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Membrane proteins and virulence pathways: new approaches to design antimicrobial peptides

Dottoranda **Ilaria Granata**

Tutor **Ch.mo Prof. Bruno Maresca**

Coordinatore **Ch.ma Prof.ssa Antonietta Leone**

Human subtlety will never devise an invention more beautiful, more simple, or more direct than does Nature-because in her inventions, nothing is lacking-and nothing is superfluous….
Leonardo da Vinci

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Abstract

In the last two decades many studies have shown that lipid composition of biologic membranes as well as their structure have a primary role in cellular signaling.

In particular, the research group in which I have worked for my PhD thesis, has demonstrated that the primary sensor of temperature variations and, in general, of other kinds of stress is localized in the membrane. The imbalance in the membrane lipid/protein ratio of the pathogen *Salmonella* Typhimurium, due to over-expression either of the protein Δ^{12}-desaturase of the cyanobacterium *Synechocistis* or individually expressed membrane regions of the enzyme, caused major changes in the MPS (Membrane Physical State) and a significant impairment of the heat shock response (Porta *et al.* 2010). These results highlighted the possibility to identify molecules that, interacting with specific membrane lipids, can modify the functionality of membrane itself and consequently the biochemical/genetical properties of the whole cell. About thirty years ago, antimicrobial peptides (AMPs), molecules that are part of an ancient mechanism of the innate immunity of all Metazoa and plants, have been identified. However, from the over 1,000 identified peptides, no *consensus* sequence has been recognized and thus, as to-day, the possibility to predict and design an active AMP is still not feasible.

Starting from these assertions, through the study of membrane proteins and virulence pathways, my project has focused on the rational design of new AMPs and identification of key rules that allow the correct insertion of short peptides inside the phospholipid bilayer.

The amino acid sequence of the first *trans*-membrane region (p200) of the Δ^{12}-desaturase, that was shown to have antimicrobial activity (AMA), has been reduced to identify the minimal active sequence. Thus, three peptides, called A2/I2, A3/I3 and A4/I4, have been designed and their corresponding oligos inserted into the vector pBAD. The plasmids were used to transform *S.* Typhimurium LT2 strain. Transformants were then tested in terms of growth and survival outside and inside host cells (murine macrophages, ϕ). The results have shown that trimming of p200 determined a reduction of its AMA.

Further, it is well acknowledged that almost all, if not all, membrane proteins have more lysine and arginine residues on the sides of *trans*-membrane protein domains, and these residues are necessary for the insertion of proteins into the membrane bilayer. Thus, to verify the importance of this rule, called “positive inside rule”, a scrambled sequence of p200 has been designed, maintaining unchanged the five aminoacids preceding the *trans*-membrane region. From the growth curve and *in vitro* infection it was observed that p200 scrambled conserved the same activity of the original sequence, as expected.

Studying the virulence mechanisms of *Salmonella* survival inside host cells, the protein PhoQ of the two components system PhoP/PhoQ has been identified as the protein mostly involved in sensing and responding to ϕ environment and as a likely target to develop a new AMP. Based on our model, an AMP has AMA if it can interfere with lipids/proteins interaction of a specific domain and if this domain has a fundamental role in the pathogen’s survival and/or growth. Thus, the original sequence of the first *trans*-membrane region of PhoQ has been modified, obtaining two potential AMP candidates. The two resulting sequences, called NUF and STA, have been cloned and expressed in *Salmonella*. In particular, the peptide NUF has been very promising in determining the decrease of *Salmonella* persistence inside ϕ.

Based on these results, to make sure that the peptide NUF interfered with PhoP/PhoQ system, real time PCR analysis has been performed to determine transcript level of *rstA*, one of the genes regulated by PhoP/PhoQ system. The experimental procedures involved the growth of the strain expressing NUF and control strain in two different conditions, low and high Mg^{2+} concentration, that determine the activation and repression of PhoQ respectively. Furthermore, RNA deep sequencing analysis has been performed using the same experimental conditions of qRT-PCR, highlighting the down- and up-regulated genes in response to the expression of the peptide NUF. Finally, PhoQ protein of *Salmonella* has been purified to be reconstituted inside proteoliposomes to develop a fast screening method for testing peptides activity.

The most promising peptides, p200, p212 and NUF have been used for *in vivo* survival experiments with C57/B6 mice. Since encouraging results with peptide p200 have been obtained, it has been tested whether *Salmonella* expressing p200 induced protection and immunological memory in mice when rechallenged with the wild type virulent strain. Mice injected with *Stm*(p200) developed *Salmonella*-specific memory Th1 response and produced elevated serum levels of anti-*Salmonella* antibodies IgG2b.

Negli ultimi due decenni numerosi studi hanno dimostrato che la composizione lipidica e la struttura delle membrane biologiche hanno un ruolo fondamentale nel *signaling* cellulare. In particolare, il gruppo di ricerca presso il quale ho svolto il mio progetto di dottorato, ha dimostrato che il sensore principale delle variazioni di temperatura, ed, in generale, di altri tipi di stress, è localizzato a livello della membrana. Il disequilibrio del rapporto lipidi/proteine di membrana del patogeno *Salmonella* Typhimurium, dovuto alla sovra-espressione sia della proteina Δ^{12}-desaturasi del cianobatterio *Synechocistis* che delle regioni di membrana dell'enzima individualmente espresse, ha causato alterazioni dello stato fisico della membrana (MPS) e un significativo danneggiamento della risposta heat shock (Porta *et al.* 2010). Questi risultati hanno messo in luce la possibilità di identificare molecole che, interagendo con specifici lipidi di membrana, siano in grado di modificare la funzionalità della membrana stessa e di conseguenza le proprietà biochimiche e genetiche dell'intera cellula. Circa trent'anni fa, sono stati identificati i peptidi antimicrobici (AMPs), molecole appartenenti ad un antico meccanismo dell'immunità innata di tutti i Metazoa e delle piante. Tuttavia, dagli oltre 1,000 peptidi identificati non è stato possibile derivare alcuna sequenza *consensus* che permetta un disegno razionale di un AMP attivo.

Partendo da queste considerazioni, mediante lo studio di proteine di membrana e *pathways* di virulenza, il mio progetto ha avuto come oggetto di studio il disegno di nuovi AMPs e l'identificazione delle regole chiave che permettono il corretto inserimento di un peptide all'interno del *bilayer* fosfolipidico.

A tale proposito, la sequenza amminoacidica della prima regione *trans*-membrana della Δ^{12}-desaturasi (p200), dotata di attività antimicrobica (AMA), è stata ridotta al fine di identificare la minima sequenza attiva. Sono stati quindi disegnati tre peptidi, denominati A2/I2, A3/I3 and A4/I4, i cui oligo corrispondenti sono stati inseriti nel vettore di espressione pBAD e i plasmidi ottenuti sono stati usati per trasformare *S.* Typhimurium LT2. I trasformanti sono stati in seguito testati in termini di crescita e sopravvivenza al di fuori e all'interno di cellule ospiti (macrofagi murini, ϕ). I risultati hanno dimostrato che la riduzione della lunghezza della sequenza di p200 ha determinato anche la riduzione della sua AMA.

Inoltre, dalla letteratura è noto che quasi tutte, se non tutte, le proteine di membrana presentano una maggiore percentuale di residui di arginina e lisina ai lati dei domini *trans*-membrana, necessari per il corretto inserimento delle proteine stesse nel *bilayer* di membrana. Al fine di verificare l'importanza di questa "positive inside rule" nel direzionamento dei peptidi in membrana, la sequenza di p200 è stata modificata alterando l'ordine degli amminoacidi della regione *trans*-membrana e mantenendo invariati i 5 amminoacidi precedenti la medesima regione. Dalle curve di crescita e dall'infezione *in vitro* è stato osservato che il peptide p200 "scrambled" ha conservato la medesima efficacia della sequenza originale, come era atteso.

Mediante lo studio dei meccanismi di virulenza responsabili della sopravvivenza di *Salmonella* all'interno delle cellule ospiti, è stata identificata la proteina PhoQ del sistema a due componenti PhoP/PhoQ quale proteina fortemente coinvolta nel *sensing* e nella risposta all'ambiente macrofagico, e quindi potenziale target per l'attività di nuovi AMPs. Secondo il modello ipotizzato, un AMP presenta AMA se in grado di interferire con le interazioni lipidi/proteine di uno specifico dominio e se questo dominio ha un ruolo fondamentale nella sopravvivenza e/o crescita del patogeno. A tal proposito, è stata modificata la sequenza della prima regione *trans*-

membrana di PhoQ, ottenendo due potenziali candidati AMP. Le due sequenze risultanti, denominate NUF e STA, sono state clonate ed espresse in *Salmonella*. In particolare, il peptide NUF ha mostrato risultati promettenti, determinando la riduzione della capacità di persistenza di *Salmonella* all'interno dei ϕ.

Al fine di verificare che l'attività del peptide NUF fosse dovuta ad un'alterazione a livello del sistema PhoP/PhoQ, è stata effettuata un'analisi di real time PCR per poter determinare i livelli di trascritto di *rstA,* uno dei geni regolati dal sistema in questione. L'esperimento è stato condotto crescendo il ceppo esprimente NUF e il ceppo di controllo in due differenti condizioni ambientali, bassa ed alta concentrazione di Mg^{2+}, che determinano rispettivamente l'attivazione e la repressione di PhoQ.

E' stata successivamente effettuata un'analisi di RNA deep sequencing, utilizzando le medesime condizioni sperimentali della qRT-PCR, che ha permesso l'identificazione dei geni sotto e sovra-regolati in seguito all'espressione del peptide NUF. Infine, la proteina PhoQ di *Salmonella* è stata purificata per essere successivamente ricostituita in proteoliposomi al fine di mettere a punto un sistema di *screening* rapido dell'attività di nuovi peptidi.

I peptidi considerati più interessanti in seguito alla sperimentazione *in vitro* sono stati utilizzati in esperimenti di infezione *in vivo* di topi C57/B6. Dal monitoraggio della sopravvivenza dei topi infettati i risultati sono stati particolarmente incoraggianti nel caso del ceppo di *Salmonella* esprimente il peptide p200.

I topi infettati con il ceppo *Stm*(p200) hanno difatti sviluppato memoria Th1 *Salmonella*-specifica e hanno prodotto elevati livelli sierici di anticorpi anti-*Salmonella* appartenenti all'isotipo IgG2b.

The capacity to treat bacterial infections is crucial for modern medicine: without antibiotics any clinical procedures would not be feasible because of the infection risk. The discovery of the antibiotics was one of the major successes of medicine that started with the introduction of sulfonamides in the 1930 followed by a golden age, between 1940 and 1960, of discoveries of natural product that determined the so called “antibiotic revolution”. After that period, there have been two decades of repeated successes in modifying natural antibiotics to enhance their activity. Subsequently, the flow of new antibiotics dwindled. The technical difficulty of discovering new antibiotics, especially those able to penetrate Gram-negative bacteria, as well as the increasing complexity of regulatory hurdles for the development of new antibiotics and, in addition, the fact that antibiotics, as short-course treatments, do not represent a valuable investment for the pharmaceutical industry unlike long-term therapies for chronic conditions, have contributed to the sharp decline of licensed antibiotics in the 1990s and in the first decade of this century (Coates *et al.* 2011). The lack of new molecules represents one of the factors that determines the continued accumulation of resistance favored by natural selection which reduces the efficacy of existing antibiotics. Resistance increases the risk of inactive antibiotics, especially in severely ill patients, leading to increased mortality, lengthy hospital care and cost.

Salmonellosis is a worldwide invasive disease in humans and animals that is associated with contaminated food and water. In particular, *Salmonella* Typhimurium is an important pathogen in food-producing animals, that represent the primary source of salmonellosis. Treatment of infections caused by multi-resistant Enterobacteriaceae is limited to chloramphenicol, ampicillin and cotrimoxazole (Butaye *et al.* 2006). Ciprofloxacin resistance strains, associated with point mutations in the *gyr* genes (Khan *et al.* 2005) are increasingly isolated in human typhoidal and non-typhoidal serotypes that have been associated with failures of treatment. In particular, in resource-poor countries such as Africa, drug resistant *Salmonella* infections may soon become untreatable and *Salmonella* species are the most frequently causative agent of septicaemia in malarial areas. Since 1998 only two drugs have been approved by FDA (Food and Drug Administration, USA). Isolation of mutants resistant to present antibiotics is a highly serious health problem, thus alternatives are badly needed.

It is a common belief that vaccines have been the best solution to decrease mortality due to infections than any other measure besides pure water. Unfortunately, methods for vaccines development also present significant limitations, thus new strategies are necessary. To date, there are no vaccines yet for many important diseases. Although in some cases commercial priorities have limited the development of new vaccines, for many of the lacking vaccines there are technical problems such as the complexity of correlates of protection and the difficulty to obtain vaccines with the correct presentation of antigens. Most of our current vaccines have been produced by growing live microorganisms in conditions that reduce their virulence, by the use of suspensions of killed microbes or through concentration of proteins or polysaccharides purified from pathogens. Luckily, the studies about virulence mechanisms and immune response, as well as the advent of molecular biology, provided us with new tools to develop novel vaccines.

Though in recent years no new molecular targets for the development of new antibiotic molecules have been identified, the current understanding of plasma membrane is providing new strategies to overcome this limitation.

It is widely recognized that the biologic membranes are highly dynamic structures that are made of heterogeneous regions, generally defined as microdomains which are required to maintain the appropriate membrane physical state (MPS) and functionality (Laude, Prior 2004).

There are numerous evidences that these domains, namely rafts, caveolae, microvilli, are directly involved in cell signaling and response to different stresses (Vigh *et al.* 2005).

Recently, a mechanism alternative to the amount of denatured proteins to explain the transcriptional activation of heat shock genes (HSG) has been proposed. This new model comprises membranes since a heat stress determines an abrupt alteration of MPS that initiates a signaling cascade that induces transcription of HSG. This model has been largely demonstrated both in prokaryotic and eukaryotic cells (Balogh *et al.* 2005; Carratù *et al* 1996; Horvàth *et al.* 1998; Saidi *et al.* 2009; Vigh *et al.* 2005).

The new appreciation of the role of membrane resulted in an increasing interest in a particular class of antimicrobial molecules that interact with biological membranes.

AMPs represent an evolutionary very ancient (>500 MY) immunological defense against microbes, and are produced by all metazoa (Cole *et al.* 1997; Lemaitre, Hoffmann 1997, Selsted *et al.* 1983, van Dijk *et al.* 2008, Zasloff 1987) and plants (Castro, Fontes 2005). Nearly all AMPs act through a direct interaction with microbial membranes causing dislocation of key membrane lipids and the permeabilization and/or disruption of the bacterial (and fungal) cell membrane. However, a certain number of AMPs penetrate inside the cell and interact with cytosolic targets while some AMPs exhibit both mechanisms (Yeaman, Yount 2003). The peptides identified so far are constituted by 7 up to 100 amino acids and, depending on structure, classified as α-helix, β-sheet, loop or extended peptides. However, the highest percentage is represented by α-AMPs (Bhattacharjya and Ramamoorthy 2009). In the last several years, a number of natural AMPs have been selected for human clinical studies. Although the exact mechanism of action of α-AMPs is not clear yet, it is generally agreed that they disrupt (may be partially) cell membrane, and that factors as peptide volume, cationic charge, 3D-arrangement and oligomeric state play an important role for their insertion into membrane (Papo, Shai 2003). Generally, AMPs are not toxic to human cells since they have lipid membrane significantly different compared to bacteria. "Landing" of a peptide on a membrane constitutes a highly dynamic mechanism (membrane perturbation, pore formation and carpet mechanism) followed by its insertion (Park, Hahm 2005). α-AMPs are universal multifunctional molecules to which not many resistance mechanisms have been reported so far. AMPs, whose mode of action is strictly associated with membrane, represent an alternative to the classical antibiotics. However, a consensus sequence from the over 1,000 known AMPs has not been identified (http://aps.unmc.edu/AP/main. html). Prediction of a sequence with potential AMA-did not succeeded so far considering that a 15 aa-long AMP is one of 20^{15} different possible peptide sequences. Though, it is recognized that a specific amino acid sequence and its 3D-conformation are critical (Mátyus *et al.* 2007), all efforts to predict sequences of active synthetic AMPs have been unsuccessful.

The increasing of antibiotics resistance is one of the more serious challenge of the modern medicine. It is well-known that bacterial resistance to various antimicrobial agents has developed as a direct response to their exposure (Cantón, Morosini 2011). Bacteria have developed different mechanisms to resist antibiotics, such as alteration of the target protein by mutation or enzymatic activation, the acquisition of genes from other bacterial species encoding for less susceptible target proteins, bypassing of the target protein, or the extrusion of the antimicrobial from the cell. These adaptations can arise in susceptible bacteria as a result of mutations or through horizontal gene transfer, either within or between genera, primarily employing mobile genetic elements such as plasmids, transposons or integrons (Giedraitienė *et al.* 2011).

Antibiotic resistance continues to develop in a wide range of microbes (Hawkey 2008; Hawkey and Jones, 2009; Livermore 2003). Surely one of the factors that largely contributes to this spread is the inappropriate use of antibiotics, determined by the overuse of powerful, broad-spectrum antibiotics, the presence of antibiotics in the food/livestock industry (Arnold *et al.* 2007; Levy 1998; Monroe, Polk 2000), and by the inclusion of antimicrobials in household products (Aiello, Larson 2003). For all these reasons, many strains of pathogenic bacteria are now multidrug-resistant (defined as resistant to three or more antibacterial drug classes) and consequently problematic to treat.

Furthermore, also vaccines development need new strategies. As we know, the success of a given vaccine depends on the stability of the antigen as well as its ability to elicit immunological memory. In modifying the pathogen or using a small part of it, some of the vaccine's immunogenicity may be lost. The ability to create genetically modified pathogens by eliminating virulence or essential genes is considered a powerful alternative in the development of an effective protective vaccine. The current genetic procedures are based on the use of specific (virulence) genes that may cause attenuation and are species specific. In other words, a given method of attenuation may be effective in a specific organism but not in other pathogens. Both these issues represents a real threat to human health and has led to efforts to develop new antimicrobial agents, including alternative drugs and strategies based on antimicrobial peptides (Hancock 2001; Lata *et al.* 2007).

CHAPTER 1

Antibiotic resistance

The discovery of antibiotics is rightly considered one of the most significant health-related events of modern era. In the 60 years since their introduction, millions of metric tons of antibiotics have been produced, for many purposes. This huge amount has certainly contributed to the selection of resistant strains. The development of generations of antibiotic-resistant microbes and their distribution in microbial populations are the results of too much years of continue selection pressure from underuse, overuse and misuse of antibiotics by humans (Julian Davies and Dorothy Davies 2010). Today the worry about the return to the pre-antibiotic era or "dark-age" is one of the main problems of modern medicine (Fig. 1.1).

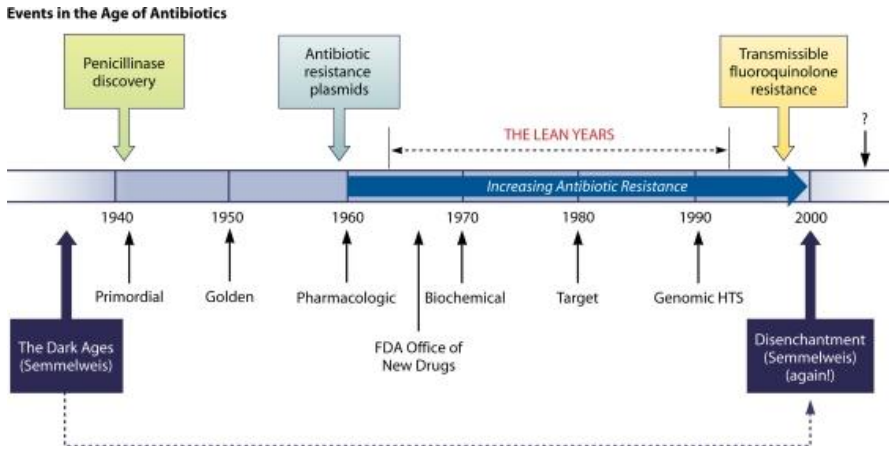

Figura 1.1- History of antibiotic discovery and concomitant development of antibiotic resistance.

A wide range of biochemical and physiological mechanisms are responsible for bacterial resistance, (Fig. 1.2):

- **Mutation of the target protein:** Bacteria can become resistant through mutations that make the target protein less susceptible to the agent. For example, fluoroquinolone resistance is mainly due to mutations in the target enzymes, DNA topoisomerases, and is easily transferred to other cells on plasmids (Hooper 2000).
- **Enzymatic inactivation of the drug:** This is a common mechanism for antibiotics of natural origin, such as aminoglycosides, which are inactivated by enzymatic phosphorylation, acetylation or adenylation (Davies, Wright 1997; Wright 1998), and β-lactames, which are inactivated by enzymatic

hydrolysis by β-lactamases (Datta 1965; Vu, Nikaido 1985), usually in the periplasm.

- **Acquisition of genes for less susceptible target proteins from other species:** Sequencing of the genes coding for the targets of penicillin, DD-transpeptidase or penicillin binding proteins (PBPs), revealed that penicillin resistance among *Streptococcus pneumoniae*, which is particularly subject to natural transformations, is due to the production of mosaic proteins, some of which coming from other organisms (Spratt 1994). MRSA (methicillin resistant *Staphylococcus aureus*) strains also contain a new methicillin-resistant PBP, whose gene is located in a large (30-60 kb) DNA segment, possibly coming from a different organism. This DNA fragment also contains additional antibiotic resistance genes (Kuroda *et al.* 2001), even though *S. aureus* does not transform in nature.
- **Bypassing of the target:** Vancomycin exerts its activity by binding to a precursor of cell wall peptidoglycan. Therefore, it has been proposed that resistance to vancomycin could emerge. However, vancomycin resistance is now prevalent among enterococci, normally living in the human's intestinal tract. Because enterococci are naturally resistant to β-lactams, aminoglycosides, macrolides, and tetracycline, these vancomycin-resistant strains of enterococci have become prevalent in a hospital's environment, colonize patients, and cause infections that are difficult to treat. Study of the resistance mechanism showed that the end of the pentapeptide, D-Ala-D-Ala, to which vancomycin binds, is replaced in the resistant strains by an ester structure, D-Ala-D-lactic acid, to which vancomycin does not bind. Production of this altered structure requires the participation of several imported genes (Courvalin 2006).
- **Preventing drug access to targets:** The active efflux of drugs plays a predominant role in the resistance. This mechanism is associated to the common tetracycline resistance, in which protein TetA of Gram-negative bacteria (Levy 1992), catalyzes a proton-motive-force-dependent outward pumping of a tetracycine-Mg complex (Tamura *et al.* 2003). Analysis of *S. aureus* strains that were resistant to multiple cationic bacteriocides and were causing nosocomial infections showed, however, that these strains contained plasmids coding for a multidrug efflux transporter QacA (or QacB), belonging to the Major Facilitator Superfamily (MFS), the first multidrug efflux pump identified in bacteria. Since then, multidrug efflux pumps belonging to various families have been discovered, and the contribution of such pumps to multidrug resistance became clear.

  Other examples of resistance determined by inhibition of drug uptake are Tet(M) or Tet(S) proteins, produced by plasmid-coded genes of Gram-positive bacteria, that bind ribosomes and change their conformation preventing tetracyclines association (Connell *et al.* 2003). Further, plasmid-coded Qnr proteins, which have become more widespread in recent years, are thought to protect DNA topoisomerases from (fluoro)quinolones (Robicsek *et al.* 2006).

  Finally, porin mutants are found in some species of Enterobacteriaceae (*Enterobacter aerogenes, Klebsiella pneumoniae*) as a last resort resistance to the more recent versions of β-lactams that withstand inactivation by common β-lactamases. Mutations within the coding sequences of the porin also have been reported, reducing possibly the permeation rates of bulky β-

lactams without affecting those of smaller nutrient molecules (Achouak *et al.* 2001).

Antibiotic	**Method of resistance**
Cloramphenicol	reduced uptake into cell
Tetracycline	active efflux from the cell
β-lactams, Erythromycin, Lincomycin	eliminates or reduces binding of antibiotic to target
β-lactams,Erythromycin	hydrolysis
Aminoglycosides, Cloramphenicol, Fosfomycin, Lincomycin	inactivation of antibiotic by enzymatic modification
β-lactams, Fusidic acid	sequestering of the antibiotic by protein binding
Sulfonamides, Trimethoprim	metabolic bypass of inhibited reaction
Sulfonamides, Trimethoprim	overproduction of antibiotic target (titration)
Bleomycin	binding of specific immunity protein to antibiotic

Figure 1.2- Mechanisms of antibiotic resistance

Multidrug resistance in bacteria derives from the accumulation of mutations in genes coding for resistance specific proteins or efflux pump, on resistance plasmids (R) or transposons (French *et al.* 1987; Paulsen 2003). Many genes present in R plasmids contain a specific tag that is necessary for the integration into a region that contain a gene coding for an integrase that catalyzes the insertion of resistance genes (Hall, Stokes 1993). In addition, R plasmids often contain genes that allow the correct partitioning of copies to daughter cells, so that these plasmids appear particularly stable and are rarely lost during replication (Nordström, Austin 1989). Moreover, their transfer among cells occurs at a very high efficiency, because of the presence of transfer genes and an efficient injection systems, now known as Type IV secretion systems (Christie *et al.* 2005; Schröder, Lanka 2005).

The population of *r* genes in nature is referred to the environmental antibiotic resistome (Baysarowich *et al.* 2008; Enright *et al.* 2002).

Cloning, PCR and gene expression are the techniques used up to know to detect natural *r* genes in random recombinant clones of DNA libraries obtained from bacteria isolated from soil and sediments (Allen *et al.* 2010; Riesenfeld *et al.* 2004). The identification requires the expression in an heterologous host and so far only *E. coli* has been used, determining perhaps the loss of many r genes that would have been found using a wide range of expression systems and hosts.

As mentioned above, since the 1940s the use of antibiotics was not restricted. The therapeutic use in humans and animals does not represent the only application, some alternative uses are: aquaculture, pest control/cloning for plants and agriculture, biocides in toiletries and in hand care and household cleaning products, culture sterility, cloning and selection in research and industry.

Today in many Western countries, antibiotic use is totally out of control and it is clear that antibiotic resistance seems to be an inevitable drawback. Over the years many solutions have been proposed, among which restricting the use of

antibiotic, requiring individual prescriptions, avoiding delivery without a doctor's prescription and controlling their uses in animals and agriculture.

Chemical modifications of classic compounds have been maden, but in many instances the target for resistance mechanisms cannot be modified or removed without reducing antimicrobial activity. Further, modifications of the molecules may lead to enhanced toxicity, and, *r* genes adapt in response to new selection pressures.

An alternative measure to the scarcity of new antibiotics and to reduce evolutionary pressure is what is usually called "cycling" antibiotics, namely the periodic replacement of front-line drugs with alternative classes. Another strategy is treatment with combinations of compound having different mode of action, but of course this approach needs a in depth study of pharmacodynamic properties of the drugs.

Also nonantibiotic approaches has been considered, involving stimulation or recruitment of innate immune system. Recently, the lack of new innovative active molecules led to re-evaluate neglected strategies as the use of bacteriophages for infections therapy.

Certainly, one of the main strategy remains the control of disease via prevention. Vaccines can drastically reduce the use of antibiotics, but up to now there are still few widely used antibacterial vaccines.

Thus, it is urgent to find new strategies and ways to cope with these problems and avoid the return to preantibiotic era.

CHAPTER 2

Methods of vaccine development

In the late 19th and early 20th century, bacterial constituents were defined as "antigen", and later as "immunogen". Paul Ehrlich in the 1897 formulated a theory that physiologically active substances interacted with specific receptors in the blood and introduced the concept of antigens ("**anti**body **gen**erators") as the ligands of antibodies. The receptors of T and B cells specifically recognize limited and unique parts of an antigen molecule during an adaptive immune response; therefore the selection and the correct presentation of an appropriate antigen is central to vaccine design. In addition to these specific antigenic components, there are several other types of pathogen constituents that are essential to the induction of innate and subsequent adaptive immune responses, that may be considered as "defensive triggers".

Pathogen-based approaches to produce vaccine antigens varies depending on the complexity of the material they contain. This may include the use of whole viruses or bacteria, in the form of reassortant, attenuated or inactivated microbes (Plotkin 2009).

Live attenuated vaccines are based on diminished virulence of pathogens, mimicking the protective immunity induced in individuals who survive infections. Attenuated pathogens are alive and replication-competent but are altered in some characteristics to reduce their virulence in the target host. Examples of live attenuated vaccines include vaccines against invariant pathogens, such as smallpox, yellow fever, measles, mumps, rubella and chicken pox. These vaccines often confer long-term immunity but there are pathogens that mutate rapidly, or that exist as multiple serotypes or that cause persistent or latent infections, thus making these kind of vaccination not appropriate.

Using inactivated pathogens to induce immunological memory requires the use of pathogens killed by thermal or chemical methods (Plotkin 2005). In the 19th century it was showed that whole organisms could be killed without losing immunogenicity, such in the case of vaccine for typhoid, colera, pertussis, influenza and hepatitis A.

Reassortant pathogens are a subtype of attenuated organisms, containing genetic material derived from at least two different strains of the same pathogen, and that express proteins derived from all component strains. When whole-pathogen approaches are not feasible, other procedures, such as the use of fractionation, subunit or recombinant antigens, are needed. The strategy to inject purified microbial proteins includes the vaccines against recombinant hepatitis B, the inactivated toxins against diphteria and tetanus, the carbohydrate vaccine against *Pneumococcus* and conjugate vaccine against *Haemophilus influenza* type B or *Meningococcus* (Plotkin 2003) (Fig.2.1). The choice of antigen depends on which one is the best in terms of safety and immunogenicity and attainable by the current standards of technology. The first vaccines using purified antigens were developed against pathogens with little antigenic variability that resulted in life immunity to further exposure to the same pathogen. Problems emerged when vaccines against bacteria with a high level of antigenic variability were utilized. For example, vaccines were developed by inducing antibodies that recognize the capsular polysaccharide (CPS), and in spite of the

problem of antigenic variability, successful vaccines were developed for *Streptococcus pneumoniae*, *H. influenzae*, and *Neisseria meningitidis*.

However, these pathogens have highly variable capsular serotypes, and rarely cross-protection between serotypes exists. Further, strains can shift in serotype, and novel serotypes can develop. Thus, for CPS-based vaccines, capsule diversity still needs to be tested continuously (Telford 2008). Furthermore in many acute infections, natural infection itself does not confer protection against reinfection, so the vaccine development may improve what nature does.

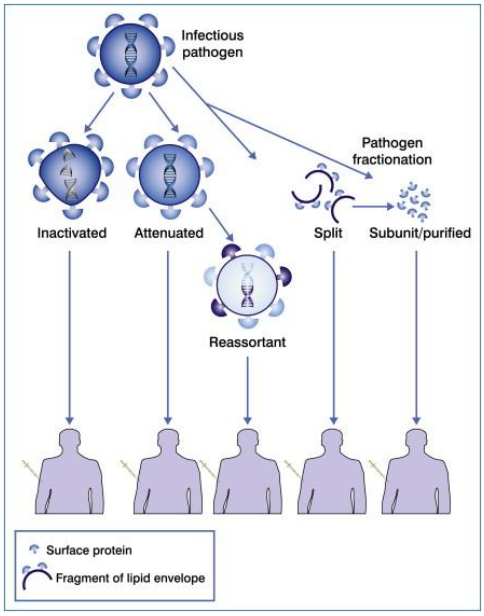

Figure 2.1 - Approaches to vaccine antigen selection. Whole pathogen-based vaccines need to undergo attenuation or inactivation processes, while subunit vaccines rely on purified fractions of pathogens derived by physical disruption of whole organisms.

Currently, thanks to improved knowledges of pathogens virulence mechanisms and immune response and to molecular biology and biotechnologies development, innovative approaches have been proposed and implemented, as those based on the production of mutant strains, expression of proteins in heterogeneous systems, purification and synthesis of antigens and induction of immune response through nucleic acids, proteins and polysaccharides (Fox *et al.* 2005; Moxon and Tang 2003; Peiser *et al.* 2006; Schweda *et al.* 2007) (Fig.2.2).

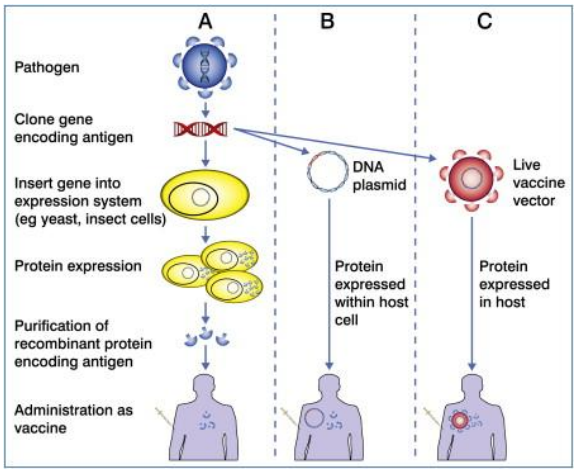

Figure 2.2 - Recombinant/DNA approaches to vaccine antigens.Protein antigens are produced using recombinant DNA technology, by which the DNA sequence coding for the antigenic protein is inserted into an expression system that produce large quantities of that specific antigen *in vitro* (panel A) or following administration to the host, e.g., using a DNA plasmid (panel B) or a live vaccine vector (panel C) as the expression system.

Moreover, new strategies using multivalent vaccines are also being employed but novel approaches need to be based on antigens that are not under immune pressure during infection. A successful acellular vaccine against *Bordetella pertussis* based on the identification of major virulence factors has been produced (Rappuoli 1996) and a vaccine containing three important virulence factors that confer protection against intragastric *H. pylori* is under study (Telford 2008). More recently, the possibility of sequencing entire genomes and the use of bioinformatics have led to the opportunity to identify genes coding for specific antigens (Mora, Telford 2010). However, though these very powerful techniques are providing important data on potential antigens to be used in vaccines, they still rely on experimental data generated on each single microorganism and results cannot be generalized to other pathogens. Thus, traditional strategies are still in use, even if though they are less safe and effective.

For example, it is very important that vaccines based on killed cells do not contain any live cells or toxic molecules, as well as vaccines with attenuated strains must not include denatured proteins unable to determine an appropriate immunological response.

In addition, vaccines made using purified antigens have often low efficacy, since they do not cover the whole antigenic *repertoire* of the pathogen.

For these reasons it is necessary to study and develop new methodologies to produce vaccines more effective and safer.

CHAPTER 3

Membranes

3.1 Membrane fluidity: new concepts of functional and structural organization

In 1972 Singer and Nicholson refined the concept of how the membrane structures of living organisms are organized, replacing the simple bilayer model of the fluid mosaic model. This new concept was based on a structure in which proteins are immersed and both lipids and proteins are free to move laterally and rotationally. The structure can be summarized as “a two-dimensional orientated solution of integral proteins…in a viscous phospholipids bilayer”.

In the last decade the concept of functional and structural organization of biological membranes has been further revisited, so that it explains why there are so many membrane lipids and why their types and concentrations are different in different species and among different organelles of the same organism (Fig.3.1).

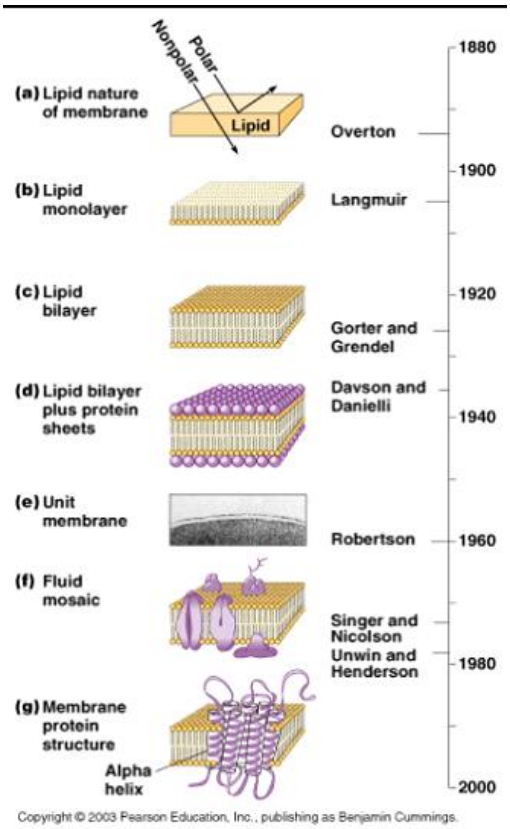

Figure 3.1- Evolution of the concept of membrane structure

A large number of studies suggest that membranes are much more than hydrophobic barriers and a home for proteins.

Lipids are natural molecules whose ability to self-assemble in dynamic macrostructures in water has been recognized as one of the important mechanisms of the origin of life (Hanczyc, Szostak 2004; Luisi *et al.* 1999). This ability depends on the amphiphilic nature of lipid molecules which tend to aggregate so that the hydrophobic (tails) and the hydrophilic (heads) parts of the lipid are well separated, and the area of the dividing surface is held by the hydrophobic effect (Tanford 1978).

Cellular membranes are strongly dynamic and undergo constant remodelling to maintain several dynamic functions (McMahon, Gallop 2005; Paiement, Bergeron 2001). However, this dynamism must not interfere with the barrier function and overall stability. This balance between flexibility and resistance is allowed by lipid bilayer and by the existence of different lipid species (Dowhan 1997). In biological membranes, there is often a mixture of liquid-disordered (Id) and liquid-ordered (Io) phases with the abundance of one or another dependent upon lipid composition.

The disordered lipid phases are characterized by rapid diffusion in the plane of membrane and a poorly ordered structure in the hydrophobic core of the bilayer, while Io is characterized by the tight packing of the extended acyl chains of lipids maintaining a certain degree of lateral mobility, as in the gel phase (Frolov *et al.* 2011). Lipids with unsaturated fatty acids (UFAs), which have kinks in their acyl chains, increase the propensity of a bilayer toward an Id organization (Thompson Jr. 1992, Beney and Gervais 2001). It is now generally accepted that this phase represents a real plasma micro-domain known as lipid raft, which fulfills an important role in signal transduction (Escriba *et al.* 2007). Membrane structure is generally in a liquid crystalline phase (Lα), characterized by high mobility of the fatty acid moieties in the central hydrophobic region of the bilayer. However, under different condition lamellar bilayers organize into more ordered states, such as the gel-like phase (Lβ), the pseudo-crystalline phase (Lc). *In vitro*, lipid curvature dominates the organization of lipids in to hexagonal (HI) or inverted hexagonal phases (HII). Lipids as phosphatidylethanolamines (PEs) as well as cholesterol can induce HII phase organization. However, non-bilayer lipids such as PE can be stabilized in a bilayer structure by the presence of bilayer preferring lipids such as phosphatidylcholine (PC), phosphatidylserine (PS) or sphingomyelin (SPM). It is usually found that between 20 and 50 mol% of the bilayer preferring HI lipids are required to maintain a net bilayer organization when mixed with HII preferring lipids such as PE. The “nonbilayer” lipids have important functions, since they mediate proteolipid interactions within the lipid bilayer (Ces, Mulet 2006; Dowhan 1997; Lee 2004) and an increase morphological plasticity of the lipid bilayer (Hafez, Cullis 2001) (Fig.3.2).

Microorganisms able to live at different temperatures can alter the ratio between these two kind of lipids when necessary (de Kruijff 1997). Large amounts of nonbilayer lipids, trans-bilayer asymmetry and lateral interactions and segregation of protein and lipid species are the factors that determine the involvement of lipids in membrane morphogenesis. The transformation of the lipid bilayer (lamellar phase) into nonbilayer structures (phases) is the classical manifestation of lipid polymorphism (de Kruijff 1997). The transformation is driven by the stored stress, which is augmented with temperature as it increases the repulsive pressure in the tail region and, thus, the bending torque.

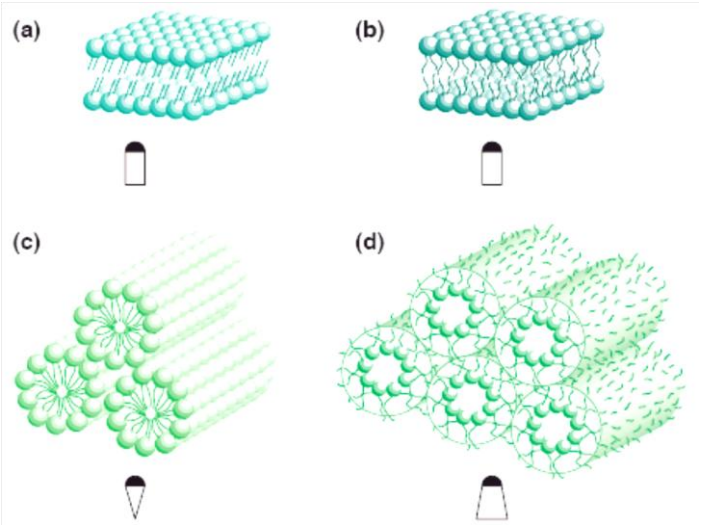

Figure 3.2. Possible arrangements of amphipathic membrane lipids. (a) Bilayer (lamellar phase) in which the acyl chains of the lipids are in the gel phase. (b) Bilayer (lamellar phase) in which the acyl chains of the lipids are in the liquid phase. At the transition temperature (Tc), membrane lipids change from the gelcrystalline to the liquid-crystalline state. (c) The hexagonal (non-lamellar) H_I phase is characterized by cylinders of lipids in which the latter's polar groups are exposed on the surface; a core of hydrocarbon chains adopt a disordered liquidlike conformation. The cylinders are hexagonally arranged in an aqueous medium. (d) The hexagonal (non-lamellar) H_{II} phase is the reverse of H_I (i.e. water forms cylinders and hydrocarbon chains form the continuous medium, and the polar groups lie at the water–hydrocarbon interface). H_{II} phases are adopted by membrane lipids that have small headgroups and whose acyl moieties occupy a large volume (e.g. unsaturated phosphatidyl-ethanolamines). H_I phases are formed by lipids that have large headgroups (e.g. monoacylphosphatidylcholine); because such molecules seldom occur *in vivo*, only the H_{II} phases are common non-lamellar arrangements. The shapes of lipids forming the different phases are shown below each diagram. Reviewed in Vigh *et al*. 1998

The presence of real non-lamellar structures *in vivo* is not very frequent and are typically restricted to membrane condition with extreme curvature like vesicle fission, during endo and exocytosis or cell division (Vigh *et al.* 1998).

Many membranes are indeed heterogeneous and direct the synthesis and positioning of lipids in individual tissues and in functionally different membranes within the cell. The most often proposed physiological significance of membrane lipids is the absolute requirement of some enzymes bound to a particular lipid to maintain functional activity, the requirement of a precisely defined physical state, or fluidity.

Each membrane is determined in a very precise way by the interactions among the individual lipids and proteins, and the direct participation of select lipids in transmembrane signaling processes.

The investigation of membrane lipid components is based on the differentiation of at least three aspects of movement:

• Physical parameters of the environment, molecular mobility and structural arrangement of the lipids;

• Biochemical processes that continuously turn over and change membrane lipid composition;

• The ultrastructure or the morphology of the bilayer, related to the shape, size, the division cycle, and the endo- or exocytotic activities of the living cells and organelles that characterize their continuous evolution.

Proteins in biological membranes reside in a complex environment in which they are surrounded by many different lipid species. The lateral organization of cell

membranes is formed by the clustering of specific lipids into highly condensed areas that form microdomains (Vigh *et al.* 2007). Such microdomains (caveolae, detergent-insoluble membranes and rafts) are distinct from other regions of membrane by their lipid structure (liquid-ordered *vs*. disordered domains) and have been identified by 2-photon (Gaus *et al.* 2003) and atomic force microscopy (Johnston 2007) (Fig.3.3). The special physical and functional activities ascribed to specific lipid in biological membranes reflect their particular organization and composition, properties that are hypothesized to depend on the differential partitioning of various membrane components between liquid-ordered and liquid-disordered lipid environments (Silvius 2005). *Trans*-membrane proteins have different affinities for different lipids, and, as a result, particular lipids or members of particular lipid classes may become enriched around a certain protein. Such differences in affinity is due to specific interactions with lipid headgroups or lipid acyl chains (Vigh *et al.* 2005).

Many of the membrane's function may derive from its microheterogeneity.

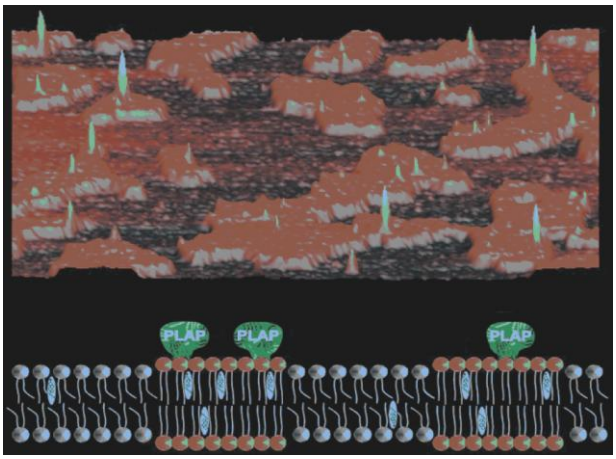

Figure 3.3- Liposome containing placental alkaline phosphatase (PLAP)**. *T**op***: AFM reveals sphingomyelin rafts (orange) protruding from a DOPC background (black) in a micasupported lipid bilayer. The height of the rafts is ~7 Å. Placental alkaline phosphatase (PLAP; green peaks), a glycosylphosphatidylinositolanchored protein, is shown to be almost exclusively raft associated. The bilayer is imaged under HEPESbuffered saline, and the width of the scan is ~2 mm. *Bottom*: an interpretation of the image at *top*. DOPC is shown by gray headgroups, and sphingomyelin is shown by orange head groups with PLAP associated. Cholesterol is incorporated in both lipid domains and is shown by the blue insertions. Reviewed in Henderson 2003.

3.2 Membrane regulation of heat shock response

The heat shock response (HSR) is a highly efficient system that protects cell functionality and the entire organism from several types of stresses, such as heat, drought, salinity, osmotic shock, membrane shearing, ischemia, etc. (Chen *et al.* 2007; Laplante *et al.* 1998; van Eden and Young 1996; Wang *et al.* 2003). The HSR involves a rapid transcriptional activation of a set of evolutionary conserved genes (hs), that code for heat shock proteins (HPSs) (Lindquist 1986). HSPs under physiological conditions act as molecular chaperones, being involved in folding, assembly and disassembly of other proteins. When a cell or an organism is subject to heat shock or other forms of stresses, HSPs are quickly accumulated in the

cytoplasm where they interact with denatured proteins protecting them from improper folding. That HSPs have crucial roles under stress is shown by the finding that HSPs are implicated also in other important physiological processes such as translocation, protection of mRNA splicing, in nucleic acid and protein syntheses, mitochondrial electron transport and photosynthesis (Broadley and Hartl 2008; Horvàth *et al.* 2008; Lee *et al.* 2007; Saibil 2008). Members of the group of small HSPs (sHSPs) have also been shown to antagonize heat-induced membrane hyperfluidization and to stabilize the membrane bilayer state under stress conditions (Saidi *et al.* 2009; Tsvetkova *et al.* 2002; Vigh and Maresca 2002). In addition, the HSR is severely altered in case of several chronic diseases, cancer, apoptosis, and during the aging process (Beere 2004; de Billy *et al.* 2012; Garrido 2011).

According to the classic model, the accumulation of denatured proteins is the signal to induce the transcription of hs genes (Morimoto 1998). However, this model does not take into proper consideration the fact that poikilotherms, plants (which constitute more than 95% of all living species on Earth), and microorganisms do not induce HSPs at a genetically determined temperature, since their response varies as their physiological temperatures adjusts during gradual temperature change (Dietz and Somero 1992; Tomanek and Somero 1999). Furthermore, this model does not explain how moderate temperature increments during temperature acclimation, which unlikely cause protein damage *in vivo*, could lead to the induction of HSPs and to the establishment of thermotolerance (Saidi *et al.* 2009). Further, in humans it is well known that HSPs are present at abnormal levels in several chronic diseases (Cappello *et al.* 2011; Flandrin-Gresta *et al.* 2012), and their synthesis decreases with aging without any evidence of a modification of the kinetics or accumulation of denatured proteins in these cases or during seasonal temperature acclimation that could explain the changes observed in hs gene transcription. Experimental evidences led to an alternative but not exclusive model. This model proposed that, besides protein denaturation, specific membrane domains acts as cellular thermometers and stress-induced membrane perturbations (Vigh *et al.* 1998, Vigh *et al.* 2007). This model suggests that subtle changes in lipid composition trigger selectively stress signaling responses via specific chemical interactions of boundary lipids with membrane proteins (Horvàth *et al.* 1998; Vigh *et al.* 2005) (Fig.3.4). This theory is supported by numerous indipendent studies. Changes in MPS of *Saccharomyces cerevisiae* obtained alterating UFAs/SFAs (saturated fatty acids) ratio by over-expression of a desaturase gene had a significant effect on the level of expression of *Hsp70* and *Hsp82* genes (Carratù *et al.* 1996). Further, it has been reported that the heat sensitivity of both heat and general stress response pathways depend critically on the fatty acid composition of membrane lipid present in the yeast cells (Chatterjee *et al.* 2000). Treatment of *Escherichia coli* with non-lethal doses of heat or benzyl alcohol caused temporary membrane fluidization and transcription of hs genes, such as unbalanced membrane lipids composition was shown to affect the expression of regulatory genes (Shigapova *et al.* 2005).

Furthermore, in *E.coli* and *Salmonella* Typhi, high-temperature signal is transduced by the phospho-relay system CpxA-CpxR, and since CpxA activity is strongly determined by the membrane lipids composition, it is likely to think that CpxA also senses changes in MPS of cells exposed to high temperature (Mikami and Murata 2003).

Taken together, these data imply that the plasmatic membrane, which is the barrier to external environment, acts also as a “cellular thermometer” and a primary regulatory interface.

From several studies it has been shown that in stress conditions the HSPs are divided into two different compartments, cytoplasmic for those that have chaperon activity and membrane for those involved in bilayer stabilization. Experimental data have shown that interactions between specific membrane domains and HSPs regulate membrane fluidity (Balogh *et al.* 2005; Nagy *et al.* 2007; Porta *et al.* 2010).

The connection between microdomain structures and physical state and regulation of HSR may provide a "unifying theory" according to which membrane microdomains are key players in gene expression and cellular functions.

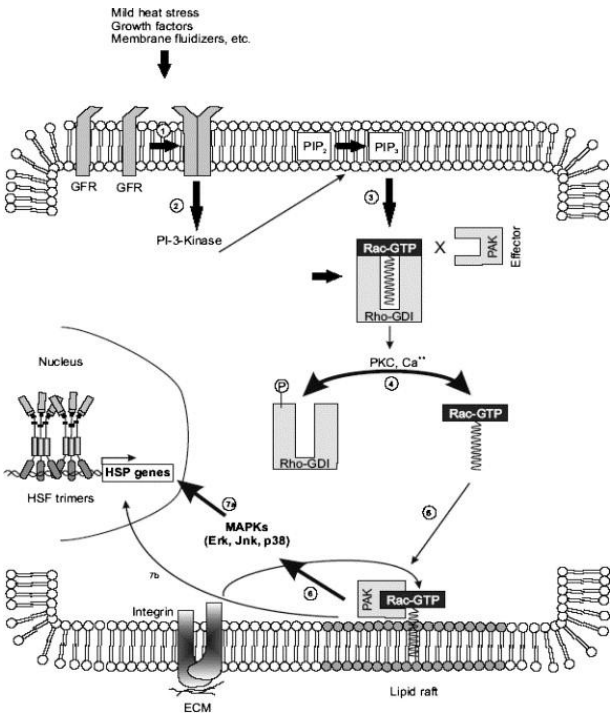

Figure 3.4- Cascade of possible heat-stress signal generation and transduction events linking plasma membrane to heat-shock genes during heat-shock protein response induced by mild heat stress and membrane fluidizers. Membrane rearrangement by mild heat stress or chemical fluidizers may activate growth factor receptor tyrosine kinases by causing their non-specific clustering (1) Activation of cell surface receptors followed by the activation of PI3K (2), which in turn activates the small GTPase Rac1 (3). In the absence of stress a Rho-GDI protein keeps solubilized Rac1 by shielding its geranyl–geranyl group in the cytosol. Ca^{2+} and PKC-dependent phosphorylation of Rho-GDI promote release of bound Rac1 (4) and its translocation to the membrane (5). Membrane localization of Rac1 is required for activation of its effector, PAK, which mediates also a downstream signaling cascade to MAP kinases (6) affecting Hsp expression (7/A). Rac1 selectively binds to lipid rafts and this binding is determined by the domain forming lipids. In addition, integrins also regulate Rac1 membrane binding sites at the cell surface. Activation of PAK may also lead to a direct activation of HSF1 (7/B)

3.3 Membranes and their role in cell signaling

Recently, it has been shown that membrane microdomains are essential for signal transduction.
Plasmatic membrane displays a wide range of functions:

- selectivity between hydrophobic hormones and hydrophilic signaling molecules;
- control of activity of membrane signaling proteins provided by the membrane lipid composition and fluidity;
- interaction of signaling proteins influenced by the net negative surface charge at the inner leaflet of the animal plasma membrane, provided mainly by phosphatidylserine;
- the hydrolysis of phosphatidylinositol bisphosphate into IP3 and diacylglycerol, which are well-known second messengers.

One role of membranes in cell signaling and the effect of interaction between lipids and proteins is represented by G protein coupled receptors (GPCRs). The function of G proteins, its localization and related signaling protein is regulated by the modulation of the membrane structure (Yang *et al.* 2005). The nature of the attached lipid moiety dictates the specific requirement of the optimal membrane lipid environment necessary for the correct localization and function of signaling proteins. It has been reported that the association of both GPCRs and G proteins to the plasma membrane makes them susceptible to the lipid environment. Vögler *et al.* have demonstrated that the HII phase propensity of membrane influences differentially the binding of G protein subunit, Gαi, Gβγ and Gαβγ in model membrane. The study showed that Gi and Gbγ are characterized markedly by preference for nonlamellar membrane, whereas Gα subunit showed opposite membrane binding behavior with a marked preference for lamellar membrane (Vögler *et al.* 2004) (Fig. 3.5). This finding is also supported by the loss of G proteins from brain plasma membranes that is observed after disruption of non-lamellar HII structures by daunomycin. (Escriba *et al.* 2007).

Recent evidences further strengthened the notion that a number of signal transduction proteins are located in special lipid microdomain, namely lipid raft (Mollinedo 2012; Staubach, Hanisch 2011).

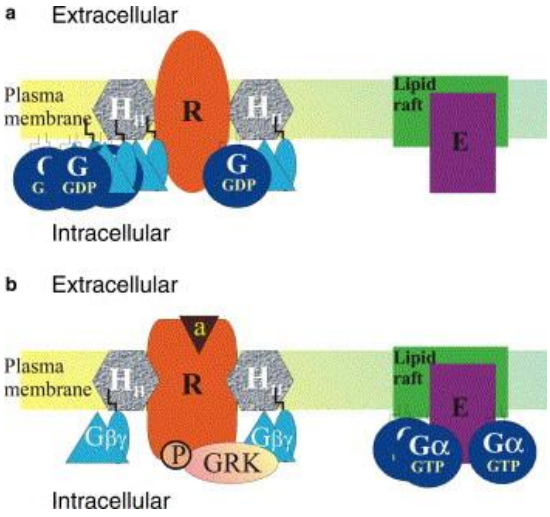

Figure 3.5- Membrane and signaling. (a) Two membrane domains are depicted. These membrane lipid domains are primarily generated and maintained by the membrane lipid composition. On the left it is shown a GPCR region, where membrane receptors (R) and G protein heterotrimers (G-GDP) are clustered in membrane areas with high H_{II}-phase propensity (H_{II}). Effectors (E) are located in membrane areas with ordered lamellar structure (e.g., lipid rafts). (b) Upon agonist binding, the receptor molecule activates several G protein molecules. Activated α subunits (Gα-GTP) move to lamellar-prone membrane domains. The dissociated Gβγ-dimer, which formerly favors the interaction of Gα subunits with the membrane, recruits GRK to the receptor's vicinity to phosphorylate and inactivate the GPCR.

CHAPTER 4

Antimicrobial peptides

4.1 Features and structure

The new concept of the structural and functional membranes organization results in an interesting consequence: the possibility to identify molecules that can modify membrane and its proteic components functionality interacting with specific lipids rather than with proteins.

In the past thirty years a new class of molecules interacting with membranes and with antibiotic activity has been identified; they are small peptides produced virtually by all living organisms, from the unicellular bacteria to higher organisms in plant and in metazoa, and act as an important part of innate immunity (Laverty *et al.* 2011).

The increasing prevalence of resistance among pathogenic bacteria to common antibiotics has become one of the most significant problems in modern medicine. The investments and development are significantly reduced in antimicrobial research among major pharmaceutical companies, thus, alternatives to existing drugs are not being produced at a sufficient rate.

In the past decade, the crisis of resistance has worsened significantly and there is an urgent need of new antimicrobials with activity against multidrug resistant pathogens.

In this contest the discovery and development of antimicrobial peptides (AMPs) has become a significant promise.

A striking feature among AMPs is their overall conservation of 3D structure and charge across different phyla.

Essential requirements for any antimicrobial agent are the selective toxicity and the affinity for one or more microbial determinants easily accessible and immutable. The majority of antimicrobial peptides are cationic, and for this reason are named cationic antimicrobial peptides (CAMPs) (Marshall, Arenas 2003). The most important features of CAMPs are charge, amphipathicity and hydrophobicity. They are often considered as individual features, but *in vivo* they cannot be pulled apart.

Since the target of CAMPs is the negatively charged bacterial membrane, it is not surprising that an increased positive net charge strongly correlates with the antimicrobial activity of CAMPs (Yeaman, Yount 2003). On the other hand, cationicity also has a strong correlation with the toxic effects of CAMPs. For example, Ovispirin-1, a peptide with a positive charge of +8 is highly toxic to mammalian cells (Sawai *et al.* 2002). The majority of naturally derived CAMPs have a net positive charge ranging from +2 to +9, which may represent an optimal charge for activity and it's due to high amount of cationic amminoacids, such as lysine, arginine or histidine and few or no acidic amminoacids such as glutamate and aspartate, (Tossi *et al.* 2000). Hydrophobic and branched residues form 30-50% of total peptide and are necessary to allow the typical amphiphilic structure that interact with membranes.

Modification of this net charge may modify activity and spectrum of activity.

Studies conducted with modified peptides have shown that within a certain range, the addition of positively charged residues can increase the efficacy of peptides. Studies with maganin 2 analogues demonstrated that an increase of charge from +3 to +5 resulted in increased antibacterial activity against both Gram-positive and Gram-negative bacteria. A further increase of charge to +6 and +7 caused loss of antimicrobial activity and led to increased hemolytic activity (Dathe *et al.* 2001). Contrasting results have shown that modulation of the net charge of cecropin/melittin analogues from +5 to +9 had no significant effect on antimicrobial activity (Scott *et al.* 1999).

Anionic antimicrobial peptides, generally with a net charge of -1 or -2, have also been identified. These often need cations, for example zinc, as cofactors for their biocidal activity (Brogden *et al.* 1996; Harris *et al.* 2009).

Nearly all CAMPs form amphipathic structures upon interaction with target membranes. In these structures, the positively charged residues are segregated on the hydrophilic face and the hydrophobic residues lie on the opposite face of the peptides. This kind of amphipathic structure can be achieved through different protein conformations. Thus, both amphipathicity and hydrophobicity are necessary for active CAMPs. The positively charged polar side helps the peptide to reach the target membrane through electrostatic interaction between CAMPs and structures on the bacterial surface, and the nonpolar side of peptides then allows the insertion into the lipid bilayer through hydrophobic interactions (Powers, Hancock, 2003; Yeaman, Yount 2003).

A high degree of amphipathicity correlates with increased toxicity to cells composed of neutral phospholipids (Dathe, Wieprecht 1999). Peptide hydrophobicity is approximately 50% for most antimicrobial peptides. Further, hydrophobicity is required for effective membrane permeabilization, but increased hydrophobicity also strongly correlates with toxicity to eukaryotic cell and loss of antimicrobial activity (Chen *et al.* 2007).

Although the secondary structure they adopt may vary, CAMPs show the same propensity to develop amphipathic structures. These structural groups include α-helical linear peptides, β-sheet peptides, peptides containing extended structures rich in certain amino acids and looped peptides (Pasupuleti *et al.* 2012) (Figure 4.1). The α-helical and β-sheet molecules are by far the most common antimicrobial peptides in nature.

Amphiphilic β-sheets contain two or three stabilizing disulphide bonds, often with a short α-helical segment and/or 2-4 β–strands. Examples of these peptides in nature include mammalian α-defensin and β-defensin.

Certain peptides are linear in hydrophilic solutions but form α-helics when they are in contact with cell membranes or in hydrophobic environments. One side of the α-helix contains hydrophobic residues while the other contains mainly polar residues fulfilling completely the requirement of an ordered amphipatic structure. Examples in nature include mellitin purified from honeybee venom, magainin from skin secretion of the frog *Xaenopus laevis* and the cecropins from insects.

Extended peptides have no defined structure due to existence of novel folds, such as tritrpticin from pork or bovine indolicidin.

The first peptide commercially available was the CAP mimetic tyrocidin, but because of blood and reproductive cells toxicity it was withdrawn from the market. Bacitracin had more success when incorporated with neomycin and polymyxin B in Neosporin® for topical use.

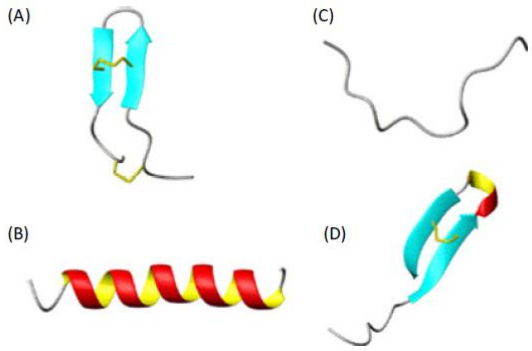

Figure 4.1 - Structural classes of CAMPs: (A) β-sheet peptides, tachyplesin I; (B) α-helical peptides, magainin 2; (C) peptides with extended structures, indolicidin; (D) looped peptides, thanatin. Adapted from Powers, Hancock (2003).

4.2 Peptide insertion and membrane permeability

The significant differences between eukaryotic and prokaryotic membranes explains the selectivity of antimicrobial peptides in bacterial cells (Glukhov *et al.* 2005).

In eukaryotic membranes, lipids as phosphatidylcoline, phosphatidylethanolamine, sphingomyelin and sterols have normally no charge leading to an overall neutral lipid bilayer. Prokariotic membranes are instead negatively charged, with a high electrical potential gradient, due to the presence of acidic hydroxylated phospholipids as phopsphatidylglycerol, cardiolipin and phosphatidylserine.

The overall positive charge of AMPs allow the initial electrostatic interaction with the bacterial cell membrane. Furthermore, acidic teichoic acids of Gram-positive and phosphate groups of lipopolysaccharides of Gram-negative improve attachment of peptides.

Thus, bacteria would have to alter several components of their membrane to develop resistance to peptides.

Fungal cell membranes are reasonable zwitterionic, so peptides with antifungal activity tend to be constituted by neutral amino acids with lipophilic regions of high polarity.

4.2.1. Membranolytic activity

Several mechanisms of action of AMPs have been recently reported, and include membrane permeabilization through the formation of stable pores, membrane thinning or formation of micelles in a detergent-like manner. Following access to the intracellular space, the sequential leakage of ions and other metabolites, loss of cytoplasmic components, dissipation of electrochemical potentials and ultimately cell death may explain the peptide's lytic action of pathogens (Shai 2002).

In the "barrel-stave model", peptide helices form pores in the membrane with a central lumen, just like a barrel composed of helical peptides staves (Vedovato, Rispoli 2007) (Fig. 4.2A). This type of mechanism is induced by strongly hydrophobic

peptides. In this mechanism, the hydrophobic domains of α-helical or β-sheet peptides constitute the outer face of the pore and interact with acyl chains of the membrane core, whereas the hydrophilic side forms the pore lining. Association of many peptide monomers may result in the expansion of membrane pores and upon phospholipid translocation or relaxation of the pore, peptides are transported to the inner membrane leaflet due to: concentration gradient of surface-bound peptide; tension exerted by peptides locally; *trans*-negative electrochemical potential.

In the "carpet model", peptides land on the external surface of the membrane the bilayer surface. This model has been associated with the activity of antimicrobial peptides such as ovispirin (Yamaguchi *et al.* 2001), dermaseptin natural analogues, cecropins, and some magainins (Papo, Shai 2002). Peptides are electrostatically bound to several heads groups of the anionic phospholipid, covering the surface of the membrane in a carpet-like manner (Fig.4.2B). At the critical threshold concentration, the peptides might form toroidal transient holes in the membrane. Membrane structure disruption occurs in a dispersive-like manner rather than channel formation, and peptides do not necessarily insert into the hydrophobic membrane core, as observed for cecropin P1 (Sitaram, Nagaraj 1999).

The "toroidal model" differs from the barrel-stave model since peptides are always associated with lipid head groups even when they are perpendicularly inserted in the lipid bilayer (Fig.4.2C). Indeed, this seems to be important since the presence of several peptide monomers in the pore would result in a thermodynamically unfavorable energy due to an excessive density of charges lining the pore. Therefore, the association with the anionic headgroup of the lipids partly neutralizes the positive net charge of peptides and favors the peptide aggregation process leading to pore formation. This type of transmembrane pore is induced by some magainins, protegrins and melittin (Sengupta *et al.* 2008).

4.2.2. Non-membranolytic activity

In some cases membrane disruption is not the main mechanism of action for AMPs. There are increasing evidences for intracellular targeting of AMPs as alternative or synergistic activity (Jenssen *et al.* 2006) (Fig.4.2).

For example, indolicidin penetrates bacterial membrane, leaving it undamaged, and once in the cytoplasm, inhibits RNA, DNA and protein syntheses (Hsu *et al.* 2005). Another mechanism could be stimulation of autolytic enzyme cascade, for example CAMPs as lactoferrin and lysozime may mimic the action of β-lactam antibiotics and activate autolytic cell wall enzymes such as muramidases.

Mammalian cathelicidins LL37 and PR39 have multiple mechanisms, both against membrane and intracellular targets and they can also stimulate immune response to pathogens (Kościuczuk *et al.* 2012). Thus, the modes of entrance, should not cause irreversible membrane damage. Three models have been proposed for CAMPs with non-membranolytic activity:

1) Aggregate channel model

Wu *et al.* (1999) have demonstrated that individual peptides have significant differences on their ability to depolarize the cytoplasmic membrane potential of *E. coli*, such as loop peptide bactenecin and the α-helical peptide CP26 being unable to cause depolarization at the MIC. This implies that membrane depolarization is not necessarily the crucial event in the killing of microorganisms by these peptides. On the basis of these results, the aggregate channel model was proposed. After binding

to the phospholipid head groups, the peptides insert into the membrane and then cluster into unstructured aggregates covering the membrane. This model is different from those previously described since only short-lived transmembrane holes are formed, allowing the peptides to transiently cross the membrane without causing significant membrane depolarization and membrane structure disruption. Once inside, the peptides may reach their intracellular targets to exert their lethal activities by acting on polyanions, such as DNA or RNA.

2) Molecular electroporation model

Molecular electroporation occurs when peptides have a sufficient charge density to generate an electric field (Fig.4.2D). This model has been proposed to explain membrane pore formation by annexin V (Karshikov *et al.* 1992), polymyxin B and melittin (Miteva *et al.* 1992). This mechanism is particularly important since the action of those peptides, that present antimicrobial activity without apparent formation of transmembrane pores, provides new insight to understand how peptides increase membrane permeability without necessarily causing its disruption.

3) Sinking-raft model

This model is based on the evidence that the biological activity of some AMPs is the result of imbalance of mass ratio for affinity of binding to particular lipid domains, locally producing a mass disproportion that directs the peptide translocation through increase in local membrane curvature (4.2E). It has been reported that δ-Lysin, an α-helical amphipathic peptide, binds more efficiently to the outer leaflet of the mammalian cell membrane, which are enriched in sphingomyelin, cholesterol and unsaturated phosphatidylcholine. Mixtures including these lipids have been shown to exhibit phase segregation between liquid-disordered (l_d) and liquid-ordered (l_o) domains, the last one particularly rich in sphingomyelin and cholesterol (lipid rafts). In such systems, Pokorny *et al.* (2005) established that the peptide preferentially binds to the l_d domains, producing a local concentration of δ-Lysin and enhancing peptide aggregation in these domains, which, in turn, creates the mass imbalance. Therefore, curvature occurs as the peptides bind to membranes, sink into the bilayer, and translocate to the cytoplasmic leaflet of the membrane perturbing it and causing the efflux of intracellular metabolites.

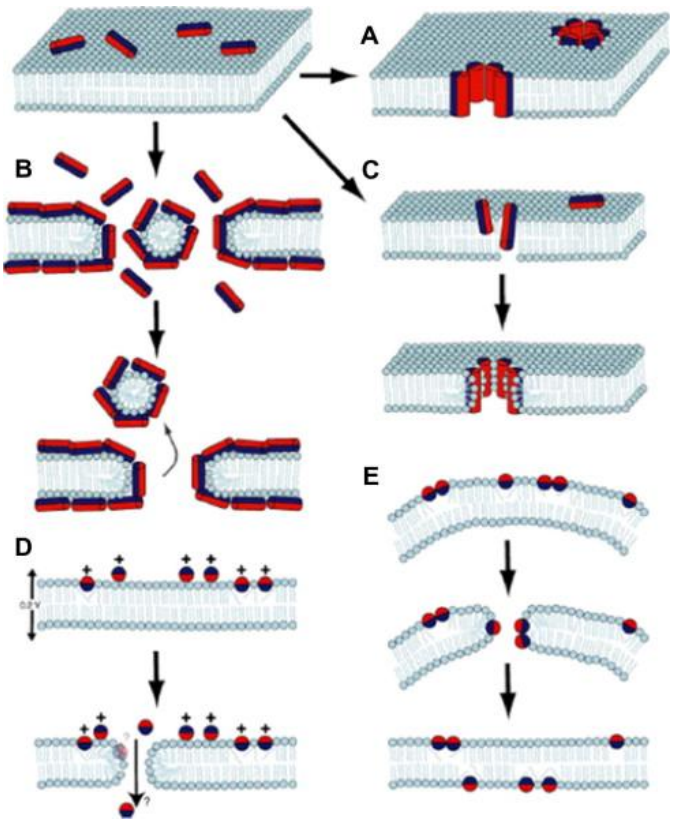

Figure 4.2- Antimicrobial peptide mechanisms of action. In this figure, various models are shown, illustrating advances in proposed mechanisms of antimicrobial peptide action. (A) In the barrel-stave model, the peptides span the membrane and form a pore with the hydrophilic portion lining the pore. (B) The carpet model is characterized by the spanning of the membrane by the peptide followed by a detergent-like action that disrupts the membrane structure. (C) The toroidal model differs from the barrel–stave mechanism as the hydrophilic portion of the peptide (in its amphipathic conformation) is associated with the lipid headgroup. (D) In the molecular electroporation model, the interaction of the cationic peptide with the pathogen membrane promotes an electrical potential difference across the membrane. When this potential reaches 0.2 V, a pore is believed to be created by molecular electroporation. (E) The sinking raft mechanism proposes a mass imbalance between the two leaflets of the membrane induced by the peptide. By creating a curvature gradient along the membrane and by self-association, peptides sink into the membrane and form transient pores that are thought to promote a transitory increase on membrane's permeability and leakage of intracellular contents. After membrane relaxation, peptides will reside on both leaflets of the membrane.

4.3 Bacterial mechanisms to resist antimicrobial peptides

Two different strategies have been described related to the resistance to AMPs: constitutive resistance or inducible resistance.

The former is related to the properties of the resistance normally expressed even in the absence of AMP; the latter inducible resistance includes the mechanisms that are activated after exposure to peptides.

Constitutive Resistance

The activity of several AMPs can be influenced by net negative charge or by transmembrane potential ($\Delta\psi$). *S. aureus* strains with constitutive decreased $\Delta\psi$ show reduced susceptibilities to some AMPs (Yeaman *et al.* 1998). Similar behavior has also been observed in the fungal pathogen *Candida albicans*: mutants of *C. albicans* deficient in respiration because of mutations of specific mitochondrial DNA genes are significantly more resistant to histatin-5 (Gyurko *et al.* 2000). Thus, fungal pathogens may suppress AMPs mechanisms by adopting a dormant metabolic status.

Furthermore it has been hypothesized that matrices such as capsule or glycocalix sequester AMPs, preventing them from accessing their targets. For example, it has been shown that in virulent strains of *Pseudomonas aeruginosa* producing alginic acid, a highly anionic capsular exopolysaccharide, purified alginate interferes with antimicrobial activity of AMPs (Friedrich *et al.* 1999).

The concept of constitutive resistance can be integrated in the niche-specific resistance model, in which some pathogens are resistant to AMPs simply because they have affinity for certain anatomical or physiological niches. One example illustrating this concept is given by small colony variants (SCVs) of *S. aureus*. This pathogen adopts this advantageous phenotype in the microenvironment of vascular endothelial cells exhibiting defects in electron transport that cause loss of membrane potential and reduction of the uptake of cationic antimicrobial agents (Proctor, Peters 1998).

Inducible resistance:

Many of the mechanisms responsible for this type of resistance involve modifications of envelope and/or extracellular portion of cytoplasmic membrane.

The essential molecules of the cell envelope can hardly be replaced without serious defects. However, some bacteria modify the charge of these molecules to prevent the attachment of CAMPs to the cell surface. Many Gram-positive bacteria such as *S. aureus*, *Streptococcus pyogenes*, *Streptococcus agalactiae* and *Listeria monocytogens* partially neutralize the negative charge of the cell wall by modifying teichoic acids with D-alanine (Bertsche *et al.* 2011; Saar-Dover *et al.* 2012; Vadyvaloo *et al.* 2004).

Reduction of the cell envelope's charge is also a common mechanism in Gram-negative bacteria and is mainly achieved by modifications of lipid A in the outer membrane. The best characterized example is the incorporation of aminoarabinose into lipid A of *S. enterica* and *P. aeruginosa* (Ernst *et al.* 2001; Moskowitz *et al.* 2004).

Moreover, it is postulated that the increased fluidity causes proton leakage out of the cells that leads to a reduced membrane potential by interfering with the permeabilization of the peptides (Bayer *et al.* 2006).

Additional strategies of bacterial cells to withstand AMPs are the production of peptidases and proteases, to which α-helical peptides are especially susceptible, and the active extrusion of peptides from the cell. The Gram-negative enteric

pathogen *S. enterica* serovar Typhimurium (*S*. Typhimurium) use two-component regulatory systems (TCRSs), such as PhoP-PhoQ (PhoPQ) and PmrA-PmrB (PmrAB), to detect environmental signals and to modulate changes in gene expression that promote survival and virulence. Indirect activation of PmrAB occurs through the PhoPQ TCRS. PhoPQ activates the expression of PmrD, which regulates PmrA activity at post-transcriptional level by binding to and stabilizing PmrA in its phosphorylated form (Richards *et al.* 2012). In addition to known *in vitro* signals such as low magnesium (Mg^{2+}), acidic pH and high concentrations of iron (Fe^{3+}), in *S.* Typhimurium PhoPQ and PmrAB and their regulons are activated by unknown environmental signals during macrophages infection, in other host cells and in the intestinal lumen (Gunn, Miller 1996).

CHAPTER 5

Salmonella

5.1 Biology of *Salmonella*

The genus *Salmonella*, belonging to the family of Enterobacteriaceae, is a morphologically and biochemically homogenous group of Gram-negative rod-shaped bacteria. They are facultatively anaerobic, non-spore forming, oxidasenegative and catalase-positive. Rods have typically a size about 0.7-1.5 x 2-5 µm, although it may vary in the case of formation of long filaments (Fig.5.1). Most strains are motile due to peritrichous flagella, but there are also non-motile sierotypes, such as *S. gallinarum* or *S. pullorum*.

Some strains produce a biofilm, a matrix of complex carbohydrates, cellulose and proteins and it is one of the virulence factors responsible for antibiotic-resistance. The ability to produce biofilm can be an indicator of dimorphism, which is the ability of a single genome to produce two distinct phenotypes in response to environmental conditions. *Salmonella* is able to grow both in aerobic and anaerobic condition. They are chemoorganotrophs, obtaining their energy from redox reactions using organic sources producing hydrogen sulfide, which is easily detected by growing them on media containing ferrous sulfate. *Salmonella* can grows from 2° to 54°C, with an optimal of 37°C within a pH range of 6.5-7.5.

Salmonella resides mainly in human and animal intestine. *Salmonella* diffuse into the environment (water, plants, soil) through feces, representing a major problem for the public health. Its chromosome organization is very similar to that of *E. coli* and consists of a single circular DNA molecule consisting of about 4 x 10^6 bp with a molecular weight of 4 x 10^9 and a total length of about 1.4 mm. Genome sequences of *S*. enterica are publicly available from NCBI as of 1 July, 2010 (Benson *et al.* 2010)

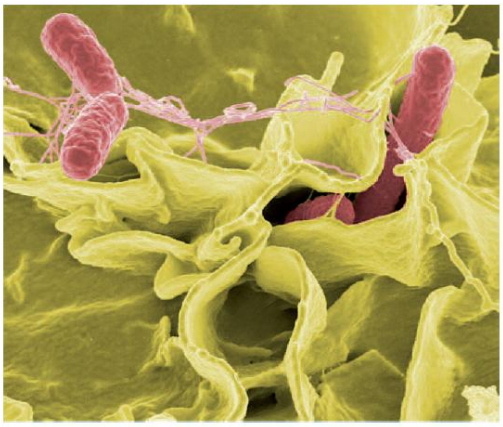

Figure 5.1–*Salmonella* Typhimurium (red) invading human cells captured by electronic microscope. Rocky Mountain Laboratories, NIAID, NIH, USA.

5.2 Pathogenesis

Salmonella enterica is mostly an intestinal pathogen, that causes a wide range of diseases, from food intoxication to serious systemic infection (typhoid fever) that needs an immediate antibiotic therapy. The course of the disease depends on the sierotypes. *S. enteric* Typhi and Paratyphi cause systemic infections representing a serious problem in developing countries and immunocompromised patients. Intestinal infections are a global problem determined mainly from sierotypes like *enteritidis* and *typhimurium.*

After oral ingestion, *Salmonella* infects cells of gastrointestinal epithelium, entering within M cells located over Peyer patches. In most cases the pathogen remains in the intestine, where the stimulation of inflammatory response causes diarrhea, but in the case of typhoid fever, *Salmonella* can reach the subepithelium where it interacts with dendritic cells and macrophages.

Furthermore, *Salmonella* is a facultative intracellular pathogen that survives and multiplies inside the phagosome of phagocitic cells. In this environment, *Salmonella* survives rapid pH decrease and lack of nutrients and then spreads in the liver and spleen through the blood or lymphatic systems (Cossart, Sansonetti 2004) (Fig.5.2).

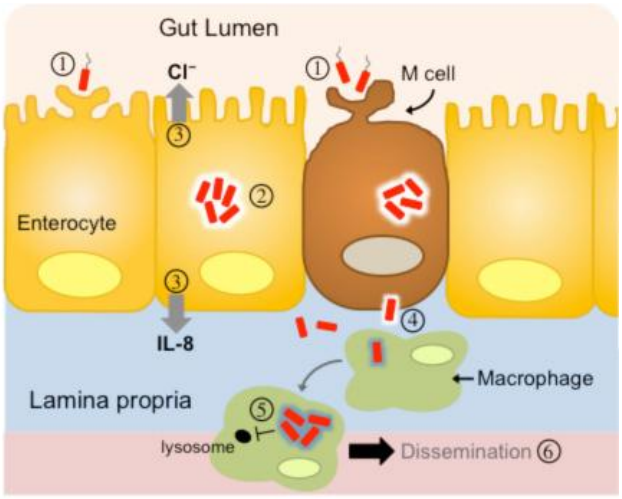

Figure 5.2- Selected events in *Salmonella* pathogenesis. (1) Once present in the gut lumen, *Salmonella* can cross the mucosal epithelial barrier by active invasion of mainly M cells (antigen sampling) but also enterocytes (absorptive epithelial cells). (2) Bacteria replicate inside the *Salmonella*-containing vacuole. (3) Invasion of the intestinal epithelium induces secretory and inflammatory responses. (4) In some cases, *Salmonella* can cross into the subepithelial lamina propria and invade phagocytes such as macrophages. (5) *Salmonella* blocks antimicrobial mechanisms of the phagosome including lysophagosomal fusion. (6) Within the phagocyte, *Salmonella* can travel throughout the lymphatic system and spread systemically (mainly to the liver and spleen).

5.3 Virulence factors

Fimbrae and flagella: Fimbriae or pili are present on the bacterial surface and seem to be mainly important for biofilm formation, colonization, and initial attachment to the host cells. However, little is known about their virulence potential (Guo *et al.* 2007). Each *Salmonella* serovar has a unique combination of fimbrial operons. Flagella are long helical filaments attached to rotary motors embedded within the membrane that enable *Salmonella* species to move through the epithelial barrier after ingestion. *In vitro*, flagellin causes upregulation of pro-inflammatory cytokines in tissue culture models. However, *in vivo* data showed that the role of flagella in virulence can be dispensable and model-dependent. Some *Salmonella* serovars (e.g., Enteritidis, Typhi) produce flagella that have always the same antigenic specificity, their flagellar antigen (H) is called monophasic. Most *Salmonella* serovars can alternatively produce flagella with two different H antigenic specificities (diphasic H antigen). These serovars have two flagella made up of flagellin which is coded by two genes, ***fliC*** and ***fliB.*** Both amino-terminal and carboxy-terminal portions of the proteins fliC and fliB are highly conserved, while the region involved in the recognition by antibodies is notably different. Every cell expresses alternatively either of the two flagellin antigens every 10^3-10^5 duplication. This phenomenon is called **variation of flagellar phase.** is The operon containing *fliB* gene codes for fliA, the repressor of fliC. Variation occurs through the reversible inversion of a DNA segment, called **segment H**, containing the promoter of genes *fliB* and *fliA* genes*.* The enzyme called **recombinase,** codified by *hin* gene, determines this inversion. When the segment H is in position ON, *fliB* and *fliA* co-transcribed so that *fliB*-expressing cells are generated. However, when segment H is in position OFF, the protein fliC is produced. A few species can be triphasic (Aldridge *et al*. 2006).

Plasmids and prophages: Integrated bacteriophages, phage remnants, or plasmids are single- or double-stranded DNA molecules that can be exchanged between bacteria by horizontal gene transfer. This process gives bacteria the opportunity to donate or receive specific genes that may enhance virulence or result in antimicrobial resistance. Certain *Salmonella* spp. have a self-transmissible virulence plasmid called pSLT (Ahmer *et al.* 1999), which harbors the *spv* genes (Guiney, Fierer 2011). spvB enzyme, which acts as an intracellular ADP-ribosylating toxin causing cytotoxicity in the host, is required for intra-macrophage survival but it is absent in *S.*Typhi and *S.* Paratyphi A. The role of plasmids carrying antimicrobial resistance genes, such as *cat*, *dhfr7*, *dhfr14*, *sul1*, and *bla*TEM-1, in the transfer and spread of antimicrobial resistance has been well described. It has been suggested that the presence of the MDR (Multi-Drug Resistance) phenotype is associated with the development of severe or fatal disease. Prophages and phage remnants can host non-essential “cargo” genes involved in bacterial virulence including several type III secretion system effectors, which play an important role in *Salmonella* virulence (Moreno Switt *et al.* 2012).

Type III secretion system and outer membrane vesicles: *Salmonella* virulence requires two type III secretion systems (TTSS) encoded on distinct *Salmonella* pathogenicity islands (SPIs). TTSS produce more than 30 effector proteins, involved in the modification of the cytoskeleton, in signal transduction, host membrane trafficking and proinflammatory response. The TTSS apparatus is highly conserved, whereas the effectors translocated by the two systems are unique and differentially regulated. SPI-1 is an important virulence factor for gastroenteritis and

intestinal colonization. Its genes are expressed in intestinal environment and their functions are necessary during the first phase of infection. SPI-2 is instead activated for virulence and survival inside phagocytic cells (Agbor, McCormick 2011).

Salmonella species are, in addition, protected from the acidic environment present in the stomach and phagosomes by a gene product that determines acid-tolerance (**ATR, acid tolerance response**) (Baik *et al.* 1996). In addition, enzymes such as catalase and superoxide dismutase protect the pathogen from the intracellular killing mechanisms. Further, bacterial outer membrane vesicles (OMV) have been recently identified as an additional method used by *Salmonella* to transfer its virulence factors into the cytoplasm of the host cells. It has also been shown that OMVs stimulates responses important to activate dendritic cells (DCs), priming *Salmonella*-specific T and B cells. Moreover, *Salmonella* possesses pro-inflammatory and antigenic functions, that make them attractive as vaccine candidates (Yoon *et al.* 2011).

5.4 *Salmonella* invasion

SPI-1 TTSS (T3SS-1) is required for bacteria-mediated endocytosis by non-phagocytic cells, while SPI-2 TTSS (T3SS-2) is associated with intracellular replication. T3SS-1 is a multiproteic apparatus with more than 20 proteins highly conserved that are shared by many Gram-negative bacteria. The central part of this structure is the *needle complex* that penetrates through the cell wall. The apical part of this complex is constituted by only one protein, PrgI, necessary for the secretion of effector proteins in the cytoplasm of host cells (Fig.5.2) (Marlovits, Stebbins 2010).

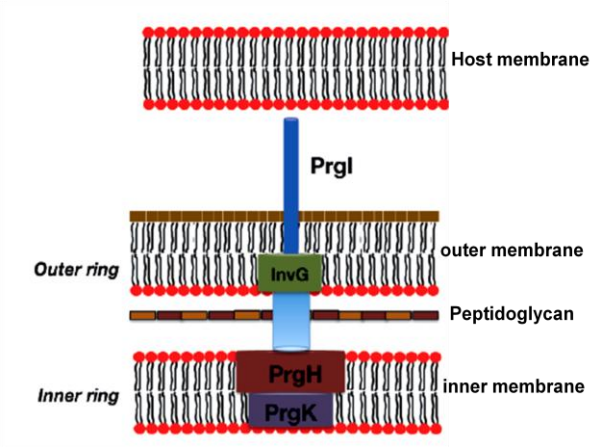

Figure 5.2-Salmonella T3SS-1 system. The protein PrgI forms a thin needle through which effector proteins are injected into host cell cytoplasm; protein InvG forms the outer ring, while the inner ring is made of two rings, namely the proteins PrgH and PrgK.

In the latest years about thirteen proteins that are released from T3SS-1 have been identified: AvrA, SipA, SipB, SipC, SipD, SlrP, SopA, SopB, SopD, SopE, SopE2, SptP and SspH1. Coordination of their functions causes the *membrane ruffling* of the host cell membrane and internalization of bacteria (Fig 5.3). The C-terminus of the SPI-1 T3SS translocon component SipC (SspC) directly nucleates

actin leading to rapid filament growth from the ends, whereas its N-terminus assembles actin bundles. Although not necessary for *Salmonella* entry, SipA (SspA) potentiates both the activities of SipC and enhances the activity of the host actin bundling protein T-plastin. SopE and SopE2, on the contrary, do not bind actin. They modulate the host actin cytoskeleton indirectly by mimicking cellular guanine exchange factors (GEFs). In particular, they catalyze the exchange of bound GDP with GTP to activate host Rho GTPases that stimulates downstream pathways driving actin cytoskeletal assembly via Arp2/3. The inositol phosphatase SopB (SigD) dephosphorylates a range of phosphoinositide phosphate and inositol phosphate substrates *in vitro* and contributes to actin remodelling during *Salmonella* entry. SopB-dependent hydrolysis of PI(4,5)P2 at the ruffled host membrane enhances the subsequent annealing of membrane invaginations to rapidly enclose bacteria within a phagosome and contributes to micropinosome formation with the cooperation of SopD. Following engulfment, *Salmonella* returns to the host cell cytoskeleton to its resting state through the downregulation of SopE/SopE2/SopB. *Salmonella* enters macrophages either by SPI-1 or non-SPI-1- induced macropinocytosis and are initially enclosed in spacious phagosomes (SPs) (McGhie *et al.* 2009).

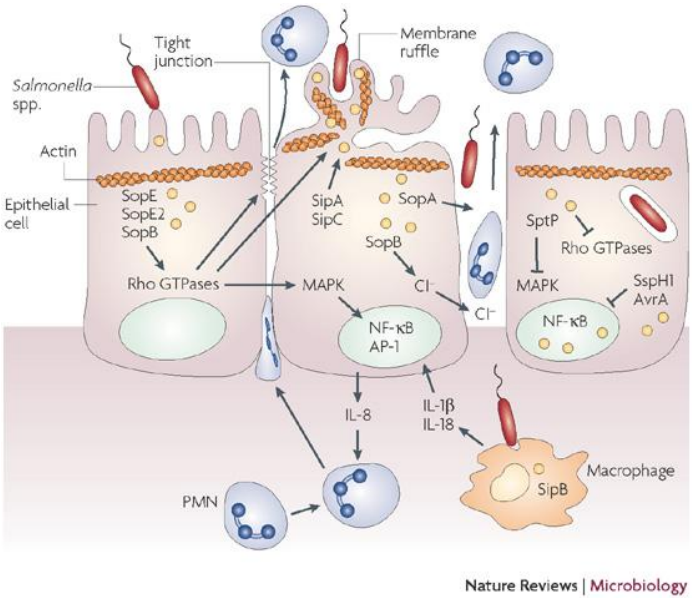

Figure 5.3- On contact with the epithelial cell, salmonellae assemble the *Salmonella* pathogenicity island 1(SPI1)-encoded type III secretion system (T3SS) and translocate effectors (yellow spheres) into the eukaryotic cytoplasm. Effectors, such as SopE, SopE2 and SopB, then activate host Rho GTPases, which results in the rearrangement of the actin cytoskeleton into membrane ruffles, induction of mitogen-activated protein kinase (MAPK) pathways and destabilization of tight junctions. Changes in the actin cytoskeleton, which are further modulated by the actin-binding proteins SipA and SipC, lead to bacterial uptake. MAPK signalling activates the transcription factors activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), which turn on production of the pro-inflammatory polymorphonuclear leukocyte (PMN) chemokine interleukin (IL)-8. SipB induces caspase-1 activation in macrophages, with the release of IL-1β and IL-18, so augmenting the inflammatory response. In addition, SopB stimulates Cl^- secretion by its inositol phosphatase activity. The destabilization of tight junctions allows the transmigration of PMNs from the

basolateral to the apical surface, paracellular fluid leakage and access of bacteria to the basolateral surface. However, the transmigration of PMNs also occurs in the absence of tight-junction disruption and is further promoted by SopA. The actin cytoskeleton is restored and MAPK signalling is turned off by the enzymatic activities of SptP. This also results in the down-modulation of inflammatory responses, to which SspH1 and AvrA also contribute by inhibiting activation of NF-κB. Figure reproduced from an original kindly provided by A. Haraga, University of Washington, USA.

Salmonella persists in SPs by fusion with macropinosomes or other SPs before forming a vacuole with the membrane. These *Salmonella*-containing vacuoles (SCVs) interact transiently with early endosomes, undergo Rab7- and phosphoinositide-dependent maturation, acidification, and acquire markers characteristic of late endosomes and lysosomes (LE/Lys), such as Rab7, vacuolar ATPase (v-ATPase) and lysosomal membrane glycoprotein (lpgs), including lysosomal-associated membrane protein 1 (LAMP-1) (McGhie *et al.* 2009). SPI2 was identified by the characterization of mutant strains that were highly attenuated in virulence in murine salmonellosis. The mutations clustered in a 40-kb region located between centisome 30 and 31 of the *Salmonella* chromosome with typical features of pathogenicity-associated island (PAI). Further analyses indicated that a second T3SS is encoded by one subset of SPI2 genes. These 31 genes are organized in two operons encoding components of the secretion system apparatus (ssa), one transcriptional unit encoding secretion system effectors (sse) and chaperones (ssc), and an operon encoding the two-component regulatory system SsrAB (secretion system regulator, ssr) (Fig. 5.4). SPI-2 is important in the latter phases, for surviving inside phagocitic cells. It appears that the main function of SPI-2 is to remodel the vacuole into an intracellular replication niche by altering endocytic trafficking, the vacuolar membrane, and manipulating the vacuolar-associated cytoskeleton including actin and microtubules. SPI-2 translocates at least 20 effector proteins across the phagosomal membrane into the eukaryotic cell cytoplasm to promote intracellular replication and systemic spread. The mechanism by which *Salmonella* stabilize its replicative niche is due to the electrostatic charge of the SCV membrane surface and the protein responsible is SopB that reduces the amount of phosphatidilserine and phosphatidilinositol. The protein SpiC is thought to avoid the fusion of macrophage late endosomes/lysosomes with the SCV. After 4-6 hours post-infection the protein SseG determines the accumulation of SCVs around Golgi vescicles, an F-actin grid assembles around the replicative SCV, then bound and stabilized by SipA. Several SPI-2 effectors may also regulate SCV-associated actin dynamics. In particular, the kinase SteC is essential for the formation of SCV-associated F-actin, while SseI and SspH2 co-localize with SCV-associated F-actin and bind the host actin crosslinking the protein filamin. Furthermore, SspH2 interacts with the cellular G-actin binding protein profilin and inhibits actin polymerization rates *in vitro.* The plasmid-encoded effector SpvB ADP ribosylates monomeric actin preventing its polymerization and inhibiting the formation of SCV-associated F-actin. All these events allow nutrients exchange, multiplication of bacteria and formation of *Salmonella* Induced Filaments (SIF) along microtubules dependent on the protein SifA (Fass, Groisman 2009).

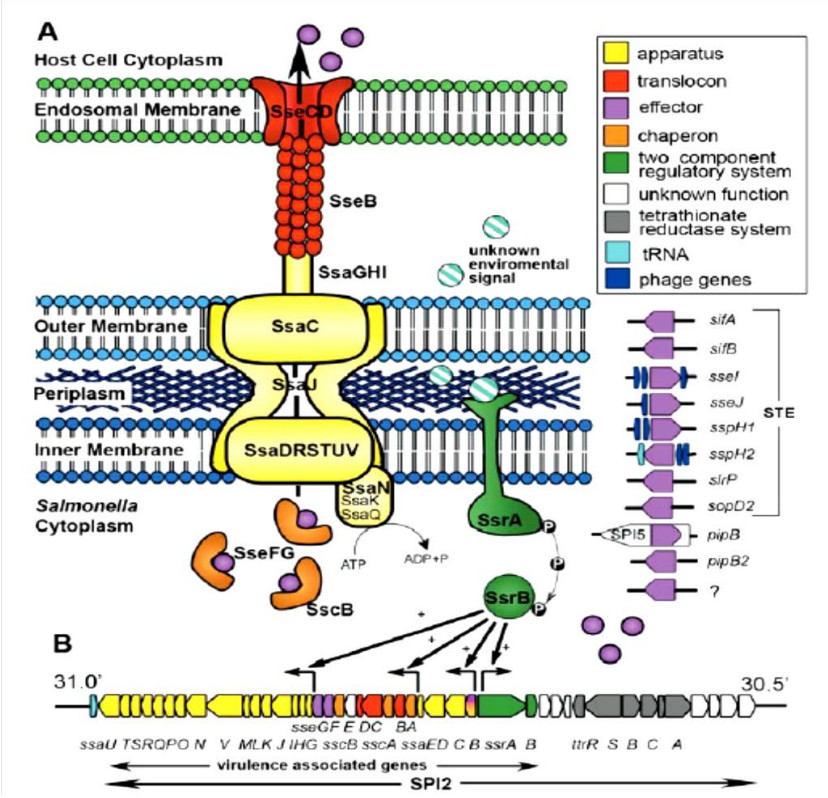

Figure 5.4- *Salmonella* pathogenicity island 2 (SPI2) and model of the SPI2-encoded T3SS. (*A*) A hypothetical model of the SPI2-encoded T3SS is shown and the subcellular localization of subunits indicated is based on experimental analyses [105] or studies on orthologs in T3SS of other bacteria [106]. The T3SS consists of the apparatus, a complex assembly of proteins (yellow) in the cell envelope, and the translocon. SseBCD (red) assemble to form a translocon with a putative pore for the translocation of effector proteins (violet) across the endosomal membrane. (*B*) Genetic organization of the SPI2 locus is depicted. Only *ssaU* to *ssrB* are required for virulence functions. Several effector proteins of the SPI2 system are encoded on separate loci outside of SPI2. Eight proteins of the family of *Salmonella*-translocated effectors (STE) share conserved N-terminal sequences. STE loci are in part associated with bacteriophage genes, while PipB is encoded by SPI5. Expression of T3SS genes in SPI2 as well as several effectors encoded outside this locus is under control of the SsrAB twocomponent regulatory system, although the precise signal sensed by SsrAB is not known.

5.5 Immune response to *Salmonella* infection

Pattern recognition receptors (PRR), particularly Toll-like receptors (TLR) and NOD-like receptors (NLR), are the first component of the immune system to detect host invasion by pathogens and initiate immune responses forming the crucial link between innate and adaptive immunity. PRRs recognize conserved motifs on pathogens termed “pathogen-associated-molecular-patterns” (PAMPs) and are also able to recognize endogenous danger signals or “danger-associated molecular-patterns” (DAMPs). Control of *Salmonella* growth in the early phases of infection requires reactive oxygen intermediates generated via the phagocyte NADPH oxidase. During invasive *Salmonella* infection, PAMPs and DAMPs initiate the innate immune system leading to activation and recruitment of neutrophils and macrophages. Phagocytosis of the pathogen by macrophages is enhanced by receptor-mediated uptake after opsonization of *Salmonella* with antibodies or complement and by the expression of *Salmonella* type III secretion system. The following formation of macrophage-rich granulomas and the production of pro-inflammatory cytokines, most notably Interleukin (IL)-6, IL-12, IL-15, IL-18, IL-1β, tumor necrosis factor (TNF)-α, and interferon-gamma (IFN)-γ, are necessary for the destruction of *S. thiphymurium*. TNF- α is involved in the formation and persistence of granulomas as well as in the regulation of NADPH oxidase-mediated killing of *Salmonella* by macrophages. IFN-γ is produced presumably by natural killer cells in response to IL-12 and IL-18 and mediates the up-regulation of nitric oxide synthase (iNOS)-dependent macrophage antibacterial mechanisms (Broz *et al.* 2012). Additional potential sources for INF-γ are macrophages, B cells, and specialized T cell populations. In particular, IFN-γ and IL-12 are crucial for host resistance to *Salmonella* infections in humans. IFN-γ or IL-12 defects or deficiency predispose humans to Salmonellosis (Picard *et al.* 2002). IL-12 is also important for the polarization of T helper cells toward the Th1 pole (Trinchieri, Gerosa 1996).
A decisive factor for the potential of macrophages activity is the expression of a functional *Nramp1* molecule, a *trans*-membrane protein related to cation channels (Vidal *et al.* 1995).
Immune response process likely involves detection of different bacterial ligands, in particular by the lipopolysaccharide (LPS) receptor TLR-4/MD-2 (Mastroeni, Ménager 2003). LPS and certain lipoproteins induce a massive inflammatory response in the surrounding tissue, leading to expression of inflammatory cytokines and a variety of chemokines involved in the recruitment of immune cells (Eckmann *et al.* 1996). Macrophages also respond to *Salmonella* through caspase-1 induction and secretion of the proinflammatory cytokine IL-1β through Ipaf (ICE protease-activating factor), a member of the NLR family. Also bacterial flagellin was recently shown to activate caspase-1 through Ipaf in a TLR-5-independent manner in infected macrophages (Simon and Samuel 2008).
Although the mechanisms of the innate immunity have a predominat role in restricting the initial growth of *Salmonella* for several days, they fail to complete the elimination of the pathogen, since the bacteria can endure the pressure of the defense response. Only the generation of a specific lymphocyte response can eventually eradicate the bacteria. In most of experimental data $CD4^+$ T cells were found to be more important than $CD8^+$ T cells (Sinha *et al.* 1997), although depletion of $CD8^+$ T cells reduce the ability to transfer protection against virulent *S.* Typhimurium (Mastroeni *at al.* 1993). Further, $CD8^+$ T cells can differentiate into cytolitic T cells, that allow the release of bacteria from their protective habitat after

cell lysis and contain granulysin with antibacterial activity (Kaufmann 1988, Stenger *et al.* 1998).
Numerous studies demonstrate that infection of mice with *Salmonella* induce a Th1 response, characterized by a large production of INF-γ, that is in part responsible for increate protection against secondary infection (Ramarathinam *et al.* 1991).
An important role for T cells is the regulation of antigen-specific B cell activation and maturation, both by contant among cells and cyotikine production.
Antibodies are largely involved in the protection during the different phases of *Salmonell*a infection. In the intestinal lumen, antibodies as IgM and IgA, block penetration of *Salmonella* into deeper tissues. Further, in Peyer's patches, lymph nodes, spleen and liver, antibodies enhance bacteria engulfment by Fc-receptor-mediated phogocytosis, that together with complement receptor-mediated uptake allow clearing of bacteria from serum through opsonization (Mittrücker and Kaufmann 2000).

5.6 The *Salmonella* PhoQ sensor: mechanism of detection of phagosome signals

Within host macrophages the bacteria encounter many antimicrobial mechanisms, such as toxic oxygen and nitrogen species, antimicrobial peptides, proteases and acidic pH. To survive in this intracellular environment, *Salmonella* must sense this stimuli and activate mechanisms to avoid killing.

Surviving of *Salmonella* inside macrophages is allowed by a two components system composed by the transcriptional regulator PhoP and the sensor PhoQ, able to regulate SPI-1 T3SS-1 and SPI-2 T3SS-2 systems (Bijlsma, Groisman 2005). Using microarray technique it has been possible to demonstrate that PhoP regulates more than 30 virulence genes involved in invasion, motility, transport of small molecules, acid tolerance, AMPs resistance and bacterial surface remodeling (Monsieurs *et al.* 2005). *Salmonella* Typhimurium strains deleted for either PhoP or PhoQ exhibit virulence defects upon infection in mice (Miller *et al.* 1989).

PhoQ is a histidine kinase found as a dimer in the bacterial inner membrane. It has two transmembrane regions, including one at the N-terminus, which span the inner membrane resulting in a periplasmic sensor domain and a cytoplasmic domain. The cytoplasmic domain contains a HAMP linker (present in Histidine kinases, Adenyl cyclases, Methyl-accepting proteins and Phosphatases) in proximity to the membrane, a conserved autophosphorylation site (histidine box) and a C-terminal domain that bind ATP to accomplish kinase and phosphatase activity. In response to specific stimuli, PhoQ activation determines the *trans*-autophosphorylation within the dimer on a conserved histidine residue of the cytoplasmic region and consequent transfer of the phosphate to an aspartate of PhoP; once phosphorylated, PhoP binds to DNA to regulate expression of PhoP-activated (pag) and PhoP-repressed (prg) genes (Prost and Miller 2008).

Twenty-one pag loci, that encode nonspecific acid phosphatase, magnesium transporters, the PhoP and PhoQ regulatory proteins, and proteins that produce active alkaline phosphatase fusions and therefore membrane or secreted proteins, have been described. PhoP-repressed genes include the SPI-1 TTSS and flagellar genes, which may result in a dampening of the host immunostimulatory activity through Ipaf and TLR-5. The negative regulation of SPI-1 TTSS system by PhoP occurs through the control of genes belonging to the *locus hil (hyper-invasion locus),*

that codify for the regulator protein HilA, HilC, and HilD, involved in the expression of SPI-1 and SPI-5 genes (Aguirre *et al.* 2006).

PhoP-activated genes modulate resistance to antimicrobial peptides and reactive oxygen species, and phagosome remodeling through regulation of the intracellular TTSS encoded by SPI-2, and are also involved in the acid tolerance response, which is particularly important within the phagosome. The expression of several operons of SPI-T3SS system is under control of the two components system SsrAB (Fig. 5.5), composed by the sensor SsrA (histidine kinase) and by the protein SsrB (response regulator). Phosphorylated PhoP binds to the SsrB promoter, inducing its transcription and influencing SsrA concentration post-transcriptionally. Unlike PhoP/PhoQ that is highly conserved among related species, SsrAB system belongs only to *S. enterica* (horizontal gene-transfer). The interaction between PhoP and SsrB is an interesting example of functional integration of genes acquired from related species and highlights the dynamism and flexibility of the regulation system during evolution (Löber *et al.* 2006).

The p*hoPQ* operon is autogenously controlled in a positive mode by the PhoP and PhoQ proteins. The *phoPQ* operon is transcribed from two promoters, one active only during growth in low Mg^{2+} and dependent on the PhoP and PhoQ proteins and another that is constitutive (i.e.,active regardless of the Mg^{2+} concentration or presence of PhoP and PhoQ proteins) (Gal-Mor *et al.* 2011).

In particular, consistent with the environment where the system is activated, PhoP/PhoQ allows *Salmonella* to grow at limited concentrations of Mg^{2+}, which results from PhoP-activated expression of the *mgtA* and *mgtB* genes, encoding Mg^{2+} transporters (Choi *et al.* 2009). The PhoQ-mediated PhoP phosphorylation also occurs at acidic pH, and transcription levels of the PhoP-activated genes *pagA*, *phoN*, and *pmrD* increase in *Salmonella* growing in acidic conditions (Prost *et al.* 2007).

A direct repeat sequence (T/G)GTTA(A/T) is present in the promoter regions of a subset of genes dependent on PhoP for expression (Lejona *et al.* 2003; Yamamoto *et al.* 2002). The promoter regions of these PhoP-dependent genes (including *phoPQ*, *mgtA*, *slyB*, *pmrD* and *pcgL*) interact directly with the PhoP protein, whereas promoter sequences from PhoP-regulated loci lacking canonical PhoP binding sites (such as *pagC*, *phoN* and *mgtCB*) do not (Lejona *et al.* 2003). This suggests that PhoP/PhoQ may indirectly control the expression of genes such as *pagC*, *phoN* and *mgtCB* by altering the expression or activity of additional transcriptional regulators.

As mentioned above, the PhoP/PhoQ system also regulates gene expression by controlling the levels and/or activity of other regulators. The RstA/RstB two-component system, which consists of a response regulator RstA and its partner sensor RstB, is one whose expression is regulated by the PhoP/PhoQ system. In *Escherichia coli*, the PhoP protein binds to the *rstA* promoter, and transcription of the *rstA* gene is repressed by the PhoP/PhoQ system in cells grown at a high concentration of Mg^{2+}. The RstA protein promotes transcription of the *asr* gene, coding for a product necessary for adaptation to acidic stress. Consequently, at acidic pH, transcription of the *asr* gene is not fully activated in a strain lacking the *phoP* gene, due to the reduced levels of the RstA protein. In addition, the *rstA* gene has been identified as a multicopy suppressor of the essential genes *yjeE*, *yeaZ*, and *ygjD*.

The *Salmonella* PhoP protein also directly binds to and activates the *rstA* promoter at low Mg^{2+} (Nam *et al.* 2010).

A subset of PhoP-activated genes is regulated by the activation of an additional two components system, called PmrA-PmrB, of which PmrA is the

response regulator and PmrB is the sensor kinase (Fig. 5.5). The low Mg^{2+} activation of PmrA-regulated genes, which requires the *phoQ, phoP, pmrD, pmrA*, and *pmrB* genes, occurs by the PhoQ protein serving as an Mg^{2+} sensor that modulates the ability of the PhoP protein to promote transcription of the *pmrD* gene. The *pmrD* gene product then activates PmrA-PmrB at a post-transcriptional level by a yet undefined mechanism. On the other hand, Fe^{3+}-promoted activation of PmrA-regulated genes occurs by Fe^{3+} binding to the periplasmic domain of the PmrB protein, which presumably promotes PmrB autophosphorylation and the ensuing phosphorylation of the PmrA protein. This would result in transcription of PmrA-activated genes because the PmrA protein binds to the promoters it regulates with higher affinity in its phosphorylated form (Richards *et al.* 2012). The PmrA/PmrB system directly controls the expression of a set of genes that mediate resistance to polymyxin B by adding 4-aminoarabinose to the lipid A portion of lipopolysaccharide (Gunn, Miller 1996).

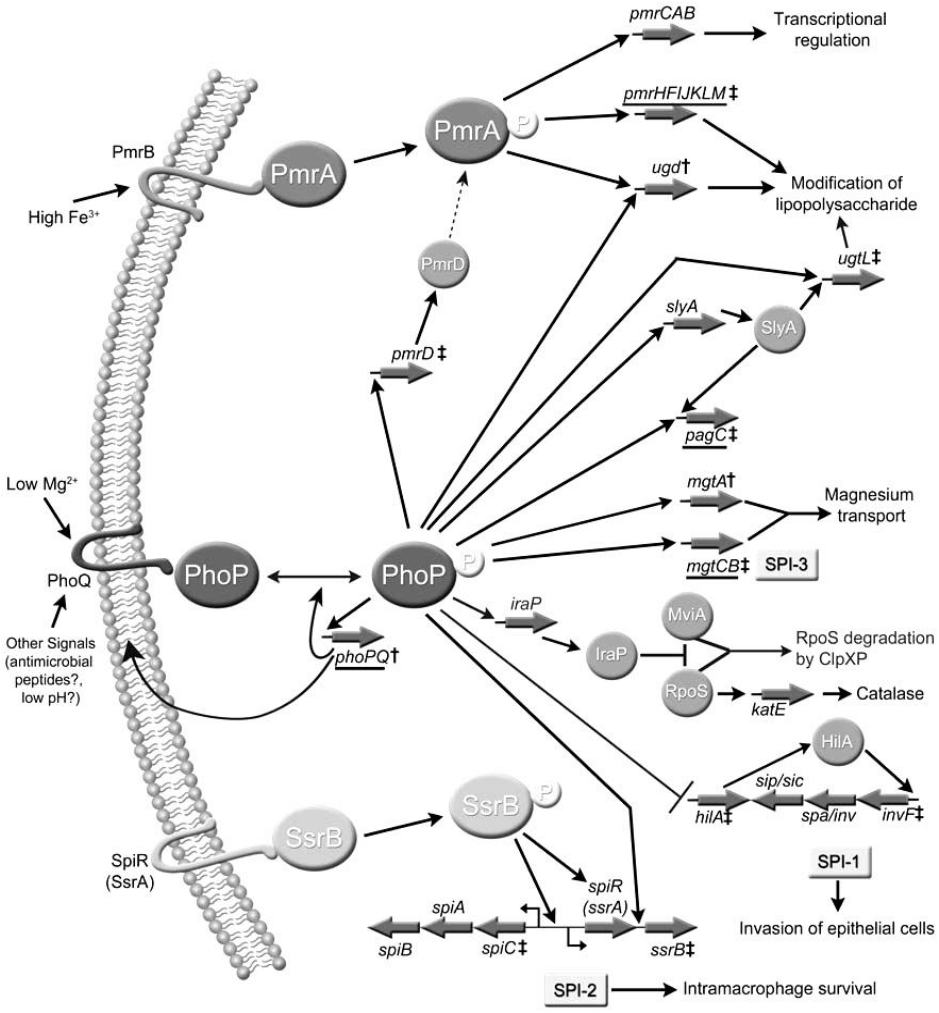

Figure 5.5- Illustration of the PhoQ/PhoP two-component regulatory system in Salmonella enterica serovar Typhimurium. PhoQ activates PhoP in response to a number of environmental signals including low magnesium. Once activated, PhoP can directly activate its own transcription and the transcription of a

number of other genes. A number of directly regulated gene products then regulate additional regulatory cascades, including PmrD, which is able to activate the PmrAB operon independent of iron; SlyA, which regulates genes important to intra-macrophage survival such as pagC and ugtL; IraP which prevents MviA-dependent degradation of RpoS leading to RpoS accumulation and its regulation of genes important for stationary phase survival and resistance to oxidative stress; HilA, which is an inducer of SPI-1 (Salmonella pathogenicity island-1), which contains genes involved in invasion of epithelial cells; and SsrB, which is an inducer of SPI-2 containing genes important in intra-macrophage survival (adapted from Groisman E. and Mouslim C. Nature Reviews Microbiology (2006) 4:705–709) [44,45]. In this figure, underlined genes denote those whose products were detected in our analysis. {: Promoter region contains a typical PhoP box defined as a dyad of (T/G)GTTTA separated by 5 nucleotides. {: Presence of an atypical PhoP box defined as a dyad of (T/G)GTTTA separated by 5 nucleotides in the promoter region, allowing four substitutions as long as the following positions were conserved: a thymine in the first dyad half (at position 3) and two conserved thymines and one conserved adenine in the second dyad half at positions 3, 4, and 6, respectively, within 300 nucleotides of the translational start site.

It has been demonstrated that *in vitro*, in rich medium supplemented with millimolar concentrations of divalent cations Mg^{2+}, Ca^{2+}, or Mn^{2+} the sensor PhoQ is repressed (Garcia *et al.* 1996).

Based on the available structural and biochemical data, it has been proposed a metal bridges model for PhoQ repression. When divalent cations are present, they form bridges between the PhoQ acidic patch and the membrane that keep the protein in a more structured state, causing PhoQ repression. In the absence of cation bridges, charge repulsion would force helices away from the membrane, thus inducing increased structural flexibility that could act as an initiation signal. Inside the macrophages, the low concentration of metal ions, in particular magnesium, is not sufficient to repress PhoQ, that is activated by factors as acidic pH, cationic antimicrobial peptides and oxygen radicals. In particular, CAMPs induce a conformational change of periplasmic domain competing with divalent cations and since they have more affinity and are structurally more ingombrant they replace the cations and force a part of the protein away from the membrane activating PhoQ (Fig.5.6). The most evidence for this event are experiments using purified PhoQ reconstituted in membrane vescicles (Bader *et al.* 2005). In addition to CAMPs, the acidic pH inside the phagosome, that ranges from 5,0 to 6,5, determines a lack of stability and makes easier the conformational change. Direct recognition of pH by the PhoQ periplasmic domain at a divalent cation concentration found within phagosome (1mM) also was demonstrated in experiment with membrane vescicles. In membrane vescicles, the effect of CAMPs and pH are additive, which suggests distinct mechanisms of sensing. Using NMR spectroscopy it has been realized that the periplasmic domain remains stable and folded at pH 3.5, indicating that it may have evolved as a pH sensor (Prost *et al.* 2007).

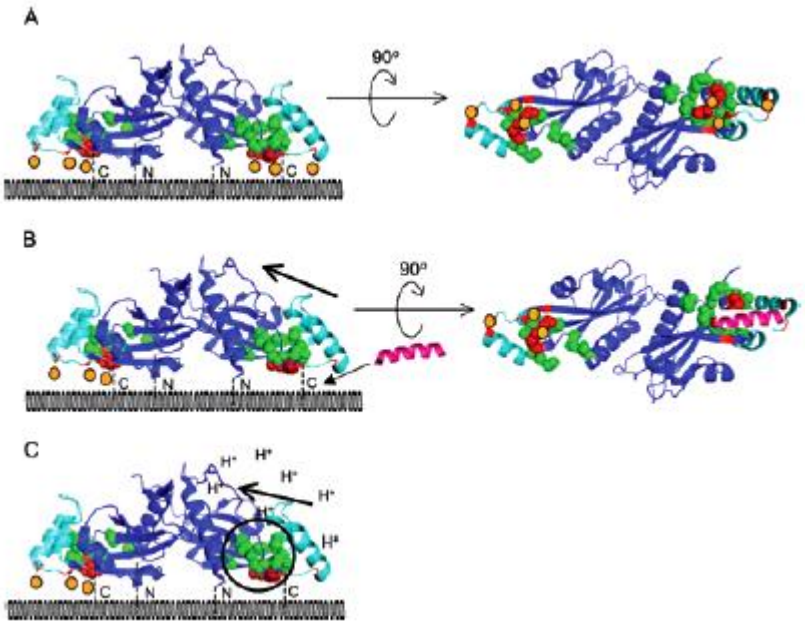

Figure 5.6- Structural mechanisms of PhoQ repression and activation. The PhoQ periplasmic domain is shown as a dimer on the inner membrane. The core of the protein is coloured blue, helices a4 and a5 are shown in cyan, the interaction network surrounding H157 is shown in green space-filling format, and residues that co-ordinate Ca2+ ions are shown in red (residues that are part of the interaction network and bind Ca2+ are shown in red space-filling format). A. Front and bottom views of the metal bridges mechanism of PhoQ repression. Ca2+ ions are shown as orange spheres. B. Front and bottom views of the proposed structural mechanism of CAMP activation. In both views, the left monomer is shown in the repressed state. On the right monomer, an a-helical CAMP, shown in magenta, binds to the same residues as divalent cation, thus forcing helices a4/a5 (cyan) away from the membrane. C. Front view of the proposed structural mechanism of acidic pH activation. The interaction network, circled, will lose structural rigidity upon exposure to acidic pH, thus allowing helices a4/a5 (cyan) to move away from the membrane.

CHAPTER 6

Previous findings

Pursuing the aim to develop a successful strategy for the production of new vaccines, a work made in the laboratory of Prof. Bruno Maresca was based on the generation of an attenuated form of the fungal pathogen *Histoplasma capsulatum.*

As discussed above, the inhibition of HSP accumulation (through changes in the MPS of the pathogen) might interfere with the process of adaptation, invasion, and disease in microbial pathogens.

Histoplasma capsulatum highly virulent strain G217B was transformed with a plasmid containing its own Δ9-desaturase gene under the transcriptional control of the upregulated Δ9-desaturase promoter and the mutant was named HcD3. Using Northern blot analysis it was shown that the Hsp70 gene was highly expressed in *H. capsulatum* G217B (from 34° to 37°C, from 34° to 42°C, and from 37° to 42°C), whereas in strain HcD3 Hsp70 transcription was detectable only from 34° to 42°C (Fig.6.1).

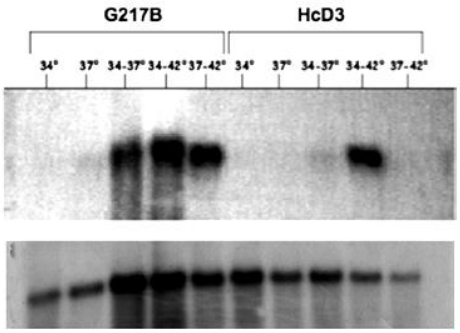

Figure 6.1- Hsp70 gene expression in the H. capsulatum HcD3 strain. Yeast cells of H. capsulatum G217B and of HcD3 strains were grown at 34° and 37°C and heat shocked for 30 min at different temperatures. The Hsp70 gene was highly expressed in strain G217B (from 34° to 37°C, from 34° to 42°C, and from 37° to 42°C), whereas in strain HcD3 Hsp70 transcription was detectable only from 34° to 42°C.

In addition, in experiments of murine macrophages (Mϕ) infection *in vitro* it was possible to observe that though *H. capsulatum* HcD3 was internalized inside Mϕ, no fungal growth was detectable at 8 h after infection, very likely because of an altered MPS and impaired heat shock response (Fig.6.2).

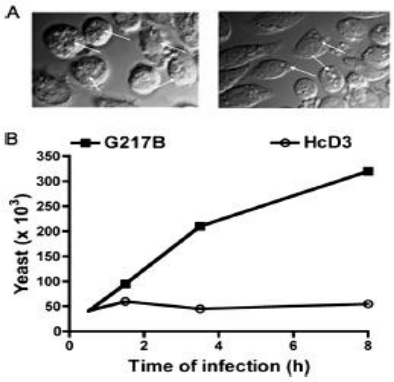

Figure 6.2- Murine Mϕ infection with H. capsulatum. (A) Yeast phase cells of strain HcD3 as well as the control G217B strain were internalized insideMϕ. (B) Yeast cells recovered from Mϕ lysed at different time points of infection were counted. G217B grew inside Mϕ while no growth was detectable for the HcD3 strain.

Afterwards, increasingly high concentrations of yeast cells of the virulent G217B or of the genetically modified HcD3 *H. capsulatum* strain (10^6, 10^7, and $5*10^7$) were injected intravenously into CD-1 mice. Mice infected with the highly virulent strain died between 23 and 35 days, while those receiving the HcD3 strain survived up to 40 days. The group of surviving mice was then challenged with a lethal dose of G217B yeast cells. After 20 days, more than 60% of mice were still alive, suggesting that the first inoculation with the attenuated strain conferred protection from the subsequent challenge with the virulent strain (Fig.6.3) (Porta *et al.* 2010).

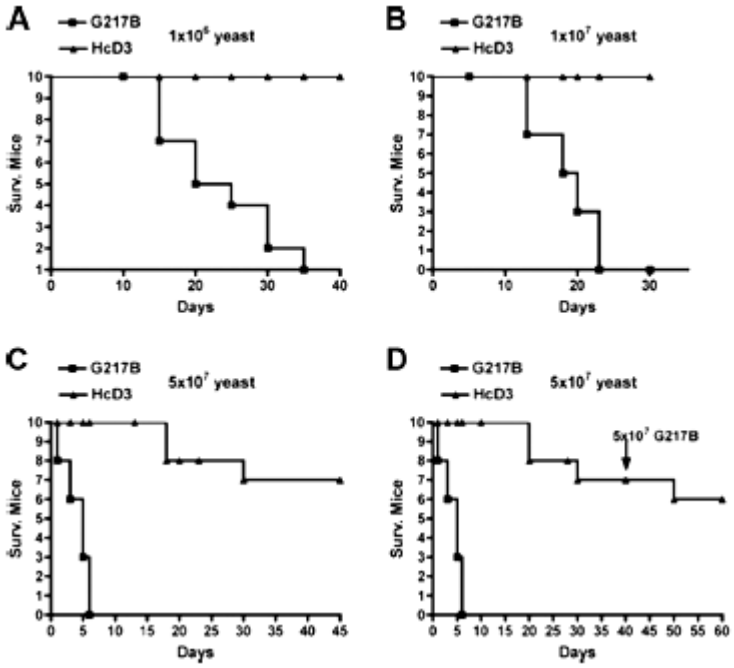

Figure 6.3- Mouse infection with H. capsulatum. (A) Mice infected with 10^6 yeast cells of strain G217B died within 40 days after infection, whereas all mice infected with strain HcD3 survived. (B) About 10^7 G217B yeast cells killed all mice after 23 days, whereas all mice infected with 107 HcD3 yeast cells survived up to 30 days after infection. (C) All mice infected with 5 * 10^7 G217B yeast cells died within 6 days, while 7 out of 10 mice infected with the same inoculum of HcD3 survived up to 45 days after infection. (D) Surviving mice were challenged with a lethal dose (5 * 107 yeast cells) of the virulent strain G217B. After 20 days (60 days from the initial infection with attenuated HcD3 strain) six mice still survived.

Thus, this procedure to obtain attenuation of virulence might be applied also to other intracellular pathogens, such as mycobacteria, staphylococci, streptococci, and parasites. Altough further studies are needed, this technique provides a new opportunity for the development of a new, safer, and more effective class of attenuated strains for human and animal use.

Recently, it has been shown that an abrupt temperature change or an exposure to other forms of stress determines a physical reorganization of lipid and protein membrane components followed by a specific gene response to compensate variations in the membrane physical state (MPS). Furthermore, by now it is known that there is a cross talk between changes in the MPS and transcriptional regulation of genes involved in lipid metabolism and heat shock genes and, as mentioned above, some sHSPs are directly involved in the remodeling of MPS.

Studies in the laboratory of Prof. Bruno Maresca have focused on the important role of membranes as primary sensor of heat stress and on the understanding of how proper lipid-protein interactions within membranes determine the observed transcriptional regulation of heat shock genes.

The SFA/UFA ratio, such as the protein/lipid ratio, is essential for the proper functioning of biological membranes and mantaining of MPS.

Insertion of *Synechocystis* Δ12-desaturase in the lipid bilayer of *S.* Typhimurium membrane (Fig.6.4), where it doesn't show enzymatic activity because of its susbstrate absence, caused a dramatic imbalance in the membrane protein/lipid ratio compared to the control strain (Fig.6.5).

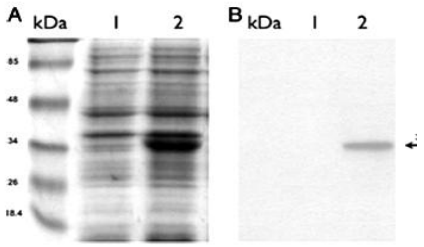

Figure 6.4- SDS-PAGE and Western blotting of S. Typhimurium strains. (A) Proteins of the membrane fraction were separated by SDS-PAGE and stained with Coomassie brilliant blue. (B) Western blot analysis using a monoclonal antibody against Synechocystis Δ12-desaturase shows that it was present in the membrane fraction of *Stm*(pΔ12). Lanes kDa, protein markers; lanes 1, membrane proteins from *Stm* wild type cells; lanes 2, membrane proteins from *Stm*(pΔ12). The arrow indicates the position of the Δ12-desaturase, with an apparent molecular mass of 36 kDa.

Strain	Lipid content (mg/ml)	Protein content (mg/ml)	Protein/lipid ratio	Membrane fluidity (mg/ml membrane extract) at:	
				30°C	40°C
Stm(pNir)	0.78 ± 0.16	3.94 ± 0.06	5.06	0.212708 ± 0.02	0.165846 ± 0.01
Stm(pΔ^{12})	0.74 ± 0.06	6.23 ± 0.06	8.42	0.220125 ± 0.02	0.185249 ± 0.01

[a] Protein and lipid were purified from cells grown at 30°C. The membrane fraction of *S.* typhimurium was labeled with DPH, and the steady-state fluorescence anisotropy was monitored as a function of temperature. The values represent the average of four independent extraction/purification procedures.

Figure 6.5- Protein and lipid content and membrane fluidity

They have shown that the overproduction of Δ12- desaturase lowered the transition temperature of certain lipid domains in the outer membranes of bacteria. Thus, *S.* Typhimurium expressing Δ12-desaturase displayed greater permeability in the outer membrane, even under nonstressing conditions.

Furthermore, *dnaK* and *ibpB* shock proteins are expressed at 30° and 37°C, with different patterns of expression when bacteria were under thermal stress and two sHSPs, ibpA and ibpB, have been found in the membrane fraction (Fig.6.6).

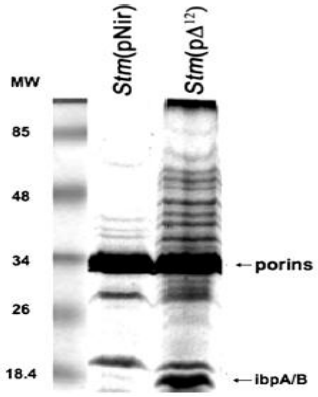

Figure 6.6- SDS-PAGE analysis of outer membrane proteins stained with Coomassie brilliant blue. A significant accumulation of IbpA and IbpB in strain *Stm*(pΔ12) grown at 30°C is detectable.

Similar changes are present in *Salmonella* wild type treated with a membrane fluidizer as benzyl alcohol (BA).

In addition, they demonstrated that the independent expression of two membrane-spanning domains of *Synechocystis* Δ12-desaturase, p200 and p212, in *Salmonella* induced a profound rearrangement of lipid membrane. Moreover, p200 caused growth defects both outside and inside murine macrophages, whereas p212 determined just inability to survive inside host cells (Fig.6.7) (Porta *et al.* 2010).

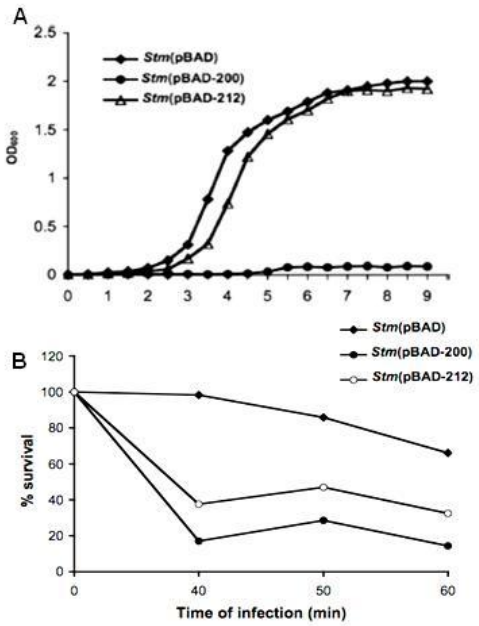

Figure 6.7- (A) Bacterial growth. Overnight cultures of Salmonella strains transformed using the plasmid pBAD containing the ORF200 or ORF212, compared to the control strain containing the empty plasmid pBAD. Expression of ORF200 strongly inhibited growth of *Stm*(pBAD-200) compared to the control strain *Stm*(pBAD) up to 9 h; expression of ORF212 had no significant effect on bacterial growth. (B) *In vitro* infection of murine macrophages with *Stm*(pBAD-200), *Stm*(pBAD-212), and *Stm*(pBAD). The number of *Salmonella* cells recovered from macrophage lysed strongly decreased when *Salmonella* expresses both ORF200 and ORF212.

CHAPTER 7

Results

7.1 Rational design of new peptides

As mentioned above, the previous work was based on the study of the effects of the expression of *Synechocystis* Δ^{12}-desaturase and its membrane domains in *Salmonella* Typhimurium. The results obtained were particularly promising in the case of the first *trans*-membrane domain coded by ORF200, since the expression of this sequence determined a significant reduction of *Salmonella* ability to survive and multiply inside murine macrophages (Porta *et al.* 2010).

It has been reported that the orientation of the membrane regions of prokaryotic and eukaryotic proteins inside the membrane is determined by the presence of arginine and lysine residues in the cytoplasmic area. This organization is also present in membrane of the endoplasmic reticulum, in the inner mitochondrial membrane, and in the chloroplast thylakoid membrane. Thus, a positive-inside rule seems to apply universally to all integral membrane proteins, with charged residues providing the topological requirement for their insertion inside membrane (Fontaine *et al.* 2011). Considering this assertion and in the search of new potential peptides with AMA, the length of ORF200 has been reduced to identify the minimal active sequence.

We identified three fragments, called A2/I2, A3/I3 and A4/I4 and derived from ORF200: A2/I2 kept both the aminoacid regions flanking the *trans*-membrane portion, A3/I3 had only the region preceding the *trans*-membrane portion and the peptide A4/I4 had neither. The aminoacid sequences are reported below. *Trans*-membrane portions are marked in red and have been identified using a *trans*-membrane topology predictor software (phobius.sbc.su.se).

p200
MAKASKAWASVLITLGAIAVGYLGIIYLPWYCLPITWIWTGTALTGAFVVGHDCGHR

A2/I2
MAKASKAWASVLITLGAIAVGYLGIIYLPWYCLPI

A3/I3
MAKASKAWASVLITLGAIAVGYLGIIYLPW

A4/I4
MAWASVLITLGAIAVGYLGIIYLPW

Further, for better confirming the importance of the positive inside rule in the correct insertion of a peptide inside the membrane bilayer, we mixed the central aminoacid sequence of p200, keeping unchanged the five aminoacids both preceding and following that sequence, obtaining the so called p200 scrambled (sc).

p200

MEKASKAWASVLITLGAIAVGYLGIIYLPWYCLPITWIWTGTALTGAFVVGHDCGHR

p200 scrambled
MEKASKATLGAGWASITGHVLILGWIWTYIYCLAVGTPWALTGAFVVIIYLP**DCGHR**

To explain the likely mechanism of action of AMPs we postulated that they may act on a specific membrane target rather than having a non specific effect on membrane.

Our model is based on the assumption that an AMP exhibits its antimicrobial activity if it interferes with lipid/protein interactions of a specific domain and, further, if this domain has a key role in pathogen survival and/or growth.

According to this model, the *Salmonella* two component system PhoP/PhoQ has been identified as target. In particular, the sensor PhoQ and the transcription factor PhoP are key proteins involved in the regulation of more than 30 virulence genes and that are required for pathogen's survival and persistence after phagocytosis by macrophages.

Through a bioinformatic approach, the aminoacid sequence of the first *trans*-membrane region of PhoQ protein (NC_003197.1) has been identified and modified, generating a peptide that kept the sequence for the correct location. Thus, two peptides have been designed. In the first peptide (named NUF) tryptophan (W) replaced all valine (V). Tryptophan was chosen because it is an aminoacid that has a major steric hindrance and its presence may determine the loss of the α-helix structure and alteration of MPS.

The second peptide (named STA) was derived by merging PhoQ sequence with that of an ABC transporter protein of *Staphylococcus epidermidis*.

PhoQ first *trans*-membrane region
SLRVRFLLATAGVVLVLSLAYGIVALVGYSVSFD

NUF
SLRVRFLLWTWGVVLVLSLWYWIVALVWYSVSFD

STA
SLRVRFLLATAMLVQVFIVFFGTTALLGLSVSFD

7.2 Cloning of A2/I2, A3/I3, A4/I4, p200scrambled, NUF and STA sequences

To investigate the effects of the expression of new peptides in *S.* Typhimurium, plasmids pBAD-A2/I2, pBAD-A3/I3, pBAD-A4/I4, pBAD-200sc, pBAD-NUF and pBAD-STA were used to electroporate *S*. Typhimurium to obtain *Stm*(A2/I2), *Stm*(A3/I3), *Stm*(A4/I4), *Stm*(p200sc), *Stm*(NUF) and *Stm*(STA) strains. A control *Stm*(pBAD) strain, carrying the empty vector pBAD, was also constructed. The promoter of the vector pBAD (Fig.7.1) is induced by arabinose and inhibited by glucose, thus it was possible to regulate the expression of peptides.

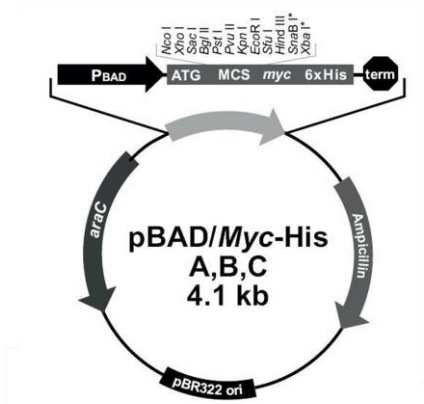

Figure 7.1-pBAD map. The figure summarizes the features of pBAD/Myc-His vector

7.3 Effect of peptides on *Salmonella* growth

Stm(p200), obtained in previous work (Porta *et al.* 2010), *Stm*(A2/I2), *Stm*(A3/I3), *Stm*(A4/I4), *Stm*(p200sc) and control strain *Stm*(pBAD) were grown in minimal medium containing 0.4% glucose or 2% arabinose as a sole carbon source to inhibit or induce respectively the transcription of inserted fragments from pBAD promoter. Growth of the strains was monitored up to 7.5 h. No difference in bacterial growth was observed in the presence of glucose at 37°C (data not shown). When arabinose replaced glucose, expression of p200 and p200sc strongly inhibited growth up to 7.5 h at 37°C (Fig. 7.2). Peptides A2/I2 and A3/I3 caused inhibition of *Salmonella* growth up to 6 h, and then resumed growth. Peptide A4/I4 showed a behavior similar to that of the control pBAD.

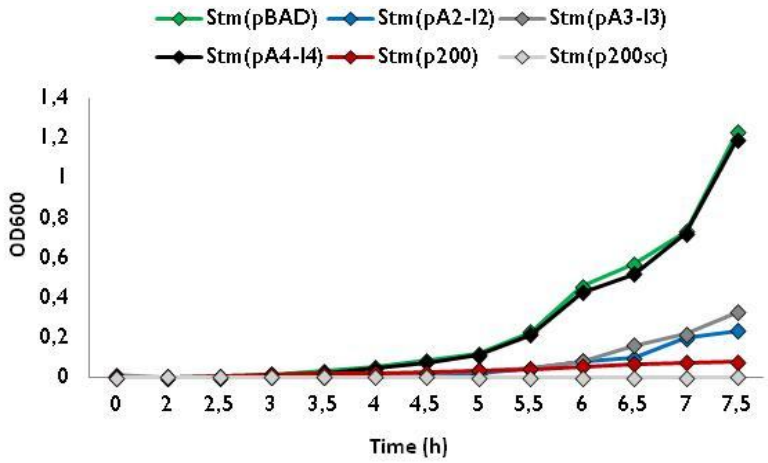

Figure 7.2- Bacterial growth. Overnight cultures of *Stm*(p200), *Stm*(A2/I2), *Stm*(A3/I3), *Stm*(A4/I4), *Stm*(p200sc) and *Stm*(pBAD) grown at 37°C in RM minimal medium containing 0.4% glucose as a sole carbon source were inoculated in fresh RM minimal medium containing 2% arabinose to induce transcription of inserted sequences from the pBAD promoter. Expression of ORF200 and 200 scrambled strongly inhibited growth of Salmonella compared to the control strain *Stm*(pBAD) up to 7.5

h;expression of A2/I2 and A3/I3 delays the log-phase, while A4/I4 had no significant effect on bacterial growth. Values are representative of three independent experiments, each performed in duplicate.

In the presence of arabinose, transcription of NUF and STA peptides had no significant effects on growth rate of *Salmonella* at 37°C, other than a slight delay of log-phase (Fig.7.3).

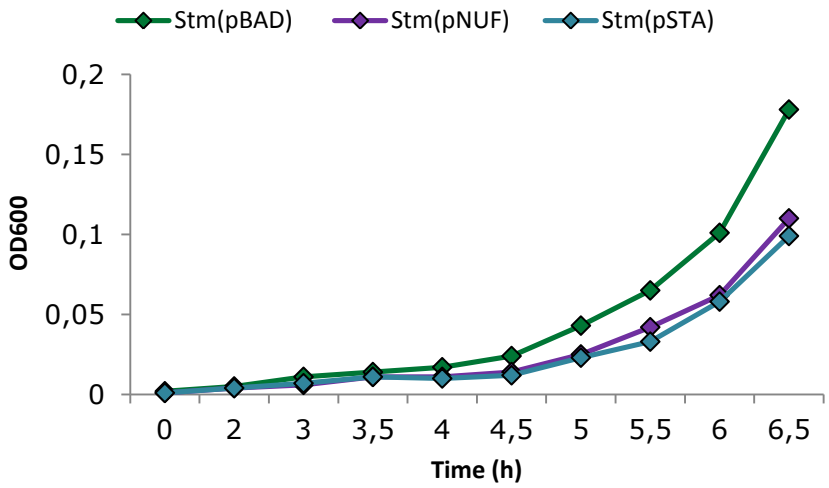

Figure 7.3-Bacterial growth. Overnight cultures of *Stm*(NUF), *Stm*(STA) and *Stm*(pBAD) grown at 37°C in RM minimal medium containing 0.4% glucose as a sole carbon source were inoculated in fresh RM minimal medium containing 2% arabinose to induce transcription of inserted sequences from the pBAD promoter. Expression of NUF and STA had no effects on growth rate of *Salmonella*, compared to control strain. Values are representative of three independent experiments, each performed in duplicate.

7.4 Murine macrophage infection with *Salmonella*

Internalization and intracellular persistence inside host cells was monitored by infecting murine macrophages with *Stm*(p200), *Stm*(A2/I2), *Stm*(A3/I3), *Stm*(A4/I4), *Stm*(NUF), *Stm*(STA) and control strain *Stm*(pBAD). Strains expressing peptides A2/I2, A3/I3 and A4/I4 did not show the same deficiency to infect and persist inside macrophages that was observed with *Stm*(p200) (Fig.7.4). Thus, the reduction of original sequence caused loss of antimicrobial activity.

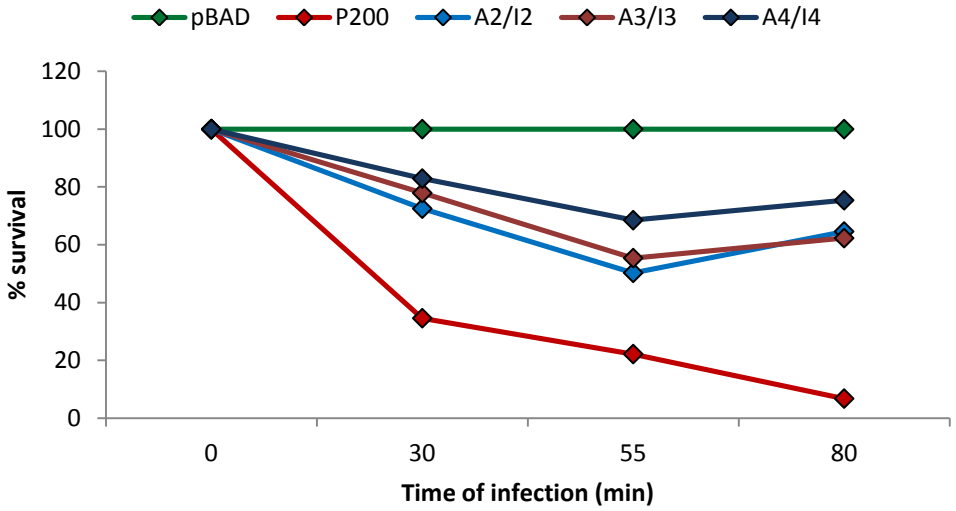

Figure 7.4-Intracellular persistence inside Mf of *Stm*(pBAD), *Stm*(p200), *Stm*(A2/I2), *Stm*(A3/I3), *Stm*(A4/I4). At different time points, macrophages were lysed, then recovered bacteria were plated on LB Amp100 agar with similar plating efficiencies. Survival is expressed as a percentage of the number of CFU at each time point (0, 30, 55, 80 min) compared to the number of CFU present at t0. *Stm*(p200) was unable to survive inside macrophages, while its smallest derived peptides did not determine any significant effect. Values are representative of three independent experiments, each performed in duplicate. Statistical differences were evaluated by a two-tailed Student's *t* test. *P* values less than 0.05 were considered significant.

Expression of p200sc caused the same antimicrobial effects of the original peptide p200. *Stm*(p200sc) entirely lost the capability to survive inside murine macrophages as *Stm*(p200) (Fig.7.5).

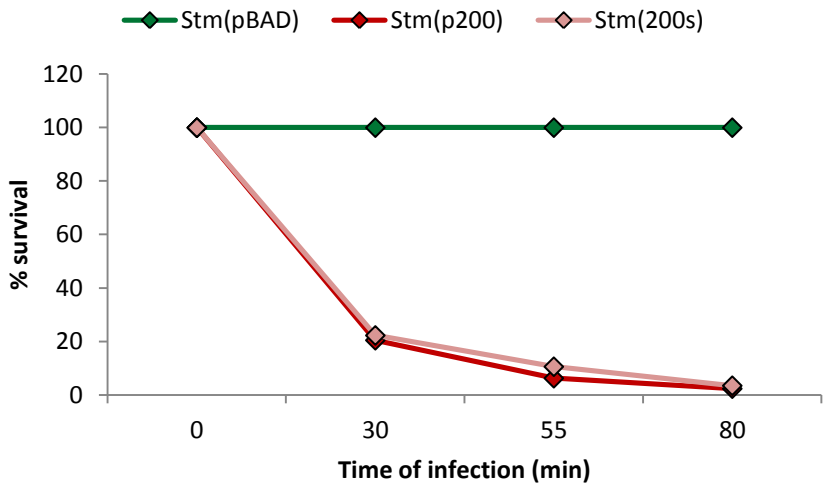

Figure 7.5- Intracellular persistence inside Mϕ of *Stm*(pBAD), *Stm*(p200), *Stm*(p200sc). The amount of CFU recovered after each time point of infection with *Stm*(p200sc) from lysed macrophages is comparable to that of *Stm*(p200). Values are representative of three independent experiments, each performed in duplicate.

The peptides designed to be targeted into PhoQ domain, NUF and STA, were tested to determine virulence of *Salmonella* inside host cells. Only the expression of NUF determined a strong inhibition (about 80%) of *Salmonella* surviving inside macrophages, expressed as low number of pathogen cells recovered after macrophages lysis (Fig.7.6). The strain carrying the peptide STA grew normally as the control strain.

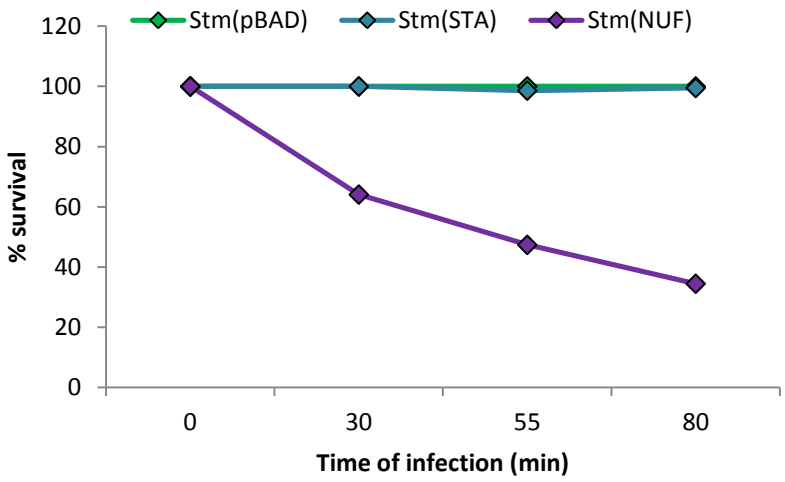

Figure 7.6- Intracellular persistence inside Mϕ of *Stm*(pBAD), *Stm*(NUF), and *Stm*(STA). A decrease of about 80% of *Stm*(NUF) cells recovered after 80min of infection was measured. Values are representative of three independent experiments, each performed in duplicate.

7.5 Expression of *rstA* gene in *Stm*(NUF)

Genome-wide transcription analysis revealed that the RstA/RstB two-component correlates with PhoQ/PhoP system. RstA/RstB leads to enhanced transcription of PhoP-activated genes in wild-type *Salmonella*. Computational approaches discovered that *rstA* promoter shares features with a group of PhoP-regulated promoters, thus the *rstA* gene seems to be a member of the PhoP regulon of *Salmonella* (Nam *et al.* 2010).

To investigate if the antimicrobial activity of NUF was due to a perturbation of PhoQ domain, the expression of *rstA,* as PhoP-dependent gene, in *Stm*(NUF) strain was analyzed.

Moreover, it is known that one of the stimuli sensed by PhoQ is a low concentration of Mg^{2+} ions which is also the condition used experimentally to induce PhoQ activation, while a high concentration of divalent cations keeps PhoQ in a repressed state.

Stm(NUF) and control strain *Stm*(pBAD) were grown in low and high magnesium condition (-Mg^{2+} and +Mg^{2+}) and total RNA was extracted from each sample to perform a real time PCR analysis.

Ribosomal 16S RNA, constitutively expressed in *Salmonella*, was used as the endogenous normalized control.

As expected, control strain *Stm*(pBAD) showed a low level of *rstA* gene expression when grown in $+Mg^{2+}$ condition and a higher level when grown in medium with low Mg^{2+} concentration, since the expression of this virulence gene regulated by PhoP is induced when the pathogen meets the phagosome environment.

In *Stm*(NUF) strain the expression level of *rstA* gene was low in both environmental conditions ($-Mg^{2+}$ and $+Mg^{2}$) (Fig.7.7), confirming the absence of PhoP-dependent regulation.

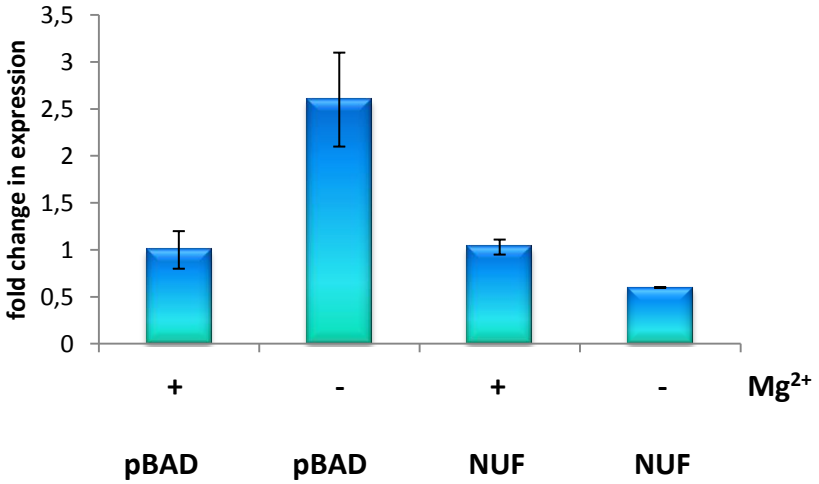

Figure 7.7-Expression of *rstA* transcripts in *Stm*(pBAD)($+Mg^{2+}$), *Stm*(pBAD)($-Mg^{2+}$), *Stm*(NUF)($+Mg^{2+}$) and *Stm*(NUF)($-Mg^{2+}$) strains. RNA was harvested from Salmonella strains grown in minimal medium in late logarithmic phase. The purified RNA was reverse-transcribed and the expression of *rstA* was examined by quantitative real-time PCR. The fold change in the abundance of transcripts in the different strains is relative to their expression in the *Stm*(pBAD)($+Mg^{2+}$) strain is presented. Expression was normalized using the housekeeping 16S rRNA gene as a control. The results represent the mean of 3 RT-PCR reactions from two independent RNA preparations with a standard error shown by the error bars.

7.6 RNA deep sequencing

To investigate more deeply the effects of expressed NUF and to confirm that they were due primarily to the interference with the activation of the major virulence system PhoQ/PhoP, the entire transcriptomes of *Stm*(pBAD) and *Stm*(NUF) strains were studied by RNA deep sequencing analysis, performed in collaboration with Prof. Alessandro Weisz. RNA was extracted from the strains grown in presence of both high ($+Mg^{2+}$) and low ($-Mg^{2+}$) magnesium concentration, since, as mentioned above, the low concentration of Mg^{2+} ions determines PhoQ activation as it occurs inside SCVs.

From the analysis of expression many genes showed different trends of expression compared to the control strain, but to limit the range of study and focus on our main goals, those genes that are known to be activated or repressed by PhoP were selected.

Four different ratio were considered: *Stm*(pBAD)(-Mg^{2+})/*Stm*(BAD)(+Mg^{2+}); *Stm*(NUF)(-Mg^{2+})/*Stm*(NUF)(+Mg^{2+});*Stm*(NUF)(-Mg^{2+})/*Stm*(pBAD)(-Mg^{2+}); *Stm*(NUF)(+Mg^{2+})/*Stm*(pBAD)(+Mg^{2+}).

The first cluster of genes analyzed was that involved in motility of *Salmonella.* It is known that decreased flagellins expression and cell motility inside host cells is dependent on activation of the PhoPQ pathway, that directly or indirectly negatively regulates transcription of flagellar genes. Results showed lack of this negative regulation in *Stm*(NUF) strain. Especially *fliZ*, which is a regulator of flagellar regulon and an activator of SPI-1 (Hung *et al.* 2012), and *fliB,* that codes for a metilase enzyme involved in flagella modification, were, as expected, down-regulated when the control strain was grown in (-Mg^{2+}) condition. On the contrary there was no difference of regulation in both environmental conditions in the case of *Stm*(NUF) and higher tanscript levels in *Stm*(NUF)(-Mg^{2+}) compared to *Stm*(pBAD)(-Mg^{2+}). *fliC* gene, constitutively expressed, also showed up-regulation in *Stm*(NUF)(-Mg^{2+}) compared to *Stm*(pBAD)(-Mg^{2+}), while its repressor *fljA* was strongly down-regulated (Fig.7.8).

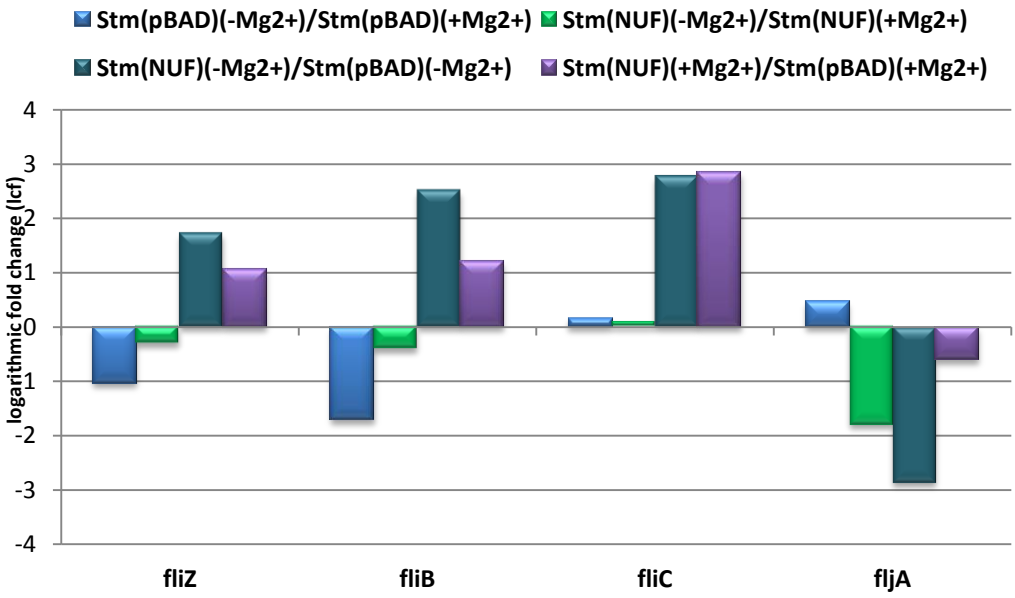

Figure 7.8- The graph shows the values in terms of logarithmic fold change of flagellin genes (*fliZ*, *fliB*, *fliC* and *fljA*)transcripts level obtained by four different ratio: *Stm*(pBAD)(-Mg^{2+}) vs. *Stm*(pBAD) (+Mg^{2+}), *Stm*(NUF)(-Mg^{2+}) vs. *Stm*(NUF)(+Mg^{2+}), *Stm*(NUF)(-Mg^{2+}) vs. *Stm*(pBAD)(-Mg^{2+}), *Stm*(NUF)(+Mg^{2+}) vs. *Stm*(pBAD)(+Mg^{2+}). Values represent the mean of three independent experiments.

The second network analyzed was that of SPI-1 related genes, which have a primary role in the entry of *Salmonella* inside non-phagocytic cells and in the initial infection phase, while they are normally repressed by PhoPQ inside SCVs. As shown in fig. 7.9, the expression of NUF, when PhoQ was activated, determined a less pronounced down-regulation of some of these genes compared to the control strain. Further, *hilD*, *prgI* and *SopE2* were down-regulated in *Stm*(NUF) strain when PhoQ was repressed. It is worth mentioning that *spaR* gene showed a major change of expression between the control and mutant strain (Fig.7.9)

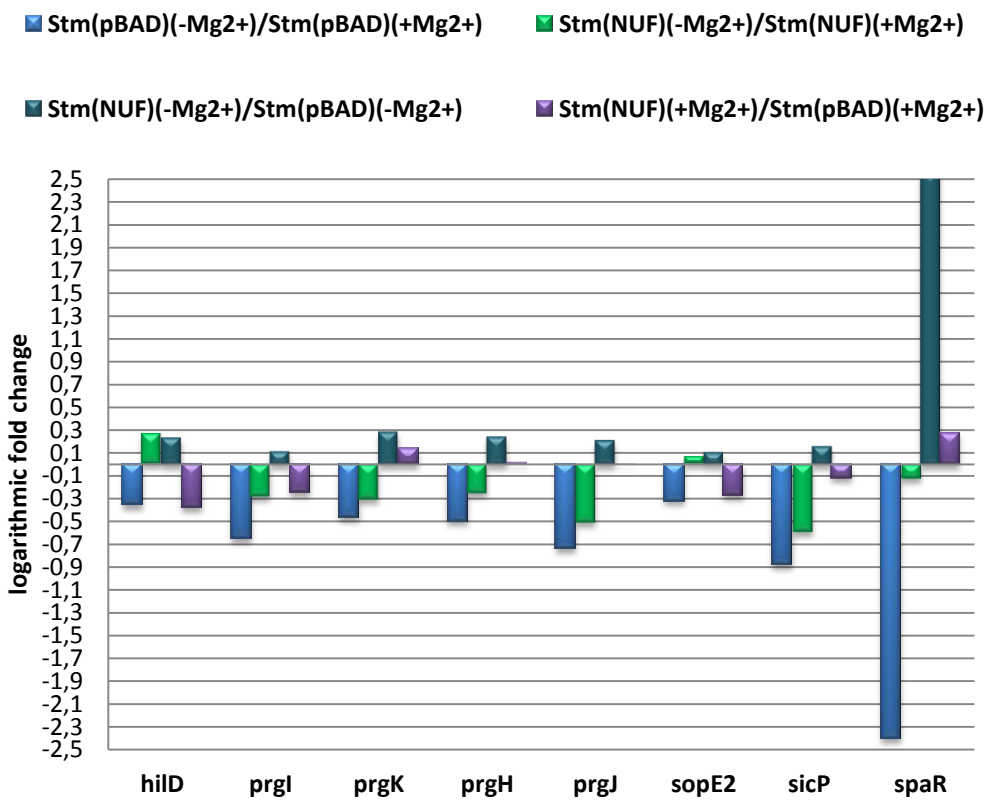

Figure 7.9- The graph shows the values in terms of logarithmic fold change of SPI-1 genes (*hilD*, *prgI*, *prgK*, *prgH*, *prgJ*, *sopE2*, *sicP* and *spaR*)transcripts level obtained by four different ratio: *Stm*(pBAD)(-Mg^{2+}) vs. *Stm*(pBAD) (+Mg^{2+}), *Stm*(NUF)(-Mg^{2+}) vs. *Stm*(NUF)(+Mg^{2+}), *Stm*(NUF)(-Mg^{2+}) vs. *Stm*(pBAD)(-Mg^{2+}), *Stm*(NUF)(+Mg^{2+}) vs. *Stm*(pBAD)(+Mg^{2+}). Values represent the mean of three independent experiments.

Furthermore, PhoP gene and those genes that normally are activated by PhoPQ system were selected. The genes belonging to SPI-2 system, which are activated by PhoP and codify for proteins involved in the virulence mechanisms operated by *Salmonella* inside phogosomes, such as *SseC*, *SseB*, *SsaD*, *SsaN*, *SsrB* and *pipB2*, were down-regulated when NUF was expressed in PhoQ activation condition (Fig.7.10).

PhoP gene was slightly up-regulated in *Stm*(NUF)(-Mg^{2+}) and its transcript level was lower than in *Stm*(pBAD)(-Mg^{2+}) but not completely off. This might be due to the fact that low Mg^{2+} concentration is not the only factor involved *in vivo*, since there are additional stimuli sensed by PhoQ in SCVs. Some of PhoP-activated genes (Pag) analyzed also showed a decreased expression in *Stm*(NUF) compared to *Stm*(pBAD) (Fig.7.11). Gene *rstA*, analyzed previously by real time PCR, also showed no difference of expression in *Stm*(NUF) between the two environmental conditions. Taken together, these data confirm a change in the pattern of expression of PhoPQ activated genes.

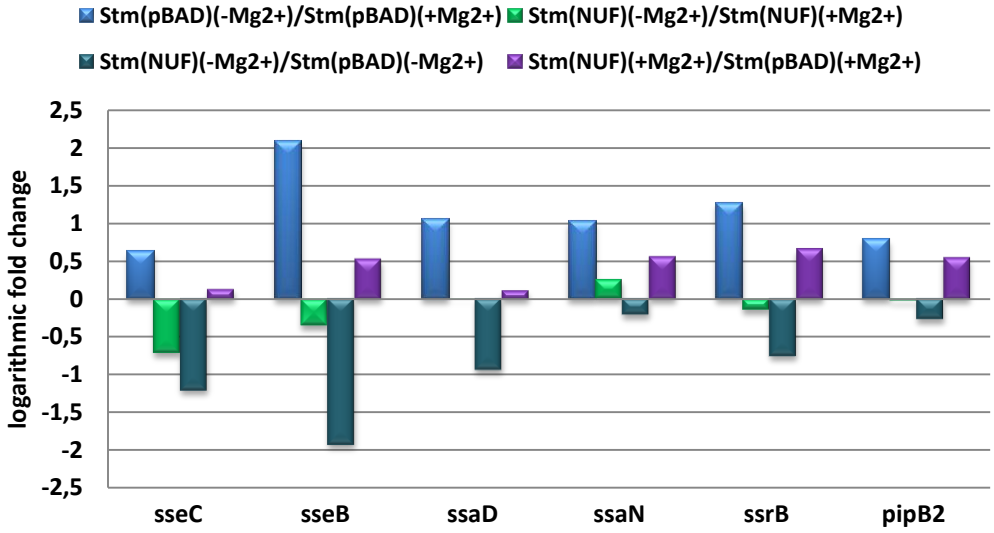

Figure 7.10- The graph shows the values in terms of logarithmic fold change of SPI-2 genes (*sseC*, *sseB*, *ssaD*, *ssaN*, *ssrB* and *pipB2*) transcripts level obtained by four different ratio: *Stm*(pBAD)(-Mg^{2+}) vs. *Stm*(pBAD) (+Mg^{2+}), *Stm*(NUF)(-Mg^{2+}) vs. *Stm*(NUF)(+Mg^{2+}), *Stm*(NUF)(-Mg^{2+}) vs. *Stm*(pBAD)(-Mg^{2+}), *Stm*(NUF)(+Mg^{2+}) vs. *Stm*(pBAD)(+Mg^{2+}). The values represent the mean of three independent experiments.

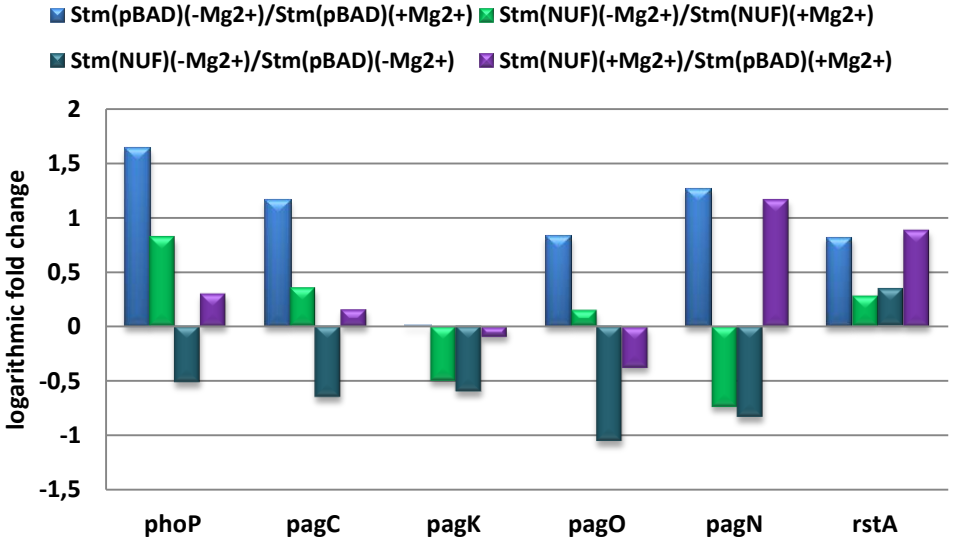

Figure 7.11- The graph shows the values in terms of logarithmic fold change of *phoP* gene, Pag genes (*pagC*, *pagK*, *pagO*, *pagN*) and *rstA* transcripts level obtained by four different ratio: *Stm*(pBAD)(-Mg^{2+}) vs. *Stm*(pBAD) (+Mg^{2+}), *Stm*(NUF)(-Mg^{2+}) vs. *Stm*(NUF)(+Mg^{2+}), *Stm*(NUF)(-Mg^{2+}) vs. *Stm*(pBAD)(-Mg^{2+}), *Stm*(NUF)(+Mg^{2+}) vs. *Stm*(pBAD)(+Mg^{2+}). The values represent the mean of three independent experiments.

Further, some genes of interest not directly regulated by PhoP but that are controlled by the stress response or necessary for survival of pathogen inside macrophages, were also evaluated. Among all genes for which a different pattern

between the two strains was found, those coding for sHSPs *ibpB* and *ibpA*, chaperones *GroES*, *GroEL*, *dnaJ* and *dnaK* and finally some enzymes involved in *Salmonella* virulence, were considered particularly interesting. Regarding the sHSPs, the expression of NUF determined a strong up-regulation of these genes when the pathogen was grown in high magnesium compared to control strain (Fig.7.12), possibly due to an effect on MPS that in presence of too high magnesium concentration required the protection by sHSPs.
Molecular chaperones as *GroES* and *GroEL* are normally also positively regulated from *SlyA*, which is in turn regulated by phosphorylated PhoP.
This study showed that molecular chaperones were down-regulated in *Stm*(NUF)(–Mg^{2+}) compared to *Stm*(NUF)(+Mg^{2+}) and to *Stm*(pBAD)(-Mg^{2+}) (Fig.7.12), condition that may determine an incorrect folding of proteins in the modified strain.

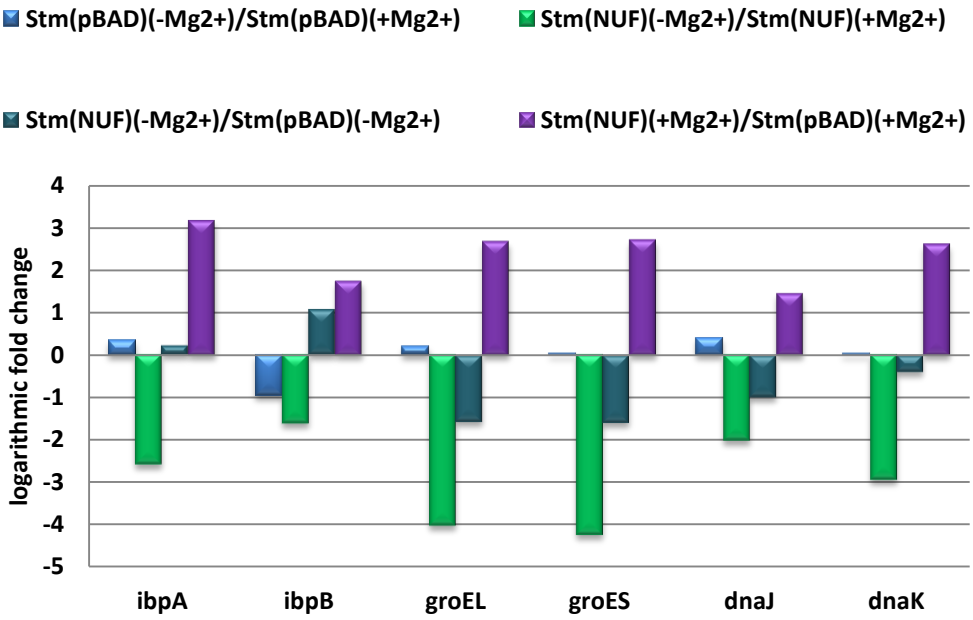

Figure 7.12- The graph shows the values in terms of logarithmic fold change of small heat shock genes *ibpa* and *ibpb*, chaperones gene *groES*, *groEL*, *dnaJ* and *dnaK*. Transcripts level obtained by four different ratio: *Stm*(pBAD)(-Mg^{2+}) vs. *Stm*(pBAD) (+Mg^{2+}), *Stm*(NUF)(-Mg^{2+}) vs. *Stm*(NUF)(+Mg^{2+}), *Stm*(NUF)(-Mg^{2+}) vs. S*tm*(pBAD)(-Mg^{2+}), *Stm*(NUF)(+Mg^{2+}) vs. *Stm*(pBAD)(+Mg^{2+}). The values represent the mean of three independent experiments.

It is also interesting to note how genes coding for enzymes necessary to the pathogen's ability to thrive in hostile environment, such as nitrite reductase (*nirBCD*), nitrate reductase (*napABCD*), superoxide dismutase (*sodA*) or anaerobic transporter (*dcuAB*), had very low transcript levels in *Salmonella* expressing NUF and growing in low magnesium environment, whereas in control strain *Stm*(pBAD) they were very strongly up-regulated (data not shown).

All results are summarized in Table 1.

	+Mg^{2+}		-Mg^{2+}	
GENES	**pBAD**	**NUF**	**pBAD**	**NUF**
Flagellins (*FliZ, FliB*)	↑↑	↑↑	↓↓	↑↑
Flagellar repressor *FljA*	↑	↑	↑	↓↓
SP-1 (*hilD, prgI, prgK, prgH, prgJ, sopE2, sicP*)	↑↑↑	↑↑↑	↓↓	↓
SpaR (SP-1)	↑↑↑	↑↑↑	↓↓↓	↑↑↑
SP-2 (*sseC, sseB, ssaD, ssaN, ssrB* and *pipB2*)	↓↓	↓↓	↑↑	↓↓
phoP	↓↓	↓↓	↑↑	↑
Pag genes (*pagC, pagK, pagO, pagN*)	↓↓	↓↓	↑↑	↓↓
sHS genes (*ibpa* and *ibpb*)	↓	↑↑	↓	↓
Chaperones (*groES, groEL, dnaJ* and *dnaK*)	↑	↑	↑	↓↓↓
Nitrite reductases Nitrate reductases Superoxide dism utase Anaerobic transporter	↑	↑	↑↑	↓↓

Table 1- Schematic representation of the results obtained by RNA deep sequencing. Transcript levels of several cluster of genes were analyzed in *Stm*(pBAD) and *Stm*(NUF) strains grown in two different environmental conditions (+Mg^{2+}; -Mg^{2+}). Induction and repression are represented by green and red narrows respectively.

7.7 Purification of PhoQ protein

Salmonella PhoQ gene was cloned into the expression vector pet21b and introduced into *E. coli* BL21 (DE3) (see Materials & Methods). To confirm the insertion of PhoQ gene inside the vector, an enzymatic hydrolysis of the plasmid, previously isolated, was performed (Fig.7.13). BL21D strain allows a better regulation of the protein expression, reducing the basal level without IPTG. After induction with 1mM IPTG (4h), PhoQ-His was detected as a strong protein band (MW~57 KDa) in the electrophoretic profile of crude lysate recovered after induction and in the soluble protein fraction, while it was absent in the lysate derived from inclusion bodies (Figure 7.14).

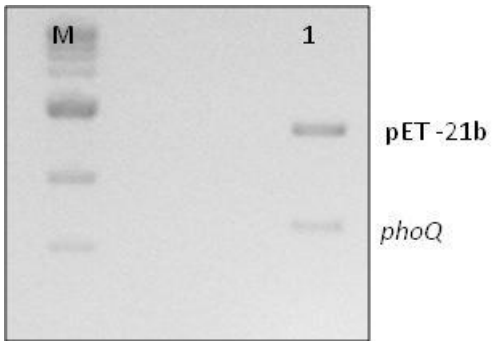

Figure 7.13- 1% Agarose gel. Marker (M); Lane 1: two bands corresponding to vector pET21b and *PhoQ* gene after hydrolysis of the plasmid with NheI and XhoI restriction enzymes.

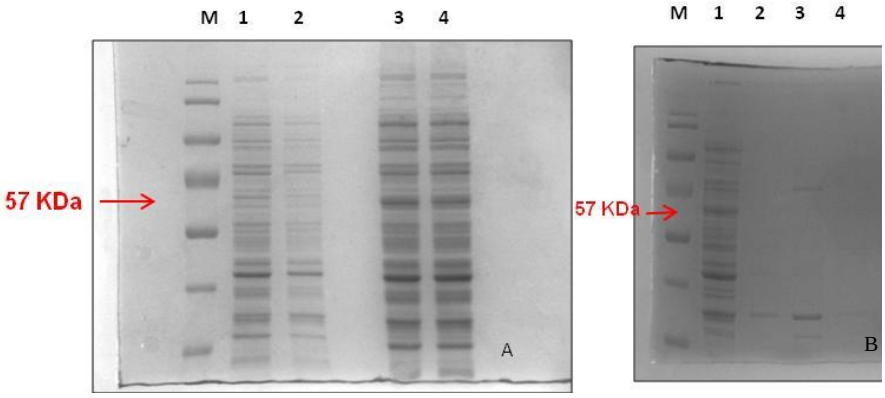

Figure 7.14- (A) M, Marker; Lanes 1-2: non induced controls; Lanes 3-4: induced controls. (B) M, Marker; Lane 1: soluble protein fraction; Lanes 2-3: washes; Lane 4: inclusion bodies.

Following purification by Ni-NTA chromatography, purified PhoQ was obtained from 1L culture of *E. coli* BL21 strain (Fig.7.15 and 7.16). Ni^{2+} ions link histidines, forming a complex that allows elution of all protein components except the recombinant protein. Using an appropriate buffer that breaks bonds between histidine and Ni^{2+}, the recombinant protein was eluted.

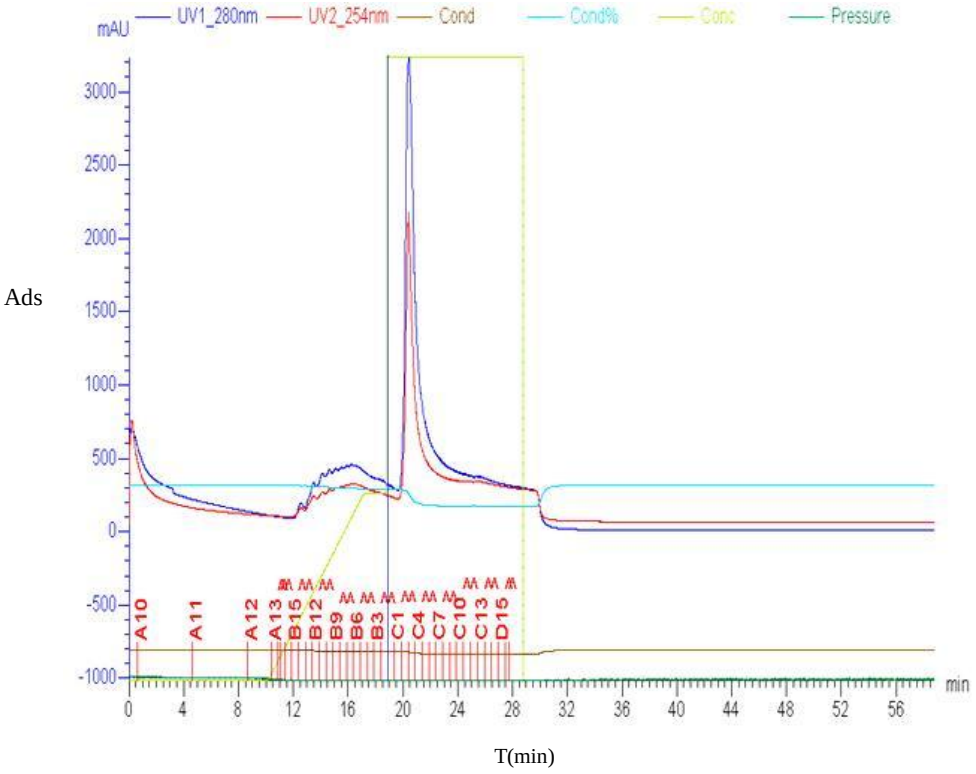

Figure 7.15- Chromatogram. Peak at 20 minutes corresponds to the eluted PhoQ protein.

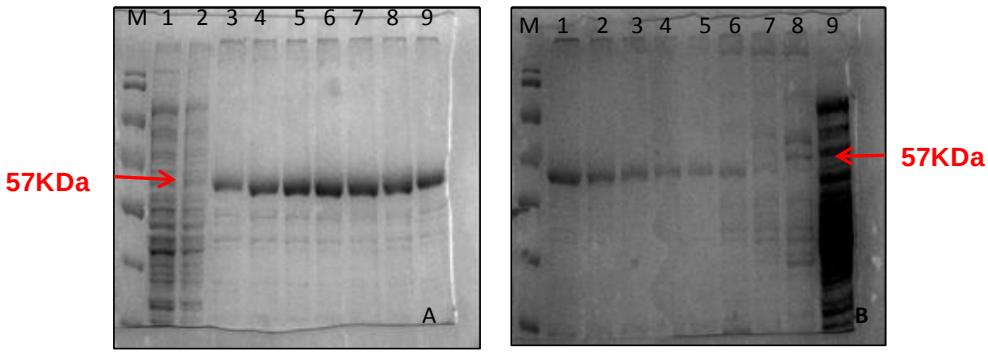

Figure 7.16- A) M, Marker; Lanes 1-2 total extract; 3, fraction B14; 4, fraction B15; 5, fraction C1; 6, fraction C2; 7, fraction C3; 8, fraction C4; 9, fraction C5. B) M, Marker; 1, fraction C7; 2, fraction C9; 3, fraction C11; 4, fraction C13; 5, fraction B12; 6, fraction B10; 7, fraction B8; 9, soluble proteic fraction.

Protein concentration (0.36µg/µL) was determined by A_{280}. Further, the purity was estimated to be about 60% by densitometric analysis (area/pixel) using a GELDOC instrument.

7.7.1 MALDI-TOF

To confirm the identity of the purified protein, *in situ* digestion was carried out. After bands excision, destaining, free cysteines block and trypsin hydrolysis (see Materials and Method section), peptides were extracted and MALDI-TOF analysis was performed (Fig.7.17). All peaks were interpretated and corresponded to tryptic peptides of the heterolog protein PhoQ. Further, the whole protein has been covered by MALDI analysis.

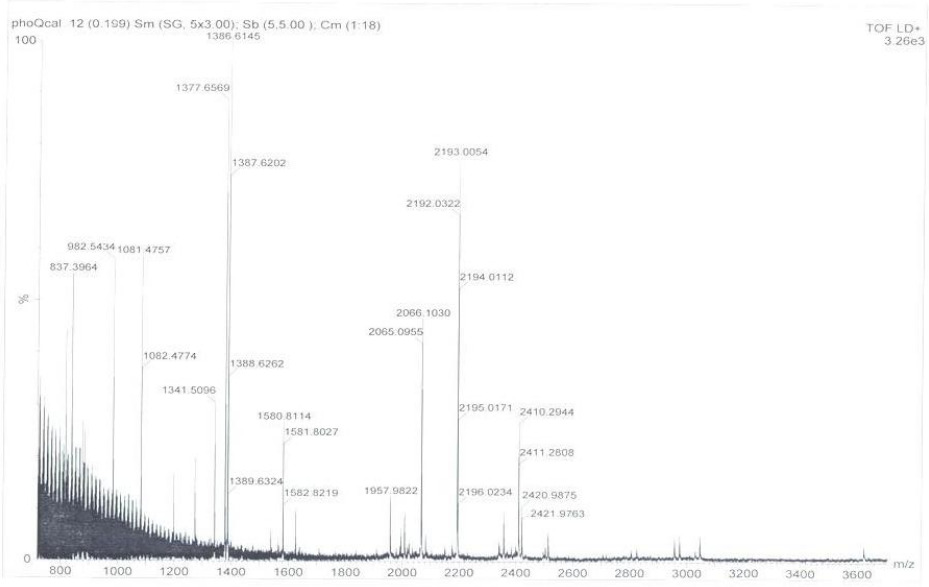

Figure 7.17- Maldi spectrum

7.8 Mouse infection

Cells in logarithmic-phase of the *Stm*(pBAD), *Stm*(p200), *Stm*(p212) and *Stm*(NUF) strains ($5x10^3$ cells/ml) were injected intravenously into C57/B6 mice (6/group). Mice infected with *Stm*(pBAD), *Stm*(p212) and *Stm*(NUF) strains began to die within 5 days, and among them only mice that received *Stm*(NUF) survived up to 18 days. Mice infected with *Stm*(p200), survived as the control group that received only the phosphate buffer saline (Fig.7.18).

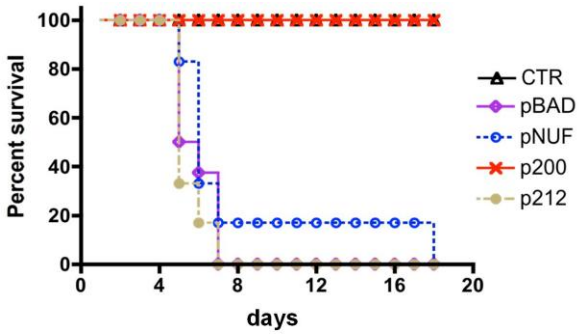

Figure 7.18-Mouse infection with *Salmonella* Typhimurium. All mice were infected with $5x10^3$ cells/ml of *Stm*(pBAD), *Stm*(p200), *Stm*(p212) and *Stm*(NUF) strains; control group (CTR) was injected with phosphate buffer saline. Mice infected with *Stm*(pBAD), *Stm*(p212) died within 8 days; mice injected with *Stm*(NUF) died within 18 days; mice infected with *Stm*(p200) survived as the control group.

Forty days after the initial infection all mice infected with *Stm*(p200) strain were alive (Fig.7.19). The survived mice and those of the control group were rechallenged with *Stm* wild type strain.

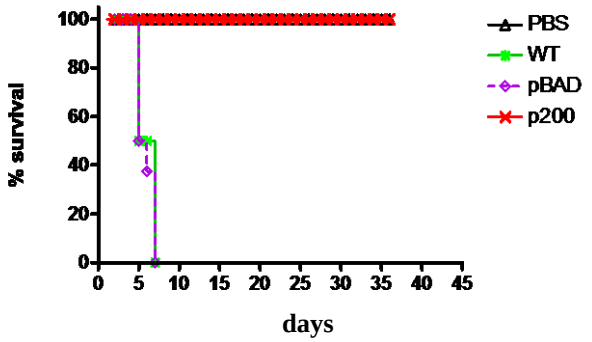

Figure 7.19- Mouse infection with *Salmonella* Typhimurium WT, *Stm*(pBAD), *Stm*(pBAD-200) and control group.

Figure 7.20 shows that 20 days after rechallenge (60 days from the initial infection) 78% of mice of the p200 initial group still survived, whereas mice of control group died within 8 days.

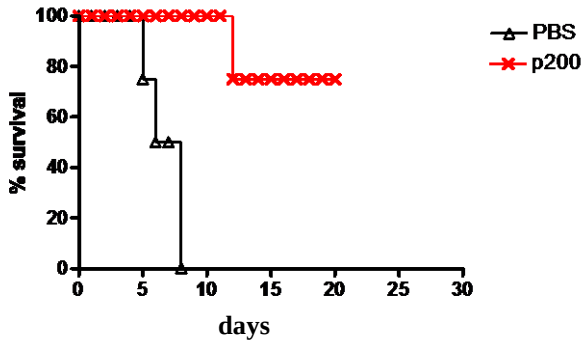

Figure 7.20- Surviving mice were challenged with *Salmonella* wild type strain. Seventy eight per cent of mice challenged with wild type *Salmonella* were still alive after 20 days (60 days from the initial infection).

7.8.1 Induction of splenomegaly and bacterial load in spleens

Infection with *Salmonella* is associated with transient splenomegaly due to recruitment of inflammatory cells. The development of splenomegaly was examined by weighing spleens harvested from mice infected with *Stm*(p200) and sacrified at different time points (3, 7, 14, 21 days) (Fig. 7.21A). One week post-infection, spleen weight increased 2 fold compared to that of saline-injected mice. This period of slight increasing spleen weight was directly related to the number of CFU recovered from spleens (Fig.7.21B). Fourteen days post infection a marked bacterial clearance was observed, to which corresponded a progressive decrease of spleen weight.

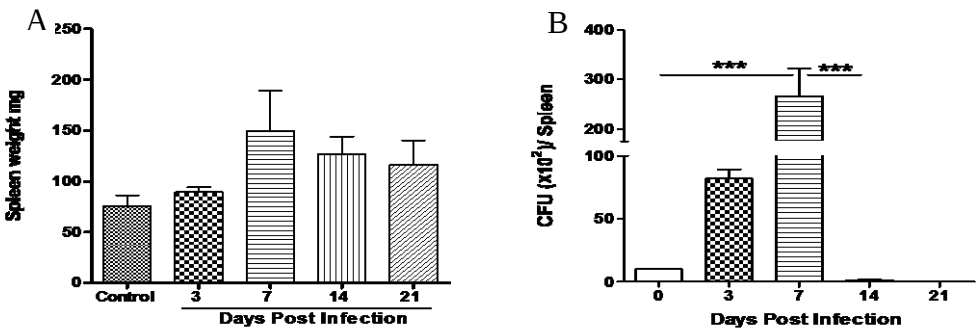

Figure 7.21- (A) Spleens of sacrifed mice were collected from mice injected with *Stm*(p200) at different time points (3,7,14,21 days) and from saline-injected mice after 21 days. (B) The supernatant from the digested spleens were plated on SSagar plates to determine the number of *Salmonella* CFUs.

7.8.2 Inflammatory cytokines production

Resistance to *Salmonella* infection is mainly dependent on a strong Th1 response that leads to macrophages activation and eradication of intracellular pathogens (Mastroeni *et al.* 1998). Thus, cytokines production both in serum and spleen cells of infected mice was analyzed at different days post infection (3,7,14,21). Saline-injected mice were used as control.

High levels of IL-12, a Th1-promoting cytokine, TNF-α and INF–γ were detected in sera and spleen cells of infected mice compared to control (Fig. 7.22). In particular, the highest levels of IL-12 and INF-g were observed in spleen supernatants collected 7 days after inoculation with *Stm*(p200), that corresponded to the highest number of *Salmonella* recovered. TNF-α accumulation in serum did not change in a time-dependent manner, unlike what was observed in the supernatants of spleen cells.

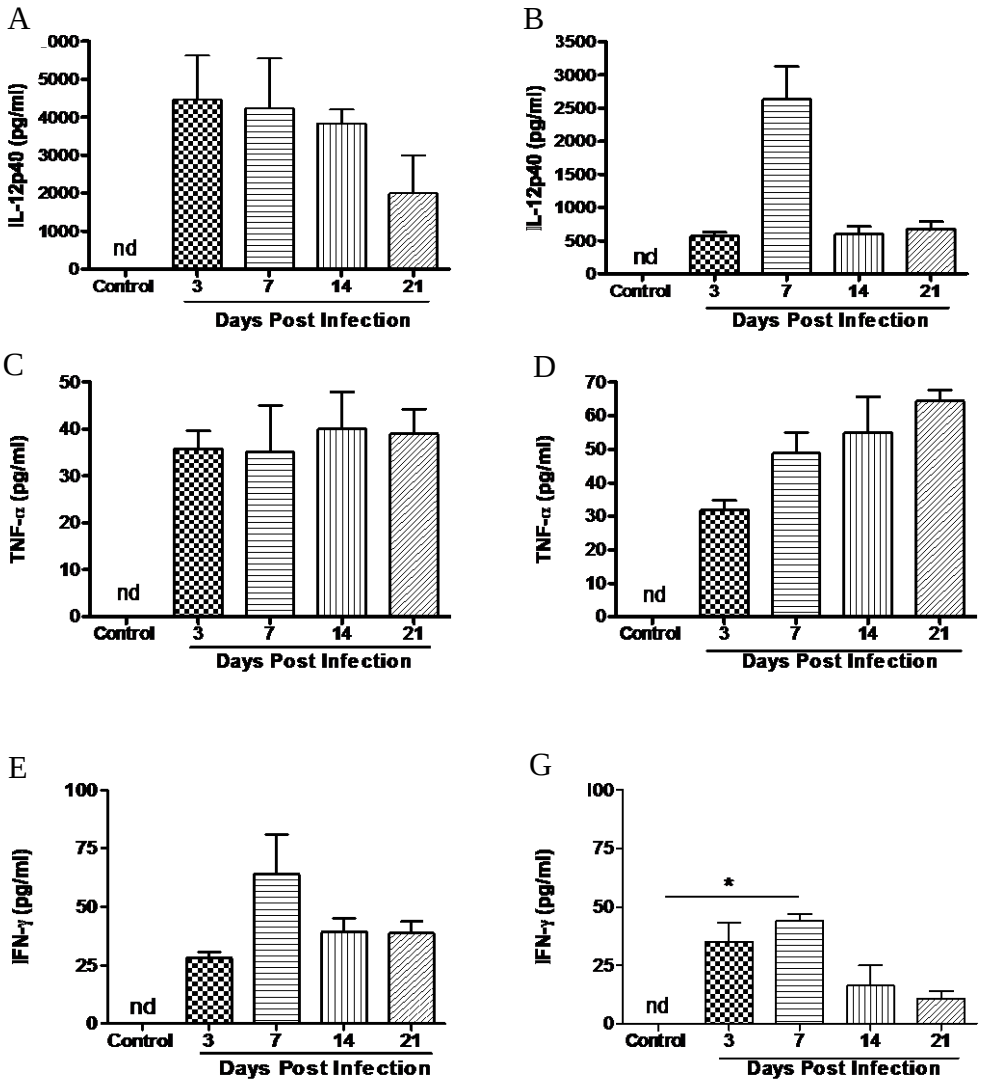

Figure 7.22- Citokine response in *Stm*(p200) infected mice. Sera and spleen supernatants were collected from mice sacrificed 3,7,14,21 days post infection. Saline injected mice were used as control and sacrificed after 21 days. Levels of IL-12p40, TNF-α and INF-γ were detected both in sera (A,C,E) and spleen supernatants (B,D,G). Each data point represents the mean ± SD of 5 mice per group.

7.8.3 Memory response

To investigate whether mice inoculated with *Stm*(p200) developed a recall T cell response to *Salmonella*, the production of INF-γ of spleen cell cultures, exposed *ex vivo* to sonicated *Salmonella* antigens (1μg/ml), was determined. Splenic cells were recovered 5 weeks following rechallenged with virulent *Stm* wild type strain. The release of INF-γ, in response to *Salmonella* antigens, was considerably high compared to that of non stimulated cells and of control (Fig. 7.23). The specificity of this response was also demonstrated by the low levels observed when cells were stimulated with LPS (1μg/ml).

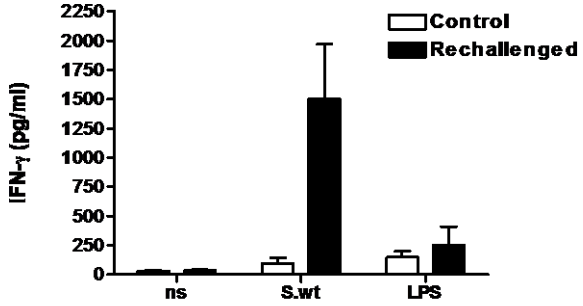

Figure 7.23- Development of memory Th1 lymphocytes. Mice were inoculated with *Stm*(p200) and after 40 days rechallenged with *Stm* wt. Five weeks after the rechallenge, spleen cells were harvested and set up in cultures alone (non stimulated, ns), in the presence of sonicated *Salmonella* antigens (1μg/ml) or LPS (1μg/ml). Cell-free cultures supernatants were collected 48h later and analyzed for INF-γ content by ELISA. Similarly processed splenocytes from saline-injected mice served as controls. Each data point represents the mean ± SD of 5 mice per group.

Systemic infection with attenuated *Salmonella* induce a strong production of IgG isotype antibodies through the expression of proinflammatory Th1 cytokines (ref). Levels of *Salmonella*-specific antibody IgG2b were measured in sera of rechallenged mice. Figure 7.24 shows a very strong IgG2b response, that, as expected, was totally absent in non infected mice.

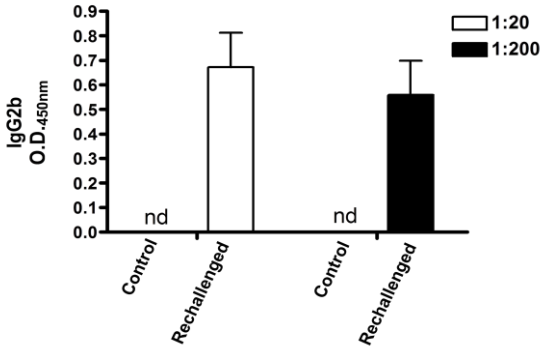

Figure 7.24- Production of *Salmonella*-specific immunoglobulins in mice infected with *Stm*(p200) and after 40 days rechallenged with *Stm* wt. Serum was collected 5 weeks post infection and, after dilution (1:20, 1:200), analyzed for the presence of IgG2b isotype. Serume from saline-injected mice was used as control. Each data point represents the mean ± SD of 5 mice per group.

CHAPTER 8

Conclusions

My PhD project focused on establishing innovative antimicrobial strategies based on peptides which exert their activity by interacting with membrane components and altered the membrane physical state (MPS). This approach allowed the development of new class of antimicrobials and a new type of vaccine.

Initially, the project started in Prof. Bruno Maresca's lab investigating the relationship between MPS and heat shock response. It was shown that in *S.* Typhimurium the imbalance in the membrane lipid/protein ratio due to overexpression either of *Synechocystis* Δ12-desaturase or individually expressed membrane regions of this protein caused major changes in the MPS and a strong impairment of the heat shock response. These effects have profound consequences on the pathogenicity of this microorganism (Porta *et al.* 2010). These, and other results in different cells, suggested that the perception of temperature is strictly controlled by membrane order and by a specific membrane lipid/protein ratio that ultimately cause transcriptional activation of heat shock genes. Moreover, other studies in the same laboratory showed that the capacity to control the level of HSPs produced pathogens with a reduced capacity to adapt to the host environment at the onset of infection (Porta *et al.* 2010). These data have encouraged additional studies.

The sequence derived from the first membrane region of *Synechocystis* Δ^{12}-desaturase, p200, was cloned and expressed in *Salmonella* Typhimurium LT2 strain. The mutant showed a strong inability to grow, survive and persist inside murine macrophages. The aminoacidic sequence of p200 was reduced to obtain the shortest still active sequence. By making different sequences, three new peptides, called A2/I2, A3/I3 and A4/I4, were derived. The oligos corresponding to these sequences were annealed and inserted individually into *Salmonella* by electroporation, and the transformed strains were tested for growth rate and macrophages infection. The reduction of the lenght of original sequence caused a decreasing of antimicrobial activity. Further, p200 sequence was scrambled to verify the importance of the positive inside rule, which states that aminoacids positively charged, as lysine and arginine, precede *trans*-membrane portion and are necessary for the correct location of proteins inside membrane. The scrambled p200 peptide, that was designed maintaining unchanged the first and the last five aminoacids flanking the *trans*-membrane sequence, showed, as expected, the same activity of p200. This result is consistent with data reported in literature, confirming the role of positive aminoacids in membrane targeting.

To confirm our model based on a specific rather than a generalized effect of AMPs, we hypothesized that an AMP to exhibit its antimicrobial activity must be inserted in a specific membrane domain and this domain have a key role in activating genes involved in virulence and in the survival of a pathogen.

We selected PhoP/PhoQ two-components system of *Salmonella*, that is the most important regulator of virulence genes in this (and other) pathogen(s). PhoQ is a sensor located in the inner membrane of *Salmonella* and other pathogens that is activated by different stimuli (such as acidic pH, low concentration of divalent cations and AMPs) when the bacterium is internalized by phagosomes. PhoQ, which is a histidine kinase, when is activated phosphorylates the regulator component of the system, PhoP, which in turn regulates the transcription of more than 30 virulence

genes, both PhoP-activated (Pag) and PhoP-repressed genes (Prg) (Kato *et al.* 2008). Thus, we designed an AMP targeted to the specific PhoQ domain to cause loss of virulence destabilizing it.

The peptide sequences were obtained from the original sequence of PhoQ, thus maintaining the necessary signal for its insertion in the proper membrane domain.

The modifications led to two peptides: the first, called NUF, tryptophan (that has an indole ring that determines more steric hindrance contributing to the destabilization of the domain) replaced all alanine residues; and a peptide, called STA, that was designed merging the PhoQ sequence with that of an ABC transporter of *S. epidermidis* (ZP_13170312.1). The oligonucleotide sequences coding for these peptides were inserted into *Salmonella.* Growth and *in vitro* infection experiments were analyzed in both *Stm*(NUF) and *Stm*(STA) strains. In particular, expression of NUF had no effects on growth rate but caused a strong reduction of *Salmonella* persistence inside murine macrophages. These data are consistent with our hypothesis, since PhoQ is activated when *Salmonella* is internalized by macrophages. *Stm*(STA) showed the same behavior of control strain in both experiments. This likely due to the loss of aminoacid properties required for the correct insertion in the target domain.

Transcriptome analysis of *Stm*(NUF) strain was performed by RNA deep sequencing to determine which genes were differentially regulated in response to expression of NUF. As mentioned above, one of the stimuli that activate PhoQ is the low concentration of divalent cations, especially Mg^{2+}. Experimental evidences showed that PhoQ can be activated or repressed by growing the cells in a medium containing 10 μM (-Mg^{2+}) or 10 mM Mg^{2+} (+Mg^{2+}) respectively. Among all genes analyzed, we focused on those sequences that are known to be regulated directly or indirectly by PhoP.

Salmonella enterica resides in a special membrane compartment of the host cell and modifies the host properties to achieve intracellular survival and proliferation. In particular, type III secretion systems have a predominant role in the interference of *Salmonella* with host cell functions. The two component regulatory system PhoPQ is an important sensor for the transition between extra- and intracellular life. Inside SCVs, PhoP positively regulates the genes of SPI2 system necessary for surviving inside phagocytic cells and down-regulates those of SPI1 system, that contribute mainly to the interaction of bacteria with the intestinal epithelium (Löber *et al.* 2006; Aguirre *et al.* 2006). Further, PhoP down-regulates flagellin genes (Adams *et al.* 2001) and up-regulates genes coding for enzymes necessary to resist the defense mechanisms of the host.

Regulation of these genes was clearly altered in *Stm*(NUF) strain, compared to control strain *Stm*(pBAD). Genes coding for flagellins (*FliZ*, *FliB* and *FliC*) and genes of SPI-1 system, that were down-regulated by PhoQ activation in *Stm*(pBAD), were up-regulated or slightly down-regulated in *Stm*(NUF). In particular, *spaR* gene, which is required for protein secretion and for the ability of *S*. Typhimurium to attach to cultured epithelial cells, was strongly down-regulated in control strain grown in (-Mg^{2+}). In *Stm*(NUF) *spar* expression did not change in the two growth conditions and it continued to be strongly up-regulated even when phoQ was activated. *spaR* gene has not been fully characterized and there are no evidence of a direct correlation with PhoPQ system. Therefore, our results suggest further studies. In addition, when *Salmonella* expressed NUF, genes of SPI-2 system were down regulated or their expression did not change when PhoQ was activated by low Mg^{2+}. *PhoP* gene was only slightly up-regulated in *Stm*(NUF) strain compared to control, However, it must

be considered that the activation of PhoQ was induced by low Mg^{2+} concentration, whereas *in vivo* additional stimuli that contributes to its activation are present. Further, we found that sHS genes were strongly up-regulated in *Stm*(NUF) grown in (+Mg^{2+}). Possibly this is due to an alteration of MPS that in addition to high Mg^{2+} concentration determined a requirement of protective proteins, as *ibpA* and *ibpB*. Molecular chaperones, such as GroES and GroEL, normally up-regulated by PhoP (Takaya *et al.* 2004) and necessary for the correct protein folding, were down-regulated in *Stm*(NUF) grown in (-Mg^{2+}). Genes coding for enzymes involved in metabolism and resistance to host defenses were also down-regulated in *Stm*(NUF), thus determining a loss of the mechanisms necessary for intracellular virulence and replication.

Taken together, these results suggest a new approach to develop AMPs derived from membrane proteins and targeted to a specific membrane domain.

Stm(pBAD), *Stm*(p200), *Stm*(p212) and *Stm*(NUF) strain were injected intravenously into C57/B6 mice and their survival monitored. Mice infected with *Stm*(p200) survived similarly to saline-injected mice.

The capacity of *Salmonella* to evade killing and replicate inside phagocytic cells determines the induction of local host defenses. Inflammatory responses in the infected tissues rapidly recruit neutrophils and inflammatory monocytes that migrate in anatomical sites containing a large population of macrophages, such as spleen and liver. One of the consequence is that the well-defined architecture of T cell and B cell zones becomes severely disrupted (Tam *et al*. 2008). These rapid changes of splenic architecture, cellular composition and cell trafficking are also accompanied by a profound increase of overall size of the organ.

Infection with *Stm*(p200) determined a slight splenomegaly 7 days post infection, and correlated to increase of bacterial CFUs recovered from digested spleens. Number of *Salmonella* after 2 weeks strongly decreased and spleen size gradually returned to normal.

The production of proinflammatory cytokines, including IL-12p40, TNF-α and INF-γ, is important for the activation of host anti-microbial defenses. *Stm*(p200) induced release of these cytokines both in sera and spleen supernatants. In particular, it was observed a very high production of IL-12p40, a Th1 promoting cytokine.

Survived mice, both *Stm*(p200) and saline-injected, were rechallenged with wild type *Salmonella* strain. Control mice died within 8 days, whereas mice that infected with *Stm*(p200) were alive after 20 days (>70%), suggesting that the initial inoculation conferred protection from the subsequent challenge with the wt strain. Since the modified *Salmonella* strain induced protection, it is reasonable to speculate that the overall antigenic *repertoire* of *Salmonella* might be unaffected or only slightly affected, thus presenting a full antigenic profile to the host immune system.

The results reported in this study show that *Stm*(p200) injection determined the activation of immunological memory, since mice developed recall T cell responses to *Salmonella*, as demonstrated by the strong production of INF-γ in response to *Salmonella* antigens, compared to saline injected mice and to LPS stimulation.

Further, anti-*Salmonella* antibodies isotype IgG2b were detected at high levels in sera collected 5 weeks post-rechallenge and exposed to heat killed *Salmonella*.

Salmonellosis is probably the most wide spread zoonosis in the world and the causative agent has a very broad animal host range, both domestic and farm animals. We can conclude that the modified *Stm*(p200) strain, obtained by the insertion of a portion of a membrane protein, represents a good candidate vaccine against Salmonellosis. Further, this methodology could be adapted to other pathogens, since the alteration of cell functionality and virulence via MPS modification is a

phenomenon shared by all organisms, as shown in previous studies (Porta *et al.* 2010).

This study open a new way for developing new class of vaccines based on live avirulent strains, which can overcome the problems correlated to the classic methods still in use.

CHAPTER 9

Materials and Methods

9.1 Media

Luria-Bertani
-10g bacto-tryptone (OXOID)
- 5g yeast extract (OXOID)
-10g NaCl (Applichem)
2% bacto-agar for plates

Minimal Medium 9 (MM9)
For 1 Liter of medium:
- 6,786 gr Na_2HPO_4
- 3 gr KH_2PO_4
- 0,5 gr NaCl
- 1 gr NH_4Cl
- 0.4% Glucose or 2% Arabinose
- 2% Casa amminoacids
- 1mM $MgCl_2$
- 1% Glycerol
- 100 μg/ml Ampicillin

Salmonella-Shigella (SS) agar (OXOID)

Dulbecco's Modified Eagle Medium (LONZA)
with 25mM HEPES and L-Glutamine w/ Na Pyruvate
properly supplemented with:
10% Fetal bovine serum (FBS)
50 U/ml Penicillin
50 mg/ml Streptomycin sulfate

9.2 Bacterial strains and growth conditions

Salmonella Typhimurium LT2 (kindly provided by R. Rappuoli, Novartis, Siena, Italy) was used as wild-type strain.
S. Typhimurium (pBAD), (A2/I2), (A3/I3), (A4/I4), (p200sc), (NUF) and (STA) were generated by insertion of fragments into pBAD vector transfected into LT2 background.
S. Typhimurium (p200) and (p212) were obtained in previous work (Porta *et al.* 2010)
Escherichia coli DH5-α, TOP10 and BL21 (Invitrogen) were used to amplify the constructs.

Bacterial cultures were routinely maintened in Luria-Bertani (LB) liquid medium or on LB agar plates supplemented with the appropriate antibiotics when necessary at 37°C with vigorous shaking in aerobic conditions.

9.3 Cell lines and growth conditions

Murine macrophages Mϕ (cell line J774A.1; ATCC TIB 67 [American Type Culture Collection, Manassas, VA]) were routinely grown in DMEM at 37°C, 5% CO2.

9.4 Plasmid construction

Using the Codon Usage of *Salmonella*, the oligos corresponding to the identified aminoacid sequences were designed.

A2/I2
Fw
5'-CATGGCGAAAGCGTCCAAAGCGTGGGCGTCCGTGCTGATTACCCTGGGCGCGATTGCGGTGG
GCTATCTGGGCATTATTTATCTGCCGTGGTATTGTCTGCCGATTTAATAACTGCA-3'
Rv
3'-CGCTTTCGCAGGTTTCGCACCCGCAGGCACGACTAATGGGACCCGCGCTAACGCCACCCG
ATAGACCCGTAATAAATAGACGGCACCATAACAGACGGCTAAATTATTG-5'

A3/I3
Fw
5'-CATGGCGAAAGCGAGCAAAGCGTGGGCGAGCGTGCTGATTACCCTGGGCGCGATTGCGGTGGG
CT ATCTGGGCATTATTTATCTGCCGTGGTAATAACTGCA -3'

Rv
3'- CGCTTTCGCTCGTTTCGCACCCGCTCGCACGACTAATGGGACCCGCGCTAACGCCACCCGATAG
ACCCGTAATAAATAGACGGCACCATTATTG-5'

A4/I4
Fw
5'-CATGGCGTGGGCGAGCGTGCTGATTACCCTGGGCGCGATTGCGGTGGGCTATCTGGGCATTATTT
ATCTGCCGTGGTAATAACTGCA-3'

Rv
3'- CGCACCCGCTCGCACGACTAATGGGACCCGCGCTAACGCCACCCGATAGACCCGTAATAAATAGA
CGGCACCATTATTG- 5'

200 scrambled
Fw
5'-CATGGAAAAAGCGAGCAAAGCGACCCTGGGCGCGGGCTGGGCGAGCATTACCGGCCATGTGCTG
ATTCTGGGCTGGATTTGGACCTATATTTATTGTCTGGCGGTGGGCACCCCGTGGGCGCTGACCGGCG
CGTTTGTGGTGATTATTTATCTGCCGGATTGTGGCCATCGCTAATAACTGCA-3'

Rv
3'-GTTATTAGCGATGGCCACAATCCGGCAGATAAATAATCACCACAAACGCGCCGGTCAGCGCCCAC
GGGGTGCCCACCGCCAGACAATAAATATAGGTCCAAATCCAGCCCAGAATCAGCACATGGCCGGTAA
TGCTCGCCCAGCCCGCGCCCAGGGTCGCTTTGCTCGCTTTTTC-5'

STA
Fw
5'-CATGGCGAGCCTGCGCGTGCGCTTTCTGCTGGCGACCGCGATGCTGGTGCAGGTGTTTATTGTGT
TTTTTGGCACCACCGCGCTGCTGGGCCTGAGCGTGAGCTTTGATTAATAACTGCA-3'

Rv

3'-GTTATTAATCAAAGCTCACGCTCAGGCCCAGCAGCGCGGTGGTGCCAAAAAACACAATAAACACCT
GCACCAGCATCGCGGTCGCCAGCAGAAAGCGCACGCGCAGGCTCGC-5'

NUF

Fw

5'-CATGGCGAGCCTGCGCGTGCGCTTTCTGCTGTGGACCTGGGGCGTGGTGCTGGTGCTGAGCCTG
TGGTATTGGATT GTGGCGCTGGTGTGGTATAGCGTGAGCTTTGATTAATAACTGCA-3'

Rv

3'-GTTATTAATCAAAGCTCACGCTATACCACACCAGCGCCACAATCCAATACCACAