *Escherichia coli* populations is higher than in their absence. Furthermore, we show that clones adapted to the presence of the macrophages also bear a general advantage when facing predators, such as protozoa and phages, and cause diarrhea upon oral gavage of mice. These results show that the rate of fitness increase is higher in the presence of the macrophages then in their absence and adaptation to macrophages lead to increased fitness in the environments outside the host.

Introduction

The rate of adaptive evolution is a key issue in evolutionary biology and it is expected to vary between species and across environments (Gordo and Sousa 2001, Eyre-Walker 2006). In particular, the rate of fitness increase in a novel environment is expected to decrease with time when environmental conditions are static (Fisher 2000) but not in an ever changing environment (van Valen 1973). This latter scenario is expected to occur when strong biotic interactions exist superimposed to a constant abiotic environment. As Dobzhansky (1950) pointed out "*The greater the diversity of inhabitants in a territory, the more adaptive opportunities exist in it*" (Dobzhansky 1950). Adaptation can be studied in real time, in controlled environments imposing different selection pressures, through experimental evolution (Elena and Lenski 2003). Such studies have demonstrated that bacterial populations have an enormous potential to adapt even to relatively simple laboratory environments (Perfeito et al. 2007, Sniegowski and Gerrish 2010). Recently it has been demonstrated that the presence of coevolving species in the environment can increase

the rate of adaptation and diversification considerably (Paterson et al. 2010) and that strong biotic interactions can affect levels of adaptive radiation (Buckling and Rainey 2002, Meyer and Kassen 2007).

In the previous work we have described several adaptive routes that commensal *Escherichia coli* can evolve in the presence of a strong biotic interaction, the presence of innate immune system cells – MΦs. For the first time we have demonstrated that just in a few hundred generations *E. coli* can acquire such specific adaptations, as increased survival in the intracellular niche of the MΦs and ability to escape phagocytosis by MΦs (see Chapter 2).

Some of those strategies to escape innate immune system cells, could be adaptive when bacteria face their environmental predators in the nature, such as protozoa, nematodes, insects and mammals (Greub and Raoult 2004, Toft and Andersson 2010). Studies of the interactions between protozoa and bacteria have revealed that many different bacterial traits (including cell surface antigens, motility and quorum sensing) are under selective pressure due to predation (Matz and Kjelleberg 2005). It is thought that some of the adaptations that evolved as defense mechanisms against predators may be important in the adaptation of bacteria to the immune system. For example, when *Legionella pneumophila* is grown in one of its natural protozoan hosts, it becomes more invasive to both epithelial and macrophage cells (Cirillo et al. 1994). Predation has also been shown to select for high levels of bacterial genetic diversity in certain loci that are antigenic. For example, Salmonella bearing certain O-antigens are differentially discriminated by protozoa species residing in host intestines (Wildschutte et al. 2004). It is not yet known if adaptation to the immune system would also confer a selective advantage to bacteria facing

its predators in nature and which types of adaptive extracellular/intracellular strategies are common in those environments.

In this study we addressed hypotheses: (1) that the rate of adaptive evolution is higher in the presence of MΦs then in their absence, (2) the level of variation between populations is higher in the presence of MΦs and (3) that certain traits evolved in the presence of MΦ cells could lead to adaptation of *E.coli* when facing natural predators.

Materials and Methods

Ethics statement

All experiments involving animals were approved by the Institutional Ethics Committee at the Instituto Gulbenkian de Ciencia (project nr. A009/2010 with approval date 2010/10/15), following the Portuguese legislation (PORT 1005/92) which complies with the European Directive 86/609/EEC of the European Council.

Strains and media

The RAW 264.7 murine macrophage cell line was maintained in an atmosphere containing 5% CO_2 at 37^{o}C in RPMI 1640 (Gibco) supplemented with 2mM L-glutamine (Invitrogen), 1mM sodium pyruvate (Invitrogen), 10mM hepes (Invitrogen), 100U/ml penicillin/streptomycin (Gibco), 50µM 2-mercaptoethanol solution (Gibco), 50µg/ml gentamicin (Sigma), with 10% heat-inactivated FCS (standard RPMI complete medium). Before infection assays, MΦs and bacteria were maintained in the same conditions, but instead of penicillin/streptomycin and gentamicin, medium was supplemented with 100µg/ml Streptomycin antibiotic (RPMI-Strep medium).

Escherichia coli strains used were MC4100-YFP and MC4100-CFP (MC4100, galK::CFP/YFP, $Amp^R Strep^R$) which contain the yellow (*yfp*) and cyan (*cfp*) alleles of GFP integrated at the *galK* locus in MC4100 (*E.coli* Genetic Stock Center #6152) and differ only by YFP/CFP locus that is constitutively expressed (Hegreness et al. 2006). MC4100-CFP strain was used for the evolution experiment and MC4100-YFP as a reference strain for the fitness assays.

Evolution experiment

Twelve populations were founded from a single MC4100-CFP clone and were therefore genetically uniform in the beginning of the experiments. 6 populations were evolved in the presence of the MΦs (1M-6M) in RPMI and 6 populations (1C-6C) in RPMI medium in the absence of the MΦs as described previously (see Chapter 2).

Fitness measurements

To estimate competitive fitness of each population (C1-C6 and M1-M6), after 285 and 450 generations of evolution, samples from each evolved population were competed against MC4100-YFP reference strain in the same conditions as used in the evolution experiment. C1-C6 populations were competed in the RPMI medium and M1-M6 populations were competed in the RPMI medium in the presence of MΦs. Both evolved and reference strains were grown separately in RPMI-Strep, then diluted in PBS and inoculated simultaneously at a ratio of 1:1. The initial and final ratios of both strains were determined by Flow cytometry. Each evolved population's relative fitness was measured 3 times and the ancestral strain 10 times to test for neutrality of the marker. The fluorescence marker was neutral, as expected. The selection coefficient, a measure of relative fitness increase, was estimated as:

$$Scoeff = \frac{Ln\left[\left(\frac{Nf_b}{Nf_a}\right)\Big/\left(\frac{Ni_b}{Ni_a}\right)\right]}{Ln\left[Nf_a/Ni_a\right]}$$ (Maree et al. 2000)

Where S_{coeff} is a selection coefficient of the strain *b* against the reference strain *a*, Nf_a and Nf_b are the numbers of evolved (*b*) and reference (*a*) bacteria after competition and Ni_a and Ni_b are the initial numbers of evolved (*b*) and reference (*a*) bacteria, before the competition.

Phage resistance assay

Overnight cultures (0.1 ml) of bacteria grown in LB broth were inoculated in 5 ml of LB containing 0.2% glucose and 5 mM $CaCl_2$ and grown for 30 min. 100 µl of P1 (MC4100) phage lysate was added to the cells and then incubated at 37°C for 2h in a shaker. Each tube was centrifuged at 13,200 rpm for 2 min, and pellets resuspended in 0.1M pH 5.5 citrate buffer and plated on LB agar plates (Ho and Waldor 2007, Thomason et al. 2007). The same procedure was repeated for the control tubes without phage lysate.

Predation by *Tetrahymena*

A sexually mature population of *Tetrahymena thermophila* mating type II (Tetrahymena Stock Center, Cornell University, NY) was grown in SPP medium (1% proteose peptone, 0,1% yeast extract, 0,22% glucose and 0,003% of EDTA ferric sodium salt) with 1% of antibiotic/antimycotic solution (Invitrogen/Life Technologies) (Barchetta et al. 2008). After 48 hours at 30 ºC, *T. thermophila* culture, at a density of a 2.5×10^5 cells/mL (acessed by Coulter counter model Z2), was centrifuged at 2200 rpm for 5 min and resuspended in RPMI-Strep medium. The experiment was performed in 48-well plates at MOI 10:1 (10^6 bacteria to10^5 *Tetrahymena*). After 7 hours, 1% Triton-X was added for 20 min, the culture centrifuged at

9000 rpm for 5 min, washed in PBS and overall number of bacteria was counted by plating. The same procedure was repeated for bacteria without *T. thermophila*.

Mouse colonization experiments

The classical streptomycin-treated mouse colonization model was used to estimate the relative fitness of *E.coli* strains in their natural environment the gut (Moller et al. 2003, Armalyte et al. 2008). Briefly, 5- to 7-week-old C57BL/6 mice were given drinking water containing streptomycin sulfate (5g/l) for one week to eliminate resident facultative bacteria. Following 3 h of starvation for food and water, each mouse were gavaged with approximately 100 µl containing 10^8 CFU of suspended bacteria grown at 37°C in brain heart infusion (Difco) to early exponential phase (OD=2). After gavage, food and water was returned to the mice. Streptomycin-containing water was given to the mice throughout the experiment. Fecal samples were homogenized, serially diluted in PBS and plated onto LB agar plates. After incubation at 37°C for 16 h, plates were scanned with a fluorescence scanner (FLA-5100 Fujifilm) and CFUs counted using the ImageJ software.

Three sets of colonization experiments were done to estimate fitness advantage of evolved clones in the gut:

I. Control group: ANC-CFP with ANC-YFP at 1:1 ratio (n=5, females, housed in cages - mice nr. 1 with 2, 3 with 4,5 alone);

II. SCV group: SCV-CFP (SCV_M1_D8 *yqiJ*::IS5,*yjbS*::IS1) with ANC-YFP at 1:1 ratio (n=6, females, housed in cages - mice nr. 6 with 7, 8 with 9 and 10 with 11);

III. MUC group: MUC-CFP (MUC_M3_D19: *lon*::IS186,*yrfF*::IS1) with ANC-YFP at 1:1 ratio (n=6, females, housed in cages - mice nr. 12 with

13, 14 with 15 and 16 with 17). Diarrhea*: mouse nr. 16 at day 1 after colonization.

* to estimate the frequency of mice that get sick after colonization with MUC_M3_D19 clone, experiment was complemented in the following way:

III.1. MUC-CFP with ANC-YFP at 1:1 ratio (n=5, males, mice nr. 11 to 15, housed all in one cage). Diarrhea: mouse nr. 12 at day 3 after colonization.

III.2. MUC-CFP with ANC-YFP at 10:1 ratio (n=10, females, nr. 1 to 10, housed all in separate cages). Diarrhea: mouse nr. 5 at day 2, nr. 6, 7 and 8 at day 3 after colonization.

III.3. MUC-CFP alone (n=5, males, nr. 6 to 10, housed all in separate cages). Diarrhea: mouse nr. 7 at day 1 and day 2 after colonization.

In total, 7 out of 26 mice developed diarrhea after colonization with MUC_M3_D19 clone.

Mapping and identification of insertion sequences

The method used to identify insertion sequences (IS) was based on the vectorette PCR technique, described before (Zhong and Dean 2004). The targets of new insertions/transposition events were determined by direct sequencing of extra bands generated with the vectorette PCR protocol.

Statistical analysis

The statistical analysis was performed using the R software: http://www.r-project.org/, except for the analysis of fitness increase in M and C populations, for which we used SAS. To test whether the rate of

adaptation in each treatment differed, we used the fitness of M populations in the biotic environment and of C populations in the abiotic environment to do a PROC MIXED with selection regime (with or without macrophages), generation and the interaction between them as fixed factors and line nested within selection regime and its interaction with generation as random factors. Given that the interaction between selection regime and generation was significant (cf. results) we then used the SLICE option to test under which selection regime was the interaction significant. We then repeated the same analysis, but this time using the data from fitness of populations from both selection regimes in the abiotic environment.

Results

Increased adaptation rate by immune pressure

We followed the evolution of 12 *E. coli* populations, all founded from the same ancestral clone, when adapting to the two different sets of environmental conditions (see Chapter 2). Six populations (C1 to C6) were allowed to evolve in the RPMI medium without MΦs – in the C environment. The other six populations (M1 to M6) were evolved in the RPMI medium where MΦs were also present – in the M environment. The size of each population was counted daily and propagation of the bacterial populations was performed with a fixed number of bacteria, such that the differences in population sizes observed between the two culture conditions (with and without MΦs) were minimized (see Materials and Methods). Any observed difference in the rate of adaptation between C and M populations is therefore not attributed to differences in bacterial population size, but to the presence or absence of the MΦs. All populations were allowed to evolve for a period of 30 days, which corresponds to

approximately 450 generations. To test if the rate of adaptation is higher in the M pops versus C pops, we performed competitive fitness assays of the bacterial populations in the environments were populations had evolved. This was done by making head-to-head competitions of each evolved population against the ancestral clonal population during 24 hours, and determining the number of bacteria for each strain by flow cytometry: the ancestral carries a YFP marker and the evolved a CFP marker. Figures 3.1A and 1B plot the relative competitive fitness of each evolved population at two different time points of the experiment: after 19 days (approximately 285 generations) and 30 days (approximately 450 generations) in the environments where populations evolved.

The results clearly show a fitness increase in all populations within a few hundred generations. More importantly, after 450 generations, the fitness increase was higher for the populations evolved in presence of MΦs than for populations evolved in their absence (P=0.01, Proc mixed, see Methods). This supports the hypothesis that the rate of bacterial adaptation is higher in presence of MΦs. On average the fitness increase observed in the M populations is of 0.27 (2SE=0.10), whereas that observed in the C populations is of 0.04 (2SE=0.02) only after 450 generations. This implies that presence of MΦs led to higher fitness increase. Also consistent with our hypotheses, Figures 3.1 A and B show that the populations evolved in presence of MΦs had significantly higher variation for fitness between populations than the C populations (F-test, P=0.03 after 285 generations; P=0.007 after 540 generations). We therefore conclude that the level of between populations variation is increased in the presence of biotic interactions, in the initial stages of adaptation.

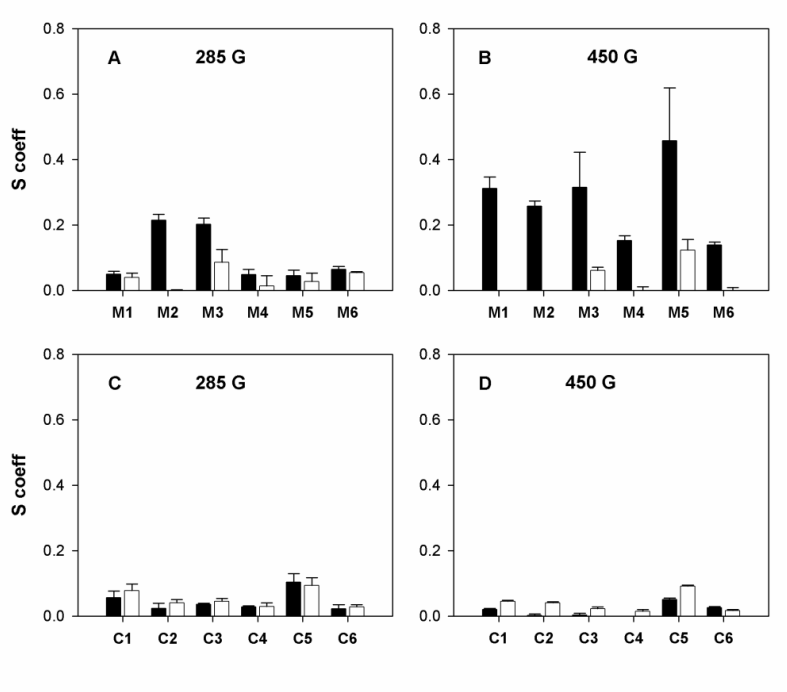

Figure 3.1. Evidence that presence of the macrophages increase the speed of adaptation in the bacterial populations. Competitive fitness of the C and M populations relative to the ancestral clone in the C (white bars) and M (black bars) environments at 285 (A and C) and 450 (B and D) generations. Error bars correspond to two time standard errors, n = 3.

We compared the fitness increase of the bacterial populations at two different time points of the evolution: at day 19 and at day 30. Interestingly no significant difference in the fitness was observed between 285 and 540 generations (P=0.73, Proc Mixed with SLICE, see Methods) for the C populations, whereas a significant change was observed in the M populations (P=0.002, Proc Mixed with SLICE). The mean fitness observed for M populations was 0.10 (2SE=0.07) at generation 285 and 0.27

(2SE=0.10) after 450 generations. The environments experienced by both sets of populations are correlated because they share the same abiotic factor, RPMI medium. To query if the adaptation to MΦs entailed any cost in adaptation to the environment where MΦs are absent we performed new competitive fitness assays of all the evolved M populations in the abiotic C environment, i.e. in RPMI medium without MΦs. The average fitness increase observed in M or C populations in the C environment was similar after 285 or 450 generations (M pop 0.04 (2SE=0.02) and C pop 0.05 (2SE=0.02) after 285 generations; M pop 0.02 (2SE=0.05) and C pop 0.04 (2SE=0.02) after 450 generations). Therefore we did not find any difference in the increase in fitness between 285 and 540 generations for M populations in the C environment (P=0.31, Proc Mixed with SLICE).

Fitness advantage when facing natural *E. coli* predators

Bacteria have evolved conserved strategies to infect and kill various evolutionary distinct eukaryotic hosts, including protozoa, nematodes, insects and mammals (Greub and Raoult 2004, Toft and Andersson 2010). It is thought that the adaptive strategies of bacteria against several natural predators may be a prerequisite to avoid MΦs and become pathogenic (Matz and Kjelleberg 2005, Hilbi et al. 2007). Under this hypothesis, one should also expect that some adaptations evolving towards escaping destruction from the professional phagocytes of the innate immune system will also confer a selective advantage to bacteria facing other predators.

To query if this hypothesis is correct we measured fitness related traits of ancestral and two evolved *E. coli* clones when facing common *E.coli* predators, namely protozoa and phages. We chose to study two clones that in the presence of MΦs acquired specific adaptations: increased survival inside MΦs (SCV – small colony variant clone) and increased resistance to escape engulfment by MΦs (MUC – mucoid

looking clone) (see Chapter 2). For the experiments, we chose MUC clone that was isolated from M3 population at day 19 of evolution experiment (MUC_M3_D19) and has two transposon insertions: an insertion of IS186 into the promoter region of *lon* and an IS1 insertion upsteam of *yrfF*. Because our previous results demonstrated that adaptation to the MΦs was mainly driven by transposable element mutations in several independently evolved MUC clones (see Chapter 2), we sought to check for transposon insertions in SCV clones (see Materials and Methods and Appendix B, Table B1). Strikingly, we did not find common insertions between SCV and MUC clones and only 14 out of 60 SCV clones had new transposon insertions. This result indicates that adaptation to the intracellular niche of the MΦs could not be solely explained by acquisition of transposable elements. For the further experiments we chose SCV clone sampled from M1 population at day 8 of evolution experiment (SCV_M1_D8). This SCV clone has two distinct insertions: an IS1 insertion within the *yjbS* gene and an IS5 insertion within *yqiJ* gene. *YjbS* is an essential gene for *E. coli* growth in rich medium, however its function is unknown. *YqiJ* gene is predicted to code for a putative oxidoreductase and has a hotspot motive 5`-YTAR-3` (Y=C or T) (R=A or G) for IS5 insertion (Mahillon and Chandler 1998) and indeed IS5 inserted in TTAA motive. It is known that *yqiJ* gene is highly up-regulated in *E. coli* strains sampled from asymptomatic bacteriuria during biofilm formation in human urine (Hancock et al. 2007).

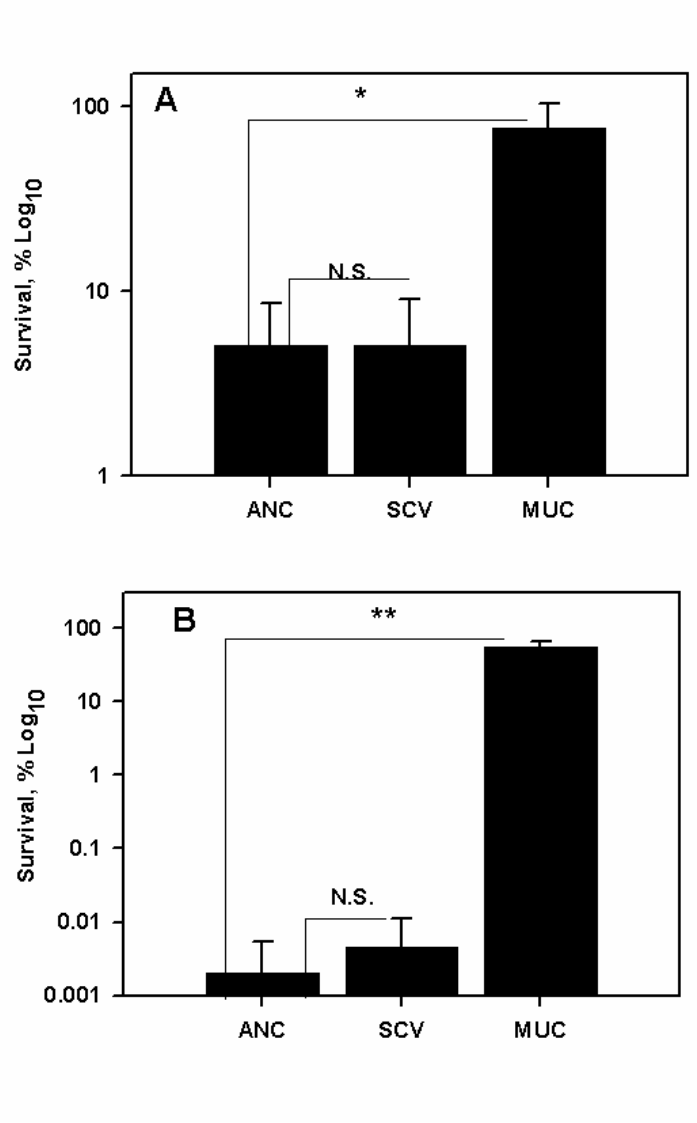

Figure 3.2. Adaptive value of the SCV and MUC clones in other ecosystems. SCV and MUC clones evolved in the presence of MΦs tested for survival under

predation by either the protozoa *T. thermophila* (**A**) or the phage P1 (**B**). *p < 0.05, **p < 0.01, Welch Two Sample t-test.

Tetrahymena thermophila share many characteristics with macrophages, such as some of the recognition mechanism and the production of oxygen radicals to kill the bacteria. If bacteria acquired mutations that make them better adapted to defense mechanisms that are common to MΦs and protozoa then we should detect a fitness advantage of the evolved bacteria. Figure 3.2A shows that MUC clones, has a fitness advantage in the presence of protozoa. With SCV clones, we did not detect any significant difference in survival compared with the ancestral clone when facing protozoa.

We also tested for fitness advantage of the evolved clones towards the bacteriophage of *E. coli*. Figure 3.2B shows that the adaptive strategy acquired by the MUC to escape phagocytosis by MΦs also provides a clear fitness advantage in the presence of phages. Other MUC clones sampled from M1 to M6 populations at generation 450 (day 30), also showed an increased resistance to the phages in a plaque assay (Appendix B, Figure B1). For the SCV no advantage relative to the ancestral bacteria was found in an environment where these specific predators (protozoa and phage) are the sole predator species, thus indicating that SCV evolved a specific adaptation towards intracellular environment of the MΦs.

Fitness advantage of SCV clone in the mouse gut

Finally we asked if the evolved bacteria show any selective advantage in an *in vivo* mammalian system, the mouse gut, which is a natural environment for *E. coli*. This is a complex ecosystem in which

competition with other species and predation by several predators occurs. In this environment a striking advantage was found for the SCV clone. By colonization of streptomycin treated mice, we show that SCV increases in frequency over the ancestral clone and is therefore better adapted *in vivo* (Figure 3.3). Fluorescent marker was neutral (Appendix B, Figure B2).

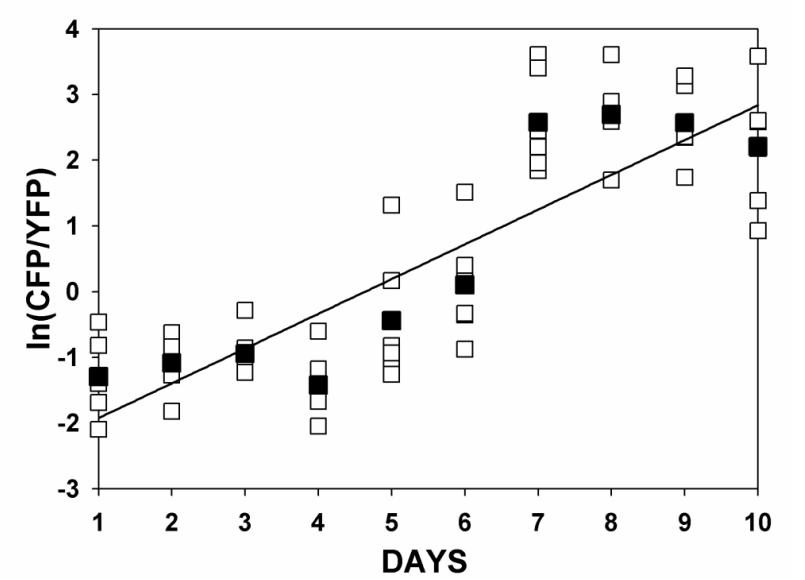

Figure 3.3. Competitive fitness assay of SCV clone in the gut. C57BL/6 mice treated with streptomycin were colonized with co-culture of 1:1 ancestral (expressing YFP) to SCV clone (expressing CFP). An increase in the ratio of CFP to YFP over time indicates selective advantage of the SCV clone (empty squares). Filled squares represent average ratio. Linear regression on ln(CFP/YFP) provides an estimate of the selective advantage per day: SCV slope 0.53 (SE=0.05), P<0.0001 adjusted R^2=0.7.

SCV clone evolves to MUC clone *in vivo*

Strikingly, we observed the emergence of MUC colonies at 7-10 days post colonization in several mice colonized with SCVs. These *in vivo* emerging MUC clones are derived from SCV clone because they have the same CFP marker. Surprisingly, we found that all MUC clones sampled from 3 independent mice, lost insertion in *yjbS* gene and acquired insertions in the promoter region of *lon* gene (Figure 3.4, Appendix B Table B2). Thus, this suggests that a mutation in *lon* gene alone or in combination with the mutation in *yqiJ*, is responsible for the transition of SCV clone into MUC clone in the gut. Moreover, MUC clone that evolved in the mouse gut also presented an increased ability to escape killing by the MΦs (Figure 3.5).

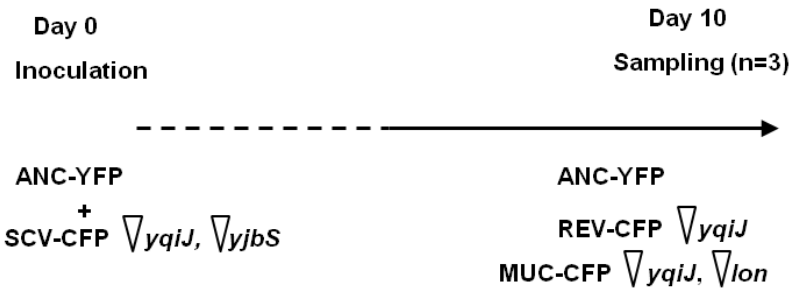

Figure 3.4. Experimental design demonstrating SCV transition to MUC clone through IS insertion in *lon*.

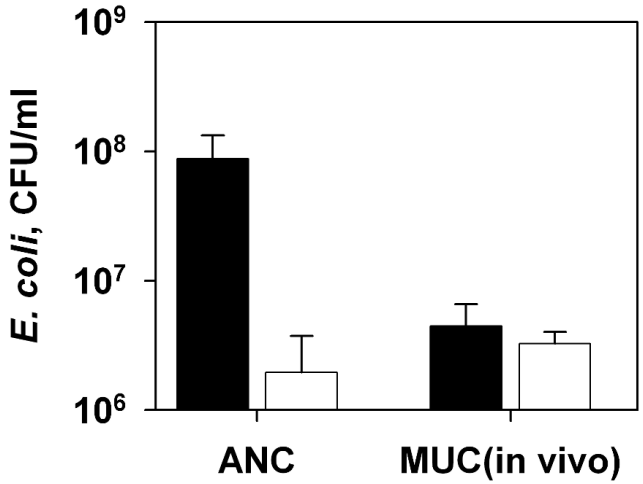

Figure 3.5. MUC clone, derived from SCV clone in the mouse gut, escapes killing by the MΦs. Black bars indicate bacterial growth density in RPMI without MΦs and white bars indicate – bacterial density after 5h at MOI (1:1) (see Materials and Methods in Chapter 2). Only extracellular bacteria are counted. MUC (in vivo) – MUC clone that was derived from SCV_M1_D8 in the competition assay *in vivo* at 10 days post colonization (clone X12, see TableS2). Initial numbers of ANC and MUC (in vivo) were not significantly different (T-test, P>0.05). Another independent MUC clone (X23, see Table S2) that emerged from SCV in the competitions *in vivo* showed similar results (not shown).

MUC clone does not show fitness advantage in the gut, but causes transient diarrhea in mice

In contrast to SCV clone, no selective advantage was revealed in the competitions of the MUC versus the ANC clone (Figure 3.6) and this morph shows lower colonization ability (not shown). Unexpectedly, however, we did observe a recurrent and important manifestation of the

colonization of mice by this MUC clone: a statistically significant proportion of the mice with MUC clones developed diarrhea within the first days of colonization (frequency of sick mice 0.27 with 95% CI [0.1; 0.4], see Materials and Methods). This was observed for at most two sequential days. Albeit small, the deleterious effect in the host caused by this evolved clone is significant in relation to the ANC strain or the other evolved SCV clone.

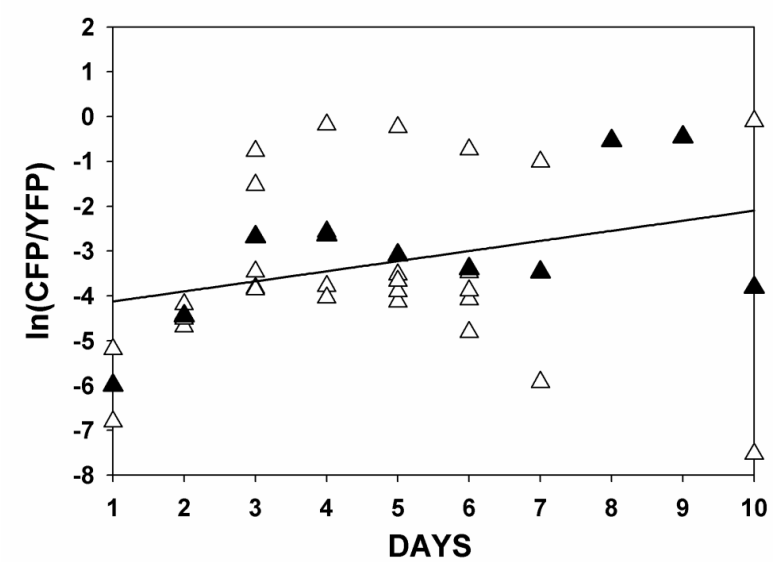

Figure 3.6. Competitive fitness assay of MUC clone in the gut. C57BL/6 mice treated with streptomycin were colonized with co-culture of 1:1 ancestral (expressing YFP) to MUC clone (expressing CFP). An increase in the ratio of CFP to YFP over time indicates selective advantage of the MUC clone (empty triangles). Filled triangles are average ratio in each experimental group. Linear regression on ln(CFP/YFP) provides an estimate of the selective advantage per day: MUC slope 0.23 (SE =0.15), P=0.13, adjusted R^2=0.08.

Discussion

Overall our results support the hypotheses that pressure imposed by the innate immunity accelerate adaptation and increase diversity. Our results show that populations adapting to simple abiotic environment, reach fitness plateau in about 285 generations. A similar pattern has been previously observed in populations of *E. coli* adapting to another abiotic environment composed of minimal media (Lenski 1991) but in a longer period of time. On the other hand M populations that are adapting to strong biotic environment do not show any signs of reaching a plateau in the tested period. Therefore the populations evolving in the presence of MΦs continue to increase in fitness at least until 450 generations.

A classical model of adaptation to a novel simple abiotic environment with a single fitness peak is Fisher's geometrical model (Fisher 2000). Under this static fitness landscape populations are expected to exhibit an initial rapid fitness increase, but as they become more adapted to the environment and approach the fitness plateau, the rate of fitness change should diminish. However, if the fitness landscape has several accessible fitness peaks, for example due to periodic environmental variation or the presence of biotic interactions, the rate of adaptation is not expected to decrease. Several other studies have shown that presence of strong biotic interactions, such as competition or predation by other species, increase adaptive radiation and lead to increase in fitness rates due to multiple coexisting high-fitness peaks (Poulin et al. 2000, Colegrave and Buckling 2005, Martin and Wainwright 2013, Williams 2013).

In the presence of the MΦs, due to complex fitness landscape,

where not only predation by MΦs, but also interspecific competition for existing resources occurs, variation for fitness and morphological diversity was observed. Some of those morphological variants that were previously characterized in Chapter 2 acquired specific adaptations to the presence of MΦs. In this study, we asked if adaptation to the MΦs would also confer a selective advantage to bacteria in other natural ecosystems, such as in the presence of bacterial predators, namely phage and protozoa or in the mouse gut, where multiple biotic interactions can be found.

We have found that clone that adapted to the intracellular niche of MΦs has a selective advantage *in vivo* in the mouse gut, observation that was surprising, given that till now there is no direct evidence that MΦs reside in the gut mucosa at high densities in the healthy individuals. Interestingly, J. M. Rhodes proposed a possibility that even non pathogenic *E. coli* during Chron's disease, may enter the lymphoid tissue, via the M cells, and subsequently persist within regional MΦs (Rhodes 2007). However question if SCV clones have a selective advantage *in vivo*, because of this phenomenon, holds for the future investigation.

MUC clone that evolved extracellular strategy to avoid engulfment by MΦs, confer resistance to predators that *E.coli* encounters in nature, namely with bacteriophages and protozoa. This demonstrates that there are some adaptive strategies to escape innate immunity that are fairly general, and can evolve very rapidly. Moreover, we observed that MUC clone can cause transient diarrhea in some animals.

Another interesting observation in this study comes from the fact that SCV can evolve to MUC phenotype in the mouse gut. Two findings lead us to believe that transition between SCV and MUC phenotype can be explained through regulation of Lon activity in SCV genetic background. Firstly, ANC clone never gave rise to the MUC clone. Secondly, only MUC

clone and not SCV revertant clone (CFP-REV) acquired insertion in the *lon* promoter region (Figure 2.4). Thus, this suggests that mutation in *lon* gene alone or in combination with the mutation in *yqiJ*, is responsible for emergence of MUC phenotype in the mouse gut. Interestingly, the promoter region of *lon* has been described as a hotspot for IS186 insertions (saiSree et al. 2001), which explains the rapid acquisition of such trait in the proper genetic background.

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M.M participated in planning the experiments, obtained and analyzed all the results for this study, participated in writing the manuscript. M. L. B participated in the mice colonization experiments, J. D was responsible for mice facility, J. D, S. M and I. G participated in planning the experiment and writing the manuscript.

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Chapter 4

Increased survival of antibiotic resistant *Escherichia coli* inside the macrophages

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Summary

Mutations causing antibiotic resistance usually incur a fitness cost in the absence of antibiotics. The magnitude of such cost is known to vary with the environment. Little is known about the fitness effects of antibiotic resistance mutations when bacteria confront the host's immune system. Here we study the fitness effects of mutations in the *rpoB*, *rpsL* and *gyrA* genes, which confer resistance to rifampicin, streptomycin and nalidixic acid, respectively. These antibiotics are frequently used in the treatment of bacterial infections. We measured two important fitness traits – growth rate and survival ability – of twelve *Escherichia coli* K-12 strains, each carrying a single resistance mutation, in the presence of macrophages. Strikingly, we found that 67% of the mutants survived better than the susceptible bacteria in the intracellular niche of the phagocytic cells. In particular, all *E. coli* streptomycin resistant mutants exhibit an intracellular advantage. On the other hand, 42% of the mutants were costly when bacteria were allowed to divide outside macrophages. This study shows that single non-synonymous changes affecting fundamental processes in the cell can contribute to prolonged survival in the context of an infection and are therefore important for the understanding of the evolution of antibiotic resistance and virulence in nature.

Introduction

A great deal of bacterial adaptation in the context of infectious diseases is their rapid evolution to tackle the immune system and antibiotics. *Escherichia coli* is both a commensal and a versatile pathogen, that can cause death (Denamur et al. 2010). Given these characteristics, it is an ideal organism to study the transition of commensalism to pathogenicity. *E. coli* colonizes the infant gastrointestinal tract within hours after birth, and typically a mutualistic relation builds up. However, even the harmless *E. coli* can cause an infection when gastrointestinal barriers are broken (Rolhion and Darfeuille-Michaud 2007) or in immunosuppressed hosts (Kaper et al. 2004). Healthy hosts are also susceptible to highly adapted *E. coli* pathogenic clones, which can cause many different types of infections. There is evidence that some of the pathogenic strains evolved from the commensal *E. coli*, through the acquisition of new genes and mutations (Denamur et al. 2010). A fundamental part of the ecology of *E. coli* during infection process is its interaction with the host immune system cells, in particular with macrophages. It is however not known if *E. coli* harbouring antibiotic resistance can have an advantage or disadvantage in the context of an interaction with the immune system. This knowledge is important given the high frequency of antibiotic resistance within commensal *E.coli* in healthy individuals (Bailey et al. 2010, Levert et al. 2010), which may lead to an increased risk of treatment failure during an infection process, because of limited therapeutic options.

Mutations that cause antibiotic resistance often produce associated fitness costs in bacteria (Andersson and Hughes 2010, MacLean et al. 2010). When the environment contains an antibiotic, resistant bacteria exhibit an advantage. However when the antibiotic is absent, resistant bacteria typically have reduced growth rates, although this depends on the genetic background (Gagneux et al. 2006, Trindade et al. 2009). This is not surprising, since mutations which cause antibiotic resistance often target physiologically important functions in the cell, such as transcription and protein synthesis, cell wall synthesis or nucleic acid synthesis (Andersson and Hughes 2010). Interestingly, the fitness effect of a resistance mutation can be detrimental in one environment and beneficial in another (Macvanin et al. 2003, Paulander et al. 2009, Bataillon et al. 2011, Hall et al. 2011, Trindade et al. 2012). For example, Trindade *et al.* showed increased variation in fitness effects of resistant mutations in *E.coli* with increased environmental stress. Similarly, Hall *et al.* demonstrated that the costs of 24 different *rpoB* mutations vary greatly among 41 environments with different carbon source. Having in mind that fitness effects of resistant mutations exhibit strong genotype-by-environment interactions, it is important to determine the effects of resistance in an environment imposed by the host. Despite its importance, to our knowledge there are only a few studies that explicitly address fitness effects of antibiotic resistant under conditions that are closer to the growth conditions in a host (Bjorkman et al. 1998, Adams et al. 2011). Furthermore, it has been shown that fitness effects of antibiotic resistant mutations vary substantially in the different *in vivo* and *in vitro* models (Bjorkholm et al. 2001, Giraud et al. 2003, Enne et al. 2004, Luo et al. 2005).

One important interaction that bacteria face in natural conditions is the interaction with cells from the immune system that are able to phagocytise them. There is little information available on fitness effects of

antibiotic resistance in this important context. The aim of the research reported here is to determine whether or not single point mutations conferring rifampicin, streptomycin and nalidixic acid resistance can affect reproduction and survival of commensal *E.coli* in the face of professional phagocytes. This study shows that commensal bacteria carrying specific resistance mutations can survive better in the intracellular environment of professional phagocytes. This has important consequences in designing therapeutic treatments and may be important to understand the spread of drug resistance and virulence.

Materials and Methods

Media and growth conditions

The RAW 264.7 murine macrophage cell line was maintained in an atmosphere containing 5% CO_2 at 37^{o}C in RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 10 mM hepes (Invitrogen), 100 U/ml penicillin/streptomycin (Gibco), 50 µM 2-mercaptoethanol solution (Gibco), 50 µg/ml gentamicin (Sigma), with 10% heat-inactivated FCS (standard RPMI complete medium). Before infection assays, MΦs were maintained in the same conditions, but in antibiotic-free RPMI media (without penicillin/streptomycin and gentamicin). Bacterial strains were grown and competed in antibiotic-free RPMI media in an atmosphere containing 5% CO_2 at 37^{o}C or in Luria-Bertani (LB) medium at 37^{o}C, with aeration (Grant-bio PHP-4 type Thermo-Shaker at 700 rpm).

Construction of strains

Susceptible MG1655-YFP and MG1655-CFP strains (MG1655, galK::CFP/YFP, ΔlacIZYA) containing yellow (YFP) and cyan (CFP)

fluorescent proteins under constitutive expression were created by moving *yfp* or *cfp* chromosomal inserts by P1 transduction from previously described strains (MC4100, galK::CFP/YFP, ampR (pZ12), strR (rpsl150)), that were kindly given by R. Kishony (Hegreness et al. 2006). To ensure constitutive expression of YFP or CFP fluorescent proteins the *lac* operon was deleted from MG1655 background. Ampicilin resistance (pZ12) was removed from the *yfp* or *cfp* locus using Wanner and Datsenko method (Datsenko and Wanner 2000). Mutations conferring resistance to rifampicin (in *rpoB* gene), streptomycin (in *rpsL* gene) and nalidixic acid (in *gyrA* gene) were previously constructed in *Escherichia coli* K-12 MG1655 background (see Appendix C, Table C1, (Trindade et al. 2009)). General transduction using P1 bacteriophage was performed as previously described (Thomason et al. 2007) in order to place resistance mutations in the new *E.coli* K-12 MG1655-YFP and MG1655-CFP background. To confirm mutations, each antibiotic resistance target gene was amplified and then sequenced. The primers used to amplify part of *rpoB* gene were: 5′-CGTCGTATCCGTTCCGTTGG-3′ and 5′-TTCACCCGGATAACATCTCGTC-3′; for *rpsL* gene: 5′-ATGATGGCGGGATCGTTG-3′ and 5′-CTTCCAGTTCAGATTTACC-3′ and for *gyrA* gene: 5′-TACACCGGTCCACATTGAGG-3′ and 5′-TTAATGATTGCCGCCGTCGG-3′. Each resistant clone was grown from a single colony in LB medium supplemented with the respective antibiotic and stored in 15% glycerol at -80^{o}C.

Competitive fitness in conditions where bacteria can divide: test for effects on reproduction

To estimate the fitness cost of resistance mutations we performed competition assays in three different environments: LB, RPMI and in RPMI with the MΦs. The resistant mutants constructed in MG1655-CFP (or in the

MG1655-YFP) strain were competed against a susceptible MG1655-YFP (or susceptible MG1655-CFP) strain in an antibiotic-free environment, at a ratio 1:1. For competitions in LB, both resistant and susceptible strains were grown separately for 48 hours for acclimatization in the media (bacteria were diluted at 1:10^{3} after 24 hours) at 37^{o}C with aeration, then mixed and 10 µl of 10^{-2} dilution was inoculated to the final volume of 150 µl of LB media in 96-well microtiter plates (Costar #3595) for 24 hour competition. Plates were arranged in a checkerboard configuration where half of the wells were without cells to control for well to-well and external contamination. For competitions in RPMI, resistant and susceptible strains were grown in antibiotic-free RPMI media for 48 hours (bacteria were diluted at 1:10^{-3} after 24 hours for acclimatization) at 37^{o}C with 5% CO_2. Competitions were performed in a 24-well cell culture tissue plates (containing 1 ml of culture media in each well), by inoculating 10 µl of 10^{-1} dilution (approximately 5×10^{4} bacteria). For competitions in the presence of the MΦs, strains were competed in the same conditions as used for competitions in the RPMI, except that MΦs were present. MΦs were seeded in a 24-well tissue culture plate at approximately 2 to 3× 10^{5} cells per well and allowed to attach overnight. Cells were then washed, re-suspended in fresh antibiotic-free RPMI media and activated with 2 µg/ml CpG-ODN 1826 (5´TCCATGACGTTCCTGACGTT 3´ - Sigma) for 24 hours. After 24 hours, cells were washed from remaining CpG-ODN, fresh antibiotic-free RPMI media was added and macrophages were infected with bacteria in the manner described before. The initial and final ratios of resistant and susceptible strains were determined by Flow Cytometry. The fitness cost of each of the resistance mutation was measured four times (twice in YFP and twice in CFP background). The selection coefficient, a measure of competitive fitness, was estimated as:

$S_{coeff} = Ln[(Nf_b/Nf_a)/(Ni_b/Ni_a)]/Ln[Nf_a/Ni_a]$ (Maree et al. 2000)

Where S_{coeff} is a selection coefficient of the resistant strain *b* against the susceptible strain *a*, Nf_a and Nf_b are the numbers of resistant (*b*) and susceptible (*a*) bacteria after competition and Ni_a and Ni_b are the initial numbers of resistant (*b*) and susceptible (*a*) bacteria before the competition (results are shown in Figure 4.1B, D and F) .

Competitive fitness inside the MΦs: test of the effect on survival

To estimate fitness effect of the resistant mutations on survival inside phagocyte cells, MΦs were prepared in the manner described above, infected with 5×10^6 bacteria (1:1 of resistant to susceptible strain) and centrifuged at 203 × g (1000 rpm) for 5 min to enhance bacterial internalization. After 2 hours of infection, MΦs were washed from extracellular bacteria and fresh cell culture medium containing 100 µg of gentamicin/ml was added to kill the remaining extracellular bacteria. After incubation for an additional hour, the medium was removed, monolayers of macrophages were washed and RPMI medium containing 20 µg of gentamicin/ml was added (0 h post-infection time point). To determine the number of intracellular bacteria after 5 h and 24 h of incubation, infected MΦs were washed 3 times with phosphate-buffered saline (PBS), 0,1% Triton-X was added for 30 min at 37°C in order to lyse the MΦs, centrifuged at 10600 × g (10000 rpm) for 5 min, washed in PBS and overall number of bacteria was counted by plating on LB agar plates. To measure intracellular survival after 48 h post-infection, fresh culture medium containing gentamicin (20 µg/ml) was added after 24 h post-infection period to the infected cells.

Survival inside the macrophages was estimated as the change in relative frequency:

$\Delta X = Nf_b/(Nf_a + Nf_b) - Ni_b/(Ni_a + Ni_b)$, where Nf_a and Nf_b are the numbers of resistant (*b*) and susceptible (*a*) bacteria after competition and Ni_a and Ni_b are the initial numbers of resistant (*b*) and susceptible (*a*) bacteria, before the competition (results are shown in Figure 4.1A, C and E).

Survival to oxidative stress of streptomycin resistant mutants

Given that all STR resistant mutants showed a survival advantage inside MΦs, we sought to determine if the mutants would also show an advantage during nutrient limitation in the stationary growth phase and under oxidative stress, which are characteristics of environment inside the MΦs.

To determine if STR resistant clones have differential fitness advantage in exponential (4 h), early stationary (24 h) and late stationary (48 h) phases, competition assays between STR resistant and susceptible strains were done. Briefly, STR resistant and susceptible strains were grown in antibiotic-free RPMI media separately for 48 hours at 37°C with 5% CO_2 (bacteria were diluted at $1:10^3$ after 24 hours for acclimatization), then mixed at a ratio of 1:1 (1 resistant to 1 susceptible strain) and 10 µl of 10^{-1} dilution inoculated to 1 ml of culture media. At 4, 24 and 48 h, a sample of bacterial suspension was plated onto LB plates to estimate the frequencies of STR resistant to susceptible strain at different growth phases (before exposure to H_2O_2 (Sigma), results are shown in Figure 2A).

To determine if STR resistant clones would show an advantage to survive oxidative stress during different growth phases, a mixture of STR resistant and susceptible strains (see description above – before exposure to H_2O_2), was treated with different concentrations of H_2O_2 (10 mM at 4 hours, 20 mM at 24 hours and 40 mM at 48 hours) for 30 min at 37°C. Appropriate dilutions were immediately plated onto LB to determine the

frequencies of STR resistant to susceptible strain after exposure to H_2O_2. Different concentrations of H_2O_2 were chosen because of higher cell mortality at exponential phase in comparison to stationary phase to the same concentration of H_2O_2 (Lim. et al. 2007). Four independent replicate experiments were done for each strain (two in YFP and two in CFP background). Survival to oxidative stress was calculated dividing relative frequencies of STR mutant after and before exposure:

$$\Delta X(H_2O_2) = (Nf_b/(Nf_a + Nf_b)) / (Ni_b/(Ni_a + Ni_b))$$

Where Nf_a and Nf_b are the numbers of resistant (b) and susceptible (a) bacteria after exposure to H_2O_2 and Ni_a and Ni_b are the numbers of resistant (b) and susceptible (a) bacteria, before exposure (results are shown in Figure 4.2B).

Statistical analysis

Wilcoxon signed rank test and Wilcoxon sum rank test with Bonferroni correction were used for statistical analysis using the R software: http://www.r-project.org/. Analysis of linear regression between survival and reproduction of antibiotic resistant mutants in the presence of the MΦs (Figure 4.2) was performed using SigmaPlot 9.0 software (Systat Software Inc., Chicago, USA) was used.

Results

We studied twelve different antibiotic resistance mutations in *rpsL*, *rpoB* and *gyrA* , conferring resistance to streptomycin (STR), rifampicin (RIF) and nalidixic acid (NAL) antibiotics, respectively (see Table 1). These mutations had been previously studied for fitness costs in Luria Bertani (LB) medium, when present in another genetic background (Trindade et al.

2009). Because fitness of antibiotic resistant clones can depend on the genetic background (Gagneux et al. 2006), we measured competitive fitness of these twelve mutants in LB media and found that all showed a cost in LB. The costs of antibiotic resistant mutations were different in the new genetic background for 33% of cases studied in the LB media (Wilcoxon sum rank test, $p<0.05$ for four out of twelve mutations, Appendix C, Figure C1).

To determine the fitness effects of antibiotic resistant clones in the presence of MΦs, competition experiments between the susceptible and the resistant mutants were performed. Two main fitness traits are important during the infection process: reproduction, which occurs outside MΦs, and survival, which is the main fitness component inside MΦs. Macrophage infection model has frequently been used to study virulence traits of many pathogenic bacteria species, where a non-pathogenic *E. coli* has served as a positive control for MΦ ability to uptake and phagocitize bacteria.

It is known that internalized non-pathogenic *E. coli* does not replicate intracellularly and thus *E. coli* survival is directly influenced by MΦs bactericidal properties and internalization time (Hamrick et al. 2000). After just 2 hours of internalization, 60% of all *E. coli* K-12 are shown to be immediately killed by RAW 264.7 macrophage cell line (Frankenberg et al. 2008). Another study also reported that a decrease in the numbers of viable bacteria can be observed as early as at 4 hours post-infection and only 5% of the initially internalized bacteria are recovered alive after 24 hours post-infection period (Glasser et al. 2001).

Table 4.1. Genotypes of single-point mutations used in the study

Gene	Genotype amino acid change	Nucleotide change	Antibiotic Resistance[1]
rpsL	K 43 N	AAA to AAC	STR
rpsL	K 43 T	AAA to ACA	STR
rpsL	K 43 R	AAA to AGA	STR
rpsL	K 88 R	AAA to AGA	STR
rpoB	S 531 F	TCC to TTC	RIF
rpoB	H 526 Y	CAC to TAC	RIF
rpoB	I 572 F	ATC to TTC	RIF
rpoB	R 529 H	CGT to CAT	RIF
rpoB	S 512 F	TCT to TTT	RIF
rpoB	H 526 D	CAC to GAC	RIF
gyrA	S 83 L	TCG to TTG	NAL
gyrA	D 87 Y	GAC to TAC	NAL

[1]- STR, streptomycin; RIF, rifampicin; NAL, nalidixic acid

The effects on both traits, reproduction outside the MΦs and survival inside the MΦs, were measured (see Materials and Methods). In order to estimate fitness effects of resistance in bacterial reproduction, competition assays in the RPMI cell culture media in the presence (+MΦs) and absence (–MΦs) of the MΦs were performed.

Global survival advantage of STR resistant mutants inside the MΦs

Figure 4.1A shows the effects on survival of *E. coli* strains carrying mutations K43N, K43T, K43R or K88R, which confer resistance to streptomycin. Surprisingly, all STR resistant mutants showed a survival advantage inside MΦs during 5, 24 and 48 hours post-infection. There was

no significant difference for survival effects of STR resistant mutants between post-infection periods (Wilcoxon signed rank sum, $p>0.05$). Contrary to the global fitness survival advantage inside MΦs, two mutants showed a cost and two other mutants were neutral when bacteria are allowed to reproduce, which was measured in competitive fitness assays against the susceptible strain in the presence and absence of the MΦs (Figure 4.1B). The cost of one mutation (K43N) differed significantly due to the presence of the MΦs (Wilcoxon sum rank test, $p=0.04$), while the costs of other three mutations were not different. In summary, single point mutations in *rpsL* gene, provide a survival fitness advantage in commensal *E. coli* in the intracellular niche of MΦs, leading to an increased risk of treatment failure during an infection process.

Variable fitness effects in RIF resistant mutants

Half of the RIF mutants (S512F, I572F and H526Y) showed a survival advantage inside the MΦs (Figure 4.1C). These were neutral or only slightly advantageous in competitive fitness assays where growth can occur outside MΦs (Figure 4.1D – white bars). Two RIF resistant mutants showed impaired survival inside the MΦs (R529H and H526D) and also showed the highest fitness costs for reproduction (Figure 4.1C and 1D). For the S531F mutation no effect was detected on survival, but a deleterious effect was measured on reproduction. There was no overall difference for effects on survival of RIF resistant mutants between different post-infection periods (Wilcoxon signed rank sum, $p>0.05$), except for one mutant (H526Y), that ceased to be advantageous for survival inside the MΦs at later time points (Wilcoxon sum rank test, between 5 and 48h post-infection, $p<0.05$). Fitness effects on reproduction of three *E. coli* RIF resistant mutants (Wilcoxon sum rank test, $p=0.01$ for H526Y, $p=0.009$ for I572F, $p=0.005$ for H526D) were significantly different due to the presence

of the MΦs, while effects for reproduction of other three mutants did not differ between presence and absence of MΦs in the environment (Figure 4.1D).

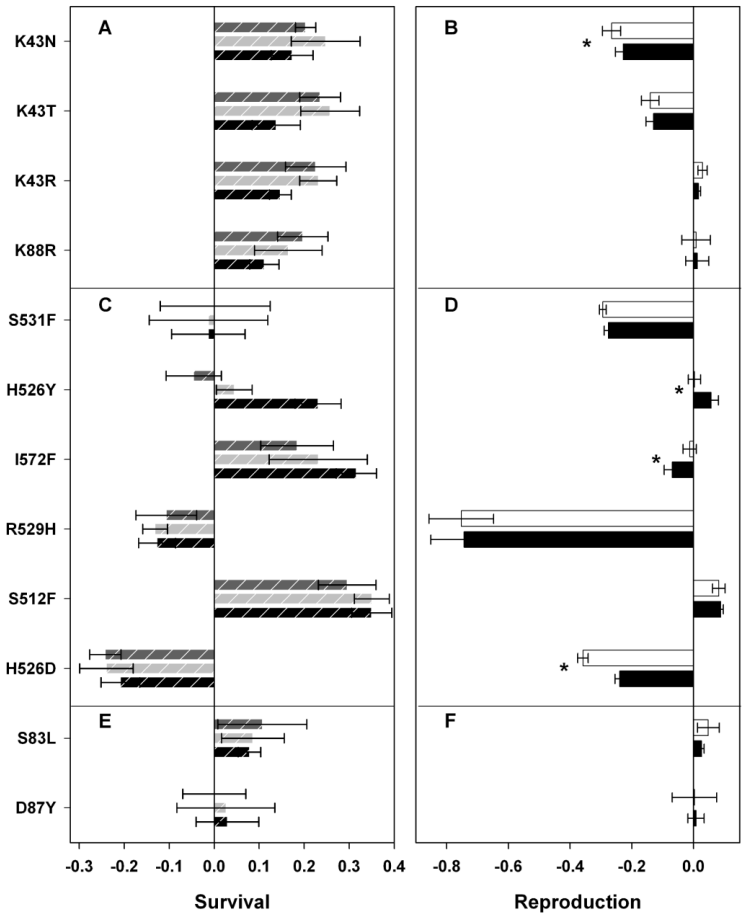

Figure 4.1. Effects of resistance on survival (left panel) and reproduction (right panel) of mutations in *rpsL* (A and B), *rpoB* (C and D) and *gyrA* (E and F) in *E. coli*.

A, C and E show fitness effects on survival inside the MΦs after 5 h (black dashed bars), 24 h (light grey dashed bars) and 48 h (dark grey dashed bars) post-infection. B, D and F show the effects of the mutations when bacteria can reproduce in the presence (white bars) and absence (black bars) of MΦs. All fitness effects were estimated using competition assays against a susceptible strain. The asterisk (*) represents significant differences ($p<0.05$) using Wilcoxon sum rank test.

NAL resistant mutants are advantageous or neutral

The fitness of S83L mutant was higher than the susceptible, for both, reproduction in the culture media and survival inside MΦs, while fitness of D87Y mutant remained neutral (Figure 4.1E and 1F). There was no effect on survival between different post-infection time points (Wilcoxon signed rank sum, $p>0.05$). We did not observe significant differences in fitness effects for reproduction for the two studied NAL resistant mutants (S83L and D87Y) due to the presence of the MΦs (Wilcoxon sum rank test, $p>0.05$ for both mutations) (Figure 4.1F).

Advantage of STR mutants to oxidative stress in the stationary phase

Given the striking survival advantage of al STR resistant mutants, we tried to determine if such results could be caused by the specific stress that bacteria face upon internalization, namely nutrient starvation and/or oxidative stress. To test this hypothesis, competition assays were performed during exponential and stationary phases of bacterial growth, and advantage to oxidative stress was measured during those phases, by addition of H_2O_2. While fitness cost for reproduction was the highest after 24 hours of bacterial growth, it was relieved after 48 hours (where bacteria experience 24 hours growth phase in addition to 24 hours stationary phase) for the two most costly STR mutants – K43N and K43T (Wilcoxon sum rank test, $p=0.03$ for K43N, $p=0.03$ for K43T, Figure 4.2A), indicating that STR resistant mutations could bear an advantage during stationary

phase induced by nutrient limitation. Interestingly, all STR resistant mutants showed an increased survival to oxidative stress after 24 hours, but not during exponential growth phase (Figure 4.2B). The results therefore indicate that nutrient deprivation and oxidative stress are key factors on the survival advantage that these mutants exhibit inside MΦs.

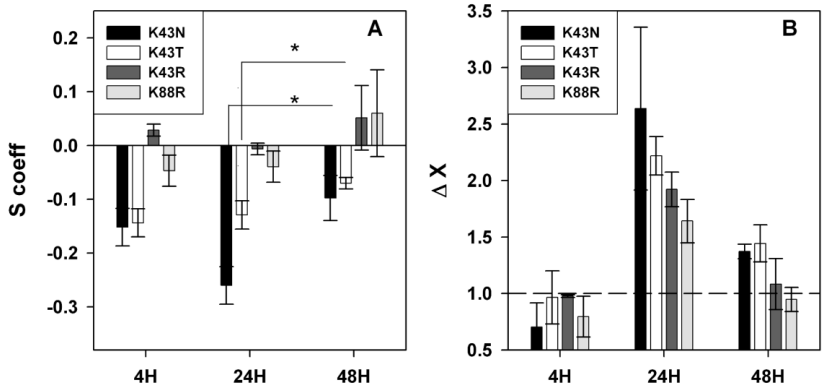

Figure 4.2. Starvation and oxidative stress diminish the fitness cost of STR resistant mutations. (A) Effects on reproduction of STR resistant mutations during 4, 24 and 48 hours competition assays against a susceptible strain in RPMI medium. (B) Advantage of STR resistant mutants against a susceptible strain after exposure to H_2O_2 at different phases of bacterial growth in RPMI medium. Bars above the dashed line represent an increased survival of the STR mutant against a susceptible strain. The asterisk (*) represents statistical significant difference ($p<0.05$) using Wilcoxon sum rank test.

Correlation between survival and reproduction

It has been proposed that resistance to stress is associated with reduced resource uptake, or growth rate (Ferenci 2005). This trade-off between self-preservation and nutritional competence, so called SPANC balance, has been observed in several studies (Ferenci 2005, De Paepe et al. 2011). Recently, SPANC trade-off has been directly linked to the growth

rate, stress resistance, outer membrane permeability, morphotype characteristics and virulence properties of antibiotic resistant *E. coli* isolates from deep and visceral infections in humans (Levert et al. 2010). In our study we tested for the existence of SPANC trade-off between survival inside the MΦs and reproduction in the presence of the MΦs (Figure 4.3). We did not observe a negative correlation, but, on the contrary, antibiotic resistant clones which survived better inside the MΦs also had a higher fitness advantage for reproduction (Figure 4.3). Overall, 67% (8 out of 12) of all studied antibiotic resistant mutants survived better than the susceptible inside the MΦs (see points that fall into S+ quarters).

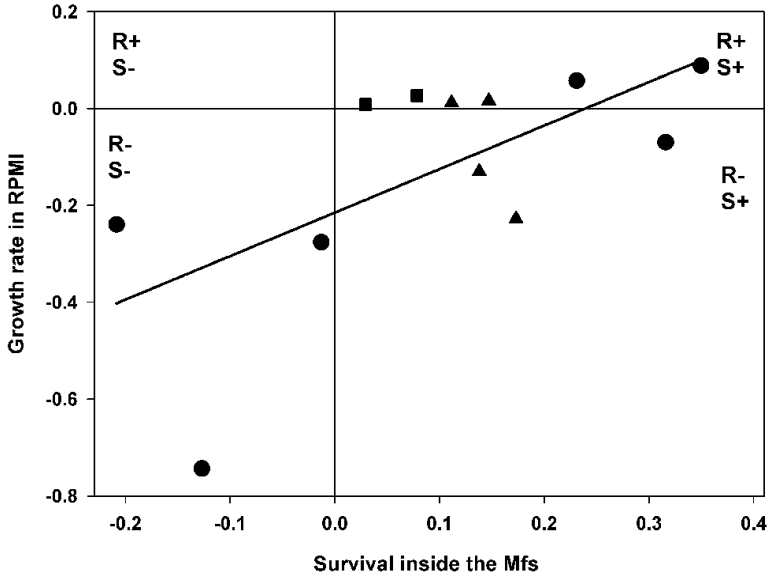

Figure 4.3. Test for correlation between the effect on survival inside MΦs and effect of growth rate. Intracellular survival of resistant mutants against a susceptible strain was measured at 5 h after bacterial internalization. Reproduction

in the RPMI media with the MΦs was measured after 24 h competition assay. The slope of the regression line (solid line) is 0.64 ± 0.2 (Std. Err) and p=0.01, with R^2=0.5. The dotted lines represent 95% confidence interval of the regression line and dashed lines 95% prediction intervals for all data points. The graph is divided into quarters where R+ and R- (or S+ and S-) means advantage and disadvantage for reproduction (or survival), respectively. Triangles represent STR, circles RIF, and squares NAL mutants.

Discussion

Drug-resistant bacteria pose a significant threat to human health, and it is important to understand how the fitness of such bacteria can be impaired during infection. Here, we studied how antibiotic resistance affects two important fitness traits: the ability to survive and reproduce in the presence of MΦs. It is known that during the entry into MΦs, bacteria experience a set of environmental stresses, such as host-induced nutrient limitation, acidification, toxic peptides, osmotic stress and reactive oxygen species (ROS), of which the latter is believed to be the major cause of bacterial killing (Schlosser-Silverman et al. 2000). To our knowledge, this is the first study that measures fitness effects for survival of several antibiotic resistant mutants in the intracellular environment of the MΦs. Surprisingly, we found that from 3 distinct groups of resistance targets 67% of all mutants had increased survival inside MΦs, 17% did not show significant differences from the susceptible and only 16% of all mutants showed reduced survival. Importantly streptomycin resistance, although carrying substantial fitness costs for growth rate, shows a global advantage for survival.

The experience of the early single use of streptomycin in 1946, for treating *Mycobacterium tuberculosis* infections indicated that resistance to

this drug could be acquired very rapidly (Crofton and Mitchison 1948). To this day STR resistant isolates have been identified in many other important pathogens, such as *Shigella flexneri*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, and even in commensal *E. coli* sampled from healthy individuals (Tseng et al. 1972, Barreto et al. 2009, Hong et al. 2010, Rahmani et al. 2012). High resistance incidence to this drug is frequently due to point mutations in *rpsL* gene, with the most common mutations at the codon K43 and K88 (Sreevatsan et al. 1996) that were included in this study. These mutations were shown here to be beneficial in the intracellular environment of MΦs in *E. coli*. One possible explanation for the fitness advantage of STR resistant mutations could be the approximately 7-fold improvement in the accuracy of ribosomes in *rpsL* mutants (Zaher and Green 2010). It was shown that streptomycin resistance mutations in *rpsL* gene often lead to hyper-accurate, but slower ribosomes (Bilgin et al. 1992). Indeed, all STR resistance mutations that were tested in our study are responsible for the increased fidelity of ribosomes (Okamoto-Hosoya et al. 2003). Though fast ribosomes are required in actively dividing cells, hyper-accurate ribosomes are advantageous in non-dividing cells during starvation, because they exhibit attenuated protein oxidation during growth arrest (Ballesteros et al. 2001) and oxidized proteins are known to be more susceptible to proteolytic degradation (Cabiscol et al. 2000). This should be extremely relevant upon entry to the MΦs, where *E. coli* not only undergoes growth arrest and nutrient starvation, but also has to deal with ROS generated by the MΦs (Schlosser-Silverman et al. 2000). Consistent with this hypothesis, we found that STR resistant mutants have reduced fitness costs when nutrients are deprived and survive better than the susceptible under oxidative stress in stationary phase (Figure 4.2). Certainly, finding that most commonly identified mutations, conferring resistance to STR,

enhanced the survival capacity of *E. coli* inside the MΦs, begs the question if that advantage could exist in other bacterial species, such as *M. tuberculosis* and other pathogenic bacteria.

Many bacterial pathogens (Sekiguchi et al. 2007, Liu et al. 2011, Yesilyurt et al. 2011) acquired resistance to RIF in the last decade. It is known that in 96% of RIF resistant clinical isolates of TB, resistance is due to the mutations in *rpoB* gene, with the most common mutations at the codon 531 and 526 in distinct geographical locations (Cavusoglu et al. 2006, Tan et al. 2012). In this study, besides prevalent mutations in codons 531 and 526 (S531F, H526Y, H526D), other mutations in codons 512, 529 and 572 (S512F, R529H, I572F) were also included. The fitness effects on survival of RIF mutants in *E. coli* varied in our study. Interestingly, different base substitutions leading to different amino acids even at the same codon position (see Figure 4.1C – H526D and H526Y) gave differential outcomes for *E. coli* survival inside MΦs. The mutation at the codon 526 has been shown to be responsible for oxidative stress sensitivity in *E. coli* and *Staphylococus aureus*. However, the molecular mechanism for this remains unknown (Kawamura et al. 2005). Several observations suggest that single point mutations in the *rpoB* gene encoding β subunit of RNA polymerase, can have an effect on RNA polymerase interaction with several promoters and transcriptional regulators, leading to different phenotypes (Zhou and Jin 1998, Maughan et al. 2004, Perkins and Nicholson 2008). For example, in *Bacillus subtilis* the RNA polymerase complex interacts with every promoter in bacterial genome, so the mutations in RNA polymerase lead to global changes in gene transcription and, hence, affect several physiological processes, such as growth and metabolism, chemotaxis, competence, spore resistance and many others (Maughan et al. 2004). Since RIF mutations have been found to affect physiological processes to different extents, it may not be surprising that

we found a great variation in their fitness effects of RIF mutants inside MΦs.

The emergence of NAL resistant isolates during treatment of *Shigella, Campylobacter* or *Salmonella* infections has been of a great concern (Wu et al. 2002, Kumar et al. 2009, Ghosh et al. 2011). Single point mutations in the quinolone resistance-determining region of the DNA subunit gene *gyrA*, at a codon 83 (42% frequency) and 87 (35% frequency) have been attributed to the high levels of resistance to this antibiotic (Hopkins et al. 2007). Although the fitness costs of those mutations appear to be low in laboratory medium (Trindade et al. 2009), it is not known how resistance to this drug may affect survival and replication of these bacteria in the context of infection. In *E. coli* we found no fitness costs (for D87Y mutation) or even slightly enhanced fitness (for S83L mutation) of NAL resistant clones for survival inside the MΦs, which is compatible with those of the previous reports showing that NAL resistance is usually associated with very small fitness costs (Bjorkman et al. 1998).

It was previously demonstrated that fitness effects for reproduction of antibiotic resistant bacteria generally increases under stressful conditions (Petersen et al. 2009, Trindade et al. 2012). Effects on reproduction of more than half (58%) antibiotic resistant mutants was either neutral or slightly advantageous in the presence of the MΦs, however this effect was mainly attributed to the growth in the RPMI cell culture medium we used for maintenance of eukaryotic cells. Still, this is altogether relevant, because RPMI cell culture medium is supposed to mimic abiotic conditions in the human host. Moreover, fitness effects for reproduction differed in 33% of antibiotic resistant cases, due to the presence of the MΦs (compare black and white bars in Figure 4.1B, D and F). It is however not surprising, given that MΦs not only inflict several different stresses on

bacteria, but can also modify composition of the extracellular media. This is consistent with earlier findings suggesting that fitness costs of antibiotic resistant mutants may vary in the different environmental conditions (Hall et al. 2011, Trindade et al. 2012).

The findings made here have several medically relevant implications. First, this work shows that the presence of macrophages can have drastic consequences for the biological fitness of antibiotic resistant *E. coli*. This conclusion points towards measuring fitness costs in such environment in other bacterial species as well as studying mutational targets of widely used antibiotics in clinics. Second, this study identifies single point mutations that are advantageous for bacterial survival in macrophages because of the environmental stresses imposed by MΦs, such as exposure to H_2O_2. Taken all together, our main finding that stressful intracellular environment of MΦs can select for antibiotic resistance has important consequences for predictions of the spread of drug resistance.

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M. M. performed all the experiments in this study and wrote the manuscript. I. G. participated in planning the experiment and writing the manuscript.

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Chapter 5

Discussion

Microbial populations constantly face a pressure imposed by hosts' immune system. However, it is not known how fast and which type of adaptive strategies commensal bacteria can evolve to circumvent the first barrier of immune system. In the preceding chapters, I demonstrate that commensal *E. coli*, under constant pressure imposed by innate immune system cells – macrophages, can evolve remarkably fast and acquire specific traits that are reminiscent to those found in pathogenic bacteria. Specifically, at least two different strategies evolved in succession: increased intracellular survival (SCV clones) and ability to escape engulfment and killing by macrophages (MUC clones).

SCV clones that emerged during the interaction with macrophages, share similar characteristics as those sampled from various bacterial species found in human clinical infections (Funada et al. 1978, Roggenkamp et al. 1998, Tappe et al. 2006). Among those are increased resistance to specific antibiotics, the ability to revert to ancestral phenotype, reduced growth in extracellular medium and longer intracellular survival, increased growth in the presence of hemin and negative catalase activity (Proctor et al. 2006). In clinical contexts, SCVs have been isolated after long and unsuccessful antibiotic exposure (Pranting and Andersson 2011, Garcia et al. 2013). This is not the only mechanism potentiating the emergence of SCVs in several pathogens, since they can be selected in the intracellular milieu of eukaryotic cells in the absence of antibiotics (Vesga et al. 1996, Cano et al. 2003). All these studies concern the emergence of SCVs in pathogenic species/strains. In contrast with all those studies, I demonstrated that even commensal *E. coli* acquire SCV phenotype in the presence of phagocytic cells. My results suggest that this

mechanism is fairly general and thus can be utilized by several bacterial species during an infection process. Moreover, this can be a common strategy that bacteria employ in order to hide inside the eukaryotic cells, which may provide shelter from host defenses and antibiotics.

The bacterial strategy evolved by MUC clones, is characterized by overproduction of EPS, increased resistance to escape macrophage phagocytosis, and increased pathogenesis *in vivo*. This increased pathogenesis in the mouse *in vivo* model is in accordance with the fact that MUC colony morphologies are frequently isolated from life-threatening infections in humans (Govan and Deretic 1996, Stollerman and Dale 2008, Bottone 2010, Djossou et al. 2010, Silva et al. 2011). In general, the mucoid phenotypes have been often associated with pathogenic mechanisms, such as resistance to phagocytosis, increased resistance to bactericidal serum factors and virulence in animal studies (Schwarzmann and Boring 1971, Wessels et al. 1991, Engleberg et al. 2001). For instance, EPS isolated from mucoid variant of *Burkholderia cenocepacia*, interfere with the function of human neutrophils *in vitro*, by inhibiting both chemotaxis and the generation of reactive oxygen species (Bylund et al. 2006). Also, during cystic fibrosis, *P. aeruginosa* produce specific EPS alginate, which protects bacteria from macrophage engulfment and killing (Leid and Jeffers 2009). Moreover, even if macrophages cannot engulf *P. aeruginosa*, they are activated and hence, secrete toxic compounds that damage healthy host tissues. Such phenomenon is called "frustrated" phagocytosis. Together, this suggests that the encounter with phagocytic cells during the infection process may explain the acquisition of mucoidy by bacteria.

To correlate phenotypic changes to genetic changes in bacterial populations adapting to the presence of the macrophages, several

randomly chosen clones from the end of experiment were sent for whole-genome sequencing. A remarkable parallelism was observed for several genetic changes, such as transposable element (TE) insertions in regulatory region of *yrfF* (a predicted inner membrane protein), in coding regions of *yiaW* (a predicted transmembrane protein), in *potA* and *potD* genes (involved in spermidine uptake), and SNPs in *fusA* (an elongation factor) gene. Such parallelism between independently evolving populations suggests that *E. coli* evolved similar mechanisms to survive phagocytosis by macrophages. Several other authors, reported the acquisition of parallel genetic changes in bacterial populations evolving to other types of selective pressures, such as simple laboratory medium, antibiotic presence or even in the human host (Woods et al. 2006, Zdziarski et al. 2010, Toprak et al. 2012). It is relevant to notice that the majority of the observed mutations, in bacterial population adapting to the macrophages, were TE insertions. While TEs sometimes are viewed as genomic parasites (Piskurek and Jackson 2011), they can also be considered as powerful facilitators of evolution, because they can be advantageous in generating variation upon which natural selection can act in novel evolutionary niches (McClintock 1984, Oliver and Greene 2011). Such adaptive process driven by TEs has been demonstrated in several other studies, in the context of adaptation to a novel environment (Papadopoulos et al. 1999, de Visser et al. 2004, Liang et al. 2011, Dragosits et al. 2013). In particular, Papadopoulos *et al.* (1999) observed that similar IS-150-mediated deletions affecting *rbs* operon occurred in all 12 parallel populations adapting to minimal medium with glucose (Papadopoulos et al. 1999). Moreover, all *rbs* deletions in this study were advantageous in the environment where populations evolved. Altogether, these results show that transposable elements are important drivers in microbial evolution and that it may be possible to predict which genetic changes underlie

adaptation to some environmental pressures.

To track the emergence of new adaptive mutations to the presence of the macrophages, I randomly sampled several clones at different time points of evolution and estimated frequencies of different haplotypes for mutations that are known. Surprisingly, insertion in *yrfF* regulatory region not only occurred in all clones that showed MUC morphology, but it was also the first mutation to be observed in all populations. After the initial sweep characterized by a common mutation in *yrfF*, populations adapting o the macrophages acquired other mutations, which were either distinct or parallel among *E. coli* populations. In the clones that were not MUC, but rather looked like ancestral phenotype (ANC), I did not detect any mutations that increased in frequency during the experiment in MUC clones. These findings strongly suggest that MUC clones acquired genotypes that are specific to the adaptation to macrophage environment. Therefore, it would be interesting to explore how each of those mutations alone and in combination contributes to *E. coli* fitness in this context.

Finally, results in Chapter 2 suggest, that clonal interference plays a major role in the evolution of the *E. coli* adaptation to the macrophages. Indeed, there is a growing body of literature suggesting that clonal interference is an important process in the dynamics of bacterial evolution (de Visser and Rozen 2006, Martens and Hallatschek 2011, Hughes et al. 2012, Herron and Doebeli 2013). And, while it is hard to exclude the possibility that other forms of selection may act during the evolution experiment, clonal interference explain reasonably well the complex dynamics in bacterial populations adapting to the macrophages.

In Chapter 3, I measured and compared fitness of populations that evolved in the presence of macrophages with populations that evolved in a constant abiotic medium. I observed that, in the presence of macrophages

the rate of adaptation in bacterial populations is higher than in their absence, and that genetic variation increases. This agrees with Dobzhansky's hypothesis (1950), who postulated that higher levels of biotic interactions are expected to exert higher selection pressures on other organisms than abiotic environments, leading to higher rates of adaptation and hence generating more diversity (Dobzhansky 1950). Surprisingly, only very recently such important hypothesis could be verified by several studies, mainly in phage-bacteria coevolutionary studies. (Paterson et al. 2010, Gomez and Buckling 2011, Hall et al. 2011b, Stern and Sorek 2011). Thus my study produced results which corroborate the findings of some of the previous work in this field, however in different experimental setup.

In the same chapter, I investigated if adaptation to the macrophages would also confer a selective advantage to bacteria in other natural ecosystems. I chose to address this in different ecological conditions, namely presence of bacteriophage and protozoa and inside the mouse gut, where multiple biotic interactions can be found. I demonstrated that SCV clones, which showed an increased survival inside macrophages, conferred a selective advantage *in vivo* in the mouse gut. However, the MUC clone did not show any advantage, but caused diarrhea in some animals. Additional work is required to confirm if the fitness advantage of SCVs *in vivo* is a consequence of the interplay between hosts immune system and SCV phenotype specific properties. When I tested evolved clones for a survival advantage against killing by protozoa and bacteriophage, MUC clones showed an advantage to both, in comparison to the ANC and SCV clones. This indicates that strategies to escape phagocytosis by macrophages and killing by *E. coli* environmental predators can be fairly general. Such observation was also found in previous studies (Cirillo et al. 1994, Wildschutte et al. 2004), however generally in a reverse order. For instance, *L. pneumophila* after growth in

one of the protozoan hosts, *Acanthamoeba castellanii*, becames 100-fold more invasive for epithelial cells and 10-fold more invasive for macrophages (Cirillo et al. 1994). Such coincidental evolution can be largely explained because of the similarities between protozoa cells and macrophages. For example, similar morphological events that follow internalization of *L. pneumophila* by amoebae are similar to those thoroughly characterized in mammalian cells (Bozue and Johnson 1996, Venkataraman et al. 1998, Cirillo et al. 2002). Moreover, *L. pneumophila* share many genes that are important to infect both amoebae and mammalian cells (Gao et al. 1997, Liles et al. 1999, Cirillo et al. 2002, Miyake et al. 2005). In many cases, evolutionary studies demonstrated that bacterial evolution in protozoa is able to select for enhanced survival and virulence in phagocytic cells. Interestingly, a recent study that resembles the experimental setup I presented in Chapter, questioned if after evolution with macrophages, bacteria can evolve to survive better in protozoa (Ensminger et al. 2012). Contrary to expectations, *L. pneumophila* showed decreased survival ability in its protozoan host after replication within macrophages for hundreds of generations (Ensminger et al. 2012). Such incompatibility between results presented by Ensminger *et al.* (2012) and my direct observations can be explained by differences in bacterial strains. While the *E. coli* strain that I used is a commensal, *L. pneumophila* is recognized as a protozoan parasite and accidental intracellular pathogen in humans. Thus, it is possible that initial adaptation to protozoa or macrophages is sufficiently general for extracellular pathogen until bacteria adapt to survive main intracellular stresses that are common between those hosts. When this happens, intracellular pathogen may become more specialized in protozoa or macrophages, in particular if growth is restricted to only one of those niches. Together, these results suggest that coincidental evolution theory can explain the acquisition of the virulence

traits in pathogenic bacteria, but cannot discriminate whether bacterial adaptation to environmental predators resulted in the increased pathogenesis in humans, or vice versa.

In Chapter 4, I measured fitness effects of antibiotic resistant clones for the ability to reproduce in the extracellular niche of macrophages and to survive inside the macrophages. I showed that 67% of all studied mutants increased survival inside macrophages, a result that is extremely relevant, given that antibiotics are widely used as tools to combat many infectious diseases. Moreover, by choosing three different antibiotics, affecting a broad spectrum of essential genes in the cell, I showed that this trait is not restricted to a specific gene, but can be quite general. Importantly, resistance to streptomycin antibiotic, although carrying substantial fitness costs for growth rate, showed a global advantage for survival inside macrophages. In this chapter I extend this finding by showing that the cost of streptomycin resistance can be alleviated during stationary phase and exposure to hydrogen peroxide, both of which constitute environmental stresses that are common in the harsh milieu of macrophages. It is known that entry into the stationary phase might trigger the expression of a unique genetic program that activates persister switch (Hu and Coates 2005, Lewis 2007). Herewith, persister cells are known to resist better many environmental challenges (Lewis 2007). It would be important to find out if mutations conferring streptomycin resistance could facilitate transition to persistent phenotype.

Although many studies had reported that fitness effects of antibiotic resistance vary in different environments and genetic backgrounds (Hall et al. 2011a, Trindade et al. 2012), there is the need to complement this knowledge with experiments focusing on possible mechanisms for this phenomenon. One possibility is to measure the costs of resistance in the

environmental conditions closest to those that bacteria has to face in nature, such as oxygen and nutrient limitation, presence of antimicrobials and others. To understand how different genes conferring resistance to antibiotics respond to new environmental challenges, it is important to know how each mutation affects gene expression and metabolism of bacterial cell. Another important task is to account for differences in the genetic backgrounds within and between bacterial species and to keep in mind that epistasis might be acting between different targets affecting resistance to various antibiotics.

The work developed in this thesis opens future avenues of research concerning adaptation of microbes to immune system. For example, while I mainly focused on *E. coli* adaptation to the macrophages, the correspondent physiological adaptation of macrophages rests unexplored. This is crucial, as several recent *in vivo* studies suggest that the functional phenotype of macrophages could change over time within a host when facing different micro-environmental signals (Stout et al. 2005, Graff et al. 2012). And while the exact mechanism responsible for this switch has not been yet identified, it is clear that this transition is linked with various important pathologies (Mosser 2003, Kinne et al. 2007, Pollard 2009). It is possible that *E. coli* could have evolved to modify some functions of macrophage cells. Do macrophages respond differently to evolved SCV and MUC clones? If so, does the localization of evolved bacteria changes within macrophages or do bacteria learn to interfere with macrophage signaling pathways? Additional work is also required to unravel the causes of increased pathogenesis caused by MUC clones in the *in vivo* model in mice. What are the possible mutations responsible for this phenotype? What is the exact mechanism by which commensal *E. coli* evolves to harm the host? Also, why SCVs appeared in the experiment only transiently and what are the selective pressures responsible for the emergence of this

phenotype? Which genetic alterations lead to this phenotype and do they confer advantage to other nonphagocytic cells? It is clear that in future investigations it might be important to complement those findings by studying other commensal bacterial species and introducing other cells present in the host.

In the broader context, the findings that I present here demonstrate the power of innate immune system as a selective pressure to help us understand the diversity of microorganism in terms of health and disease. Ultimately, such knowledge is crucial in order to predict the emergence of bacteria with increased pathogenic potential and resistance to antibiotics, and to effectively come up with new therapeutic strategies.

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Appendices

Appendix A

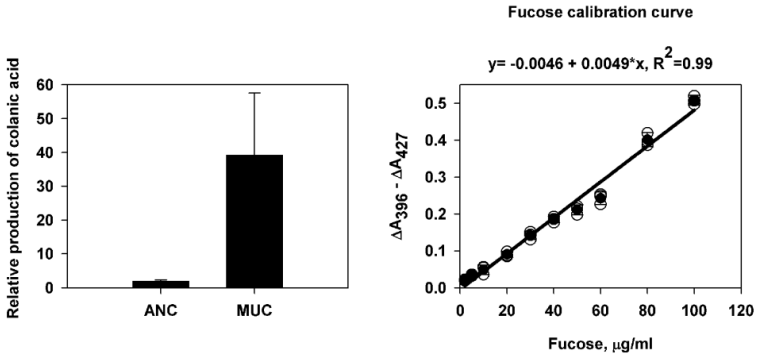

Figure A1. Quantification of colanic acid in MUC_M3_D19 clone. After purification from the growth medium of each clone, the amount of colanic acid was determined by measuring non-dialyzable methylpentose (fucose) absorbance at 396 and 427 nm after reaction with sulfuric acid and cysteine hydrochloride. Measurements were repeated three times for each clone. Obtained values (ΔA396–ΔA427) were directly correlated with fucose calibration curve and normalized for CFUs.

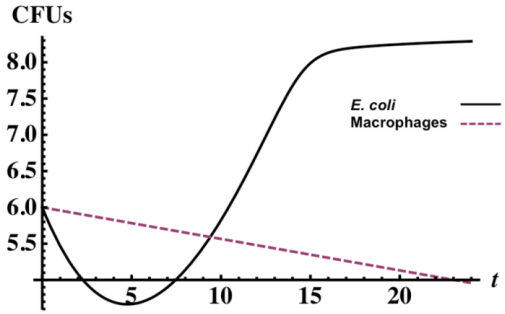

Figure A2. Infection dynamics of the ancestral strain. Simulated dynamics of a population of bacteria dividing in the presence of macrophages for 24 hours,

according to the model $\frac{dB}{dt} = B(r - \frac{B}{K} - a_m M_0 e^{-\delta t})$ (see main text), using the following parameter values: $B_0=10^6$; $m_0=10^6$; r=2.3; $K=10^8$; a_m= -3.7*10^{-6}; δ=0.1.

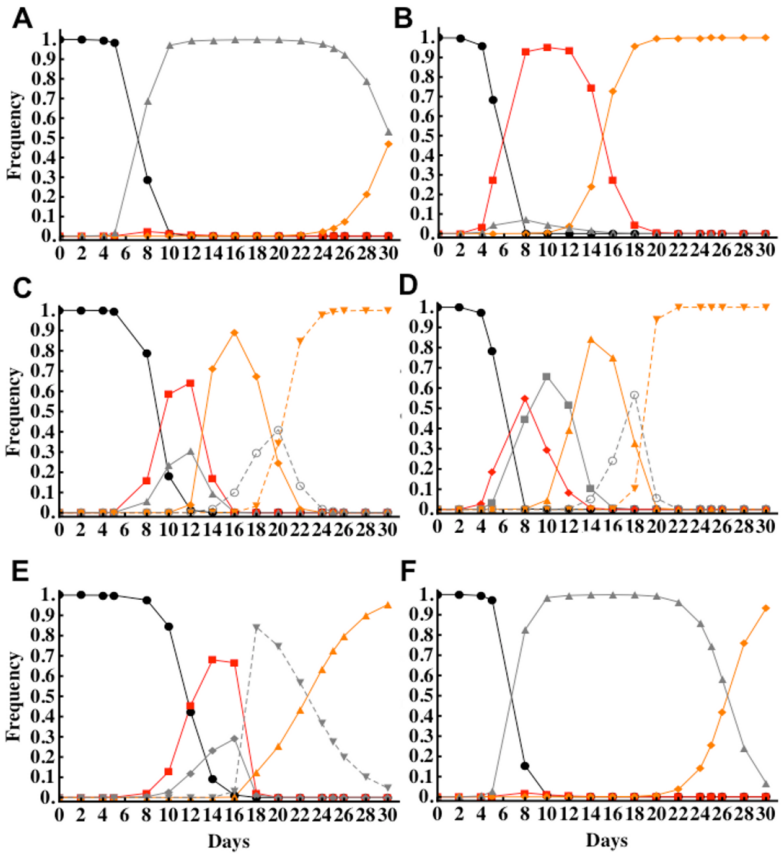

Figure A3. Dynamics for the different haplotypes under the model of clonal interference. Simulated frequencies of the different haplotypes which result in the

frequencies of the mucoid phenotypes of Figure 2.5. r=2.3, am =-3.7x10^{-6} and the other parameters used are as follows:

	U (x10^{-7})	Uis (x10^{-7})	r_m	a_{mm} (x10^{-6})	Uis' (x10^{-7})	r'	a_{mb} (x10^{-6})	r_m'	$a_{mm'}$ (x10^{-6})
A	3	4	2.1758	-3.23	12	2.369	-3.7	2.2171402	-3.23
B	0.96	4.45	2.21122	-3.2	3.84	2.392	-3.7	2.2554444	-3.2
C*	0.43	4	2.185	-3.24	1.72	2.36095	-3.7	2.2724	-3.24
D*	0.1	10	2.1988	-3.2	5	2.4104	-3.7	2.2691616	-3.2
E*	0.43	6.94	2.180768	-3.28	0.1075	2.346	-3.7	2.180768	-2.86
F	3	4	2.1758	-3.23	12	2.3736	-3.7	2.2247555	-3.23

Marked as * are dynamics where more haplotypes were assumed to reproduce the experimental dynamics and, therefore, additional parameters are required:

	U''(B'->B'')	r''	$a_{mb'}$ (x10^{-6})	Uis''(M'->M'')	$r_{m''}$	$a_{mm''}$ (x10^{-6})
C*	4.3 x 10^{-8}	2.47309513	-3.7	1.72 x 10^{-7}	2.3428444	-3.24
D*	1 x 10^{-8}	2.494764	-3.7	1 x 10^{-8}	2.32021774	-2.99
E*	4.3 x 10^{-8}	2.482068	-3.7			

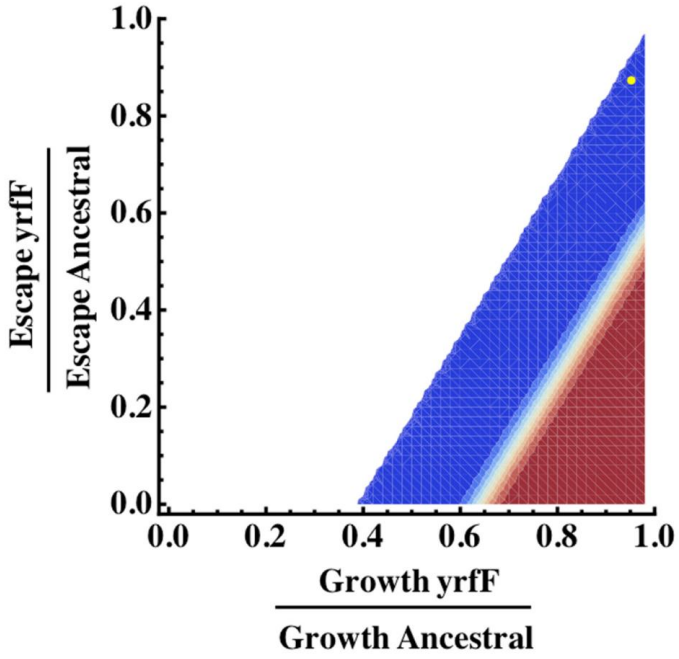

Figure A4. Region of parameter space theoretically expected for the invasion of first mucoid morph. Colored areas show the parameter region (r_m/r and a_{mm}/a_m) where a mucoid genotype (mimicking the IS insertion upstream of *yrfF* in the experiment) that has emerged is able to increase in frequency so that it can survive the bottleneck imposed every 24 hours in the experiment. The equations for these simulations are:

$$\frac{dB}{dt} = B\left(r - \frac{(B+M)}{K} - a_m M_0 e^{-\delta t}\right)$$

$$\frac{dM}{dt} = M\left(r_m - \frac{(B+M)}{K} - a_{mm} M_0 e^{-\delta t}\right)$$

with initial conditions M(0)=1, B(0)= 10^6 and the other parameter values as in Figure A4: M_0=10^6; r=2.3; K=10^8; a_m= 3.7*10^{-6}; δ=0.1. Warmer colors show higher frequency of the mucoid genotype in the population after 24 hours of its

emergence as a single copy. The black dot indicates the value of r_m and a_{mm}, of the first mucoid haplotype assumed to emerge in the 6 models that produced the dynamics in Figures 2. 5 and A5.

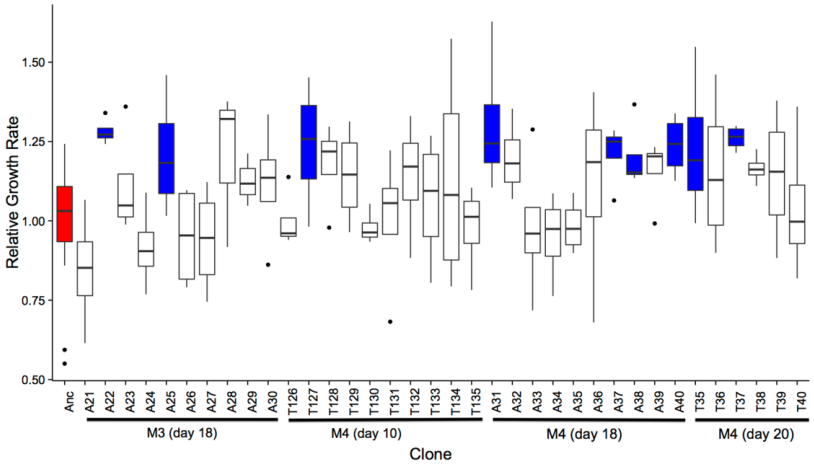

Figure A5. Relative growth rate for clones with an ancestral colony morphology (non-mucoid). Multiple clones were randomly isolated from two populations at different time points, as indicated in the x-axis, below the clone numbers. Replicate measures for the maximum growth rate of each clone were obtained from independent cultures and divided by the mean growth rate of the original ancestral. The ancestral for the main experiment (Anc) is highlighted in red, the evolved clones whose growth rate is significantly different from the ancestral are highlighted in blue (P < 0.05, ANOVA; white: not significantly different from ancestral). Anc: 16 replicates; evolved clones: 3-4 replicates

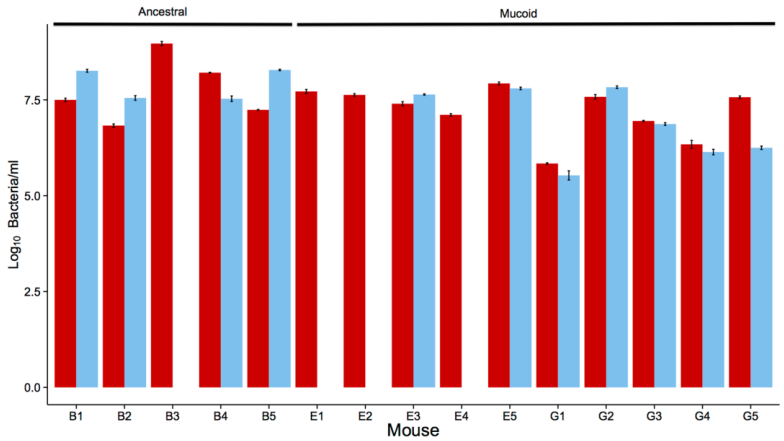

Figure A6. Bacterial densities for animals that failed to survive infection. Bacterial counts were obtained from plated samples of blood (red) or peritoneal contents (blue). Mice were infected with either the ancestral (B1-B5) or a mixture of mucoid clones (E1-E5 and G1-G5; see methods). For some animals it was impossible to obtain samples for the peritoneal contents (missing blue bars).

Table A1. Clones that were typed for existing mutations in M1 to M6 pops.

Population	Day	Morphs	Nr. of clones	*yrfF*	*lon*	*fusA*	*yegh*	*yiaW*	*potA*	*PotD*	*folD*	*trkH*	*hipA*	*wzc*
M1	18	ANC	10	-	-	-	-							
	30	MUC	11	+	-	+	+							
	30	ANC	10	-	-	-	-	-	-	-				
M2	5	MUC	8	+	-	-								
	5	ANC	2	-	-	-								
	30	MUC	11	+	-	+		-	-	-				
M3	18	ANC	10	-	-			-	-		-	-		
	18	MUC	6	+	-			+	-		-	-		
	18	MUC	2	+	-			+	-		-	-		
	18	MUC	2	+	-			-	-		-	-		
	30	MUC	7	+	-			-	-		-	-		
	30	MUC	2	+	-			+	+		-	+		
	30	MUC	1	+	-			+	-		-	+		
	30	MUC	1	+	-			+	+		+	+		
M4	8	MUC	8	+	-			-		-				
	8	ANC	2	-	-			-		-				
	16	ANC	6	-	-			-		-				
	16	MUC	3	+	-			-		-				
	16	MUC	1	+	-			+		-				
	20	ANC	6		-			-		-				
	20	MUC	2	+	+			+		+				
	20	MUC	2	+	-			-		-				
	30	MUC	2	+	-	-		-		-				
	30	MUC	1	+	-	-		+		-				
	30	MUC	8	+	-	-		+		+				
M5	8	ANC	10	-	-								-	-
	14	ANC	4	-	-								-	-
	14	MUC	6	+	-								-	-
	18	ANC	10	-	-								-	-
	20	ANC	6	-	-								-	-
	20	MUC	4	+	-								-	-
	30	MUC	1	+	-	-		-	-	-			+	+
	30	MUC	5	+	-	-		-	-	-			-	+
	30	MUC	5	+	-	-		-	-	-			-	-
M6	18	ANC	10	-	-			-		-				
	26	ANC	6	-	-			-		-				
	26	MUC	2	-	-			-		-				
	26	MUC	2	+	-			+		+				
	30	MUC	1	+	-	-		-		-				
	30	MUC	10	+	-	-		+		+				

Table A2. Primers used in this study.

Amplified region	Primers (5` - 3`)	PCR product (bp)
fusA	F: CAATTACGGCCTGAGCAACG R: CTGCACCTCGACATCATCGT	709
yegH	F: CGGATGATTGAGCGGGTACT R: ATCTCGCCATCCTTACGCAG	704
folD	F: CCCGACTTCTTCACAAGCCT R: AGAGAGCCCCGTTAGATGAA	518
trkH	F: CGTTGGACTACTGGTCATCTT R: CAGGTAGGAGGCAATCTCGC	1500
wzc	F: CGGCTTATGAGCTGGTTTGC R: AAAGCCCGCAAGTACTGGAA	883
hipA	F: ATAGCGATATCCTGCGACCT R: AATCATGACCGCCAACGACT	773
lon	F: GCAATACGGGGATTTCAATG R: GGAAGACGTCGAAAAAGTGG	408
yrfF	F: TTGCTCATCGTGGGATCTATGCTGT R: TCGGCATTCAGCGCCAGAGA	578
yiaW	F: TCACCACACCAGTCACCTGT R: CCTGGACTATTTTGCACTGGGA	547
potA	F: CATCAGCCAGTACGACCTCC R: CAATTGGCGGGAATTCGCAA	1069
potD	F: GCCTTCGGTATGAATCGCGT R: ATTACTGGTGCTGTCGCTGG	1314
cycA[1]	F: TTTCCAGCCACATGATGAAA R: GTTTATGGGGTCTGGCAAAA	1250

[1] – primers used to amplify *cycA* gene to test for the increase in general mutation rate.

Appendix B

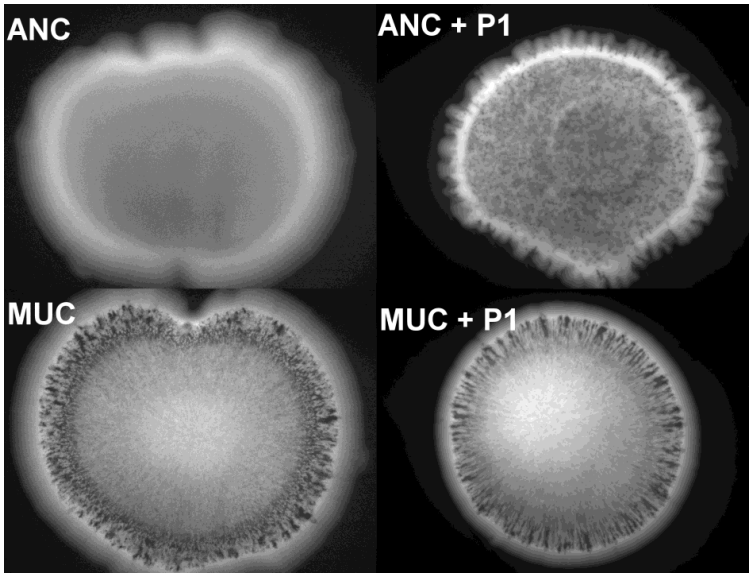

Figure B1. The effect of phage lysis in different clones. On the left panel, ANC and representative MUC clone (MUC clone isolated at 450 generations after evolution in the presence of the MΦs) growth without phage, on the right side, ANC and MUC clone growth with phage. Assay was done on soft agar plates. Visible phage plaque formation is clearly seen in the ANC clone with phage, but not in the MUC clone with phage.

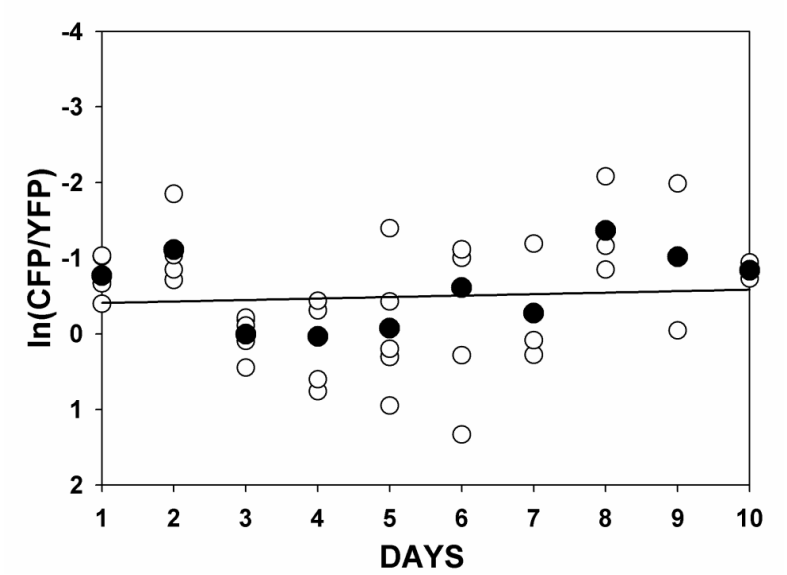

Figure B2. Competitive fitness assay of ANC clone in the gut. Control experiment (circles) to check for neutrality of the fluorescent marker was done by colonization of 1:1 ANC (expressing YFP) to ANC (expressing CFP). Filled symbols represent average ratio per day. Linear regression on ln(CFP/YFP) provides an estimate of the selective advantage of ANC-CFP per day: slope -0.02 (SE =0.05), P=0.069, adjusted R^2=0.0.

Table B1. Genetic characterization of SCV clones.

Population	Day	Nr. of clones	*yqiJ*[1]	*yjbS*[1]	*ycbU*[1]	*pepP*[1]	*yrfF*[2]	*lon*[2]
M1	8	9	-	-	-	-	-	-
	8	2	+	+	-	-	-	-
M2	4	9	-	-	-	-	-	-
	4	1	-	-	+	-	-	-
M3	5	5	-	-	-	-	-	-
	5	3	+	-	-	-	-	-
	5	1	+	+	-	-	-	-
M4	5	10	-	-	-	-	-	-
M5	8	8	-	-	-	+	-	-
	8	2	-	-	-	-	-	-
M6	4	10	-	-	-	-	-	-

[1] – targets of new insertions/transposition events were determined by direct sequencing of extra bands generated with the vectorette PCR protocol.
[2] – additional genes that were typed by amplification PCR (see Chapter 2 for primers that were used).

Table B2. Clones that were typed for existing mutations in 1:1 SCV to ANC co-inoculation.

Mice nr.	Clone	Day	Nr. of clone	*yiaW*	*yrfF*	*lon*	*yqiJ*	*yjbS*
-	SCV-CFP	0	S61	-	-	-	+	+
-	ANC-YFP	0	X0	-	-	-	-	-
7	**MUC-CFP**	10	X1	-	-	**+**	**+**	-
7	**MUC-CFP**	10	X2	-	-	**+**	**+**	-
7	**MUC-CFP**	10	X3	-	-	**+**	**+**	-
7	**MUC-CFP**	10	X4	-	-	**+**	**+**	-
7	**MUC-CFP**	10	X5	-	-	**+**	**+**	-
7	REV-CFP	10	X6	-	-	-	**+**	-
7	REV-CFP	10	X7	-	-	-	**+**	-
7	REV-CFP	10	X8	-	-	-	**+**	-
7	ANC-YFP	10	X9	-	-	-	-	-
7	ANC-YFP	10	X10	-	-	-	-	-
7	ANC-YFP	10	X11	-	-	-	-	-
9	**MUC-CFP**	10	X12	-	-	**+**	**+**	-
9	**MUC-CFP**	10	X13	-	-	**+**	**+**	-
9	**MUC-CFP**	10	X14	-	-	**+**	**+**	-
9	**MUC-CFP**	10	X15	-	-	**+**	**+**	-
9	**MUC-CFP**	10	X16	-	-	**+**	**+**	-
9	REV-CFP	10	X17	-	-	-	**+**	-
9	REV-CFP	10	X18	-	-	-	**+**	-
9	REV-CFP	10	X19	-	-	-	**+**	-
9	ANC-YFP	10	X20	-	-	-	-	-
9	ANC-YFP	10	X21	-	-	-	-	-
9	ANC-YFP	10	X22	-	-	-	-	-
10	**MUC-CFP**	10	X23	-	-	**+**	**+**	-
10	**MUC-CFP**	10	X24	-	-	**+**	**+**	-
10	**MUC-CFP**	10	X25	-	-	**+**	**+**	-
10	**MUC-CFP**	10	X26	-	-	**+**	**+**	-
10	**MUC-CFP**	10	X27	-	-	**+**	**+**	-
10	REV-CFP	10	X28	-	-	-	**+**	-
10	REV-CFP	10	X29	-	-	-	**+**	-
10	REV-CFP	10	X30	-	-	-	**+**	-
10	ANC-YFP	10	X31	-	-	-	-	-
10	ANC-YFP	10	X32	-	-	-	-	-
10	ANC-YFP	10	X33	-	-	-	-	-

Appendix C

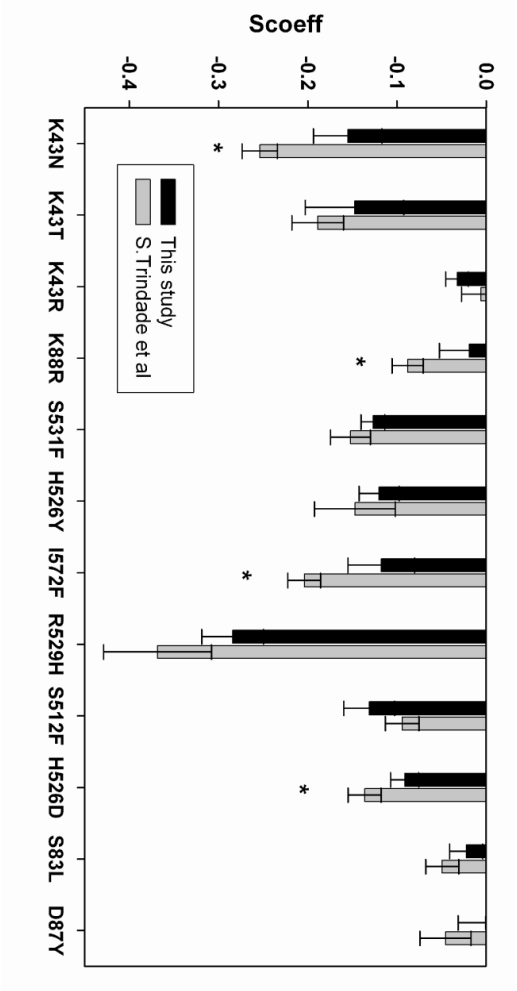

Figure C1. Fitness of antibiotic resistant clones in LB media. The asterisk (*) represents statistical significant difference ($p<0.05$) using Wilcoxon sum rank test without Bonferroni correction.

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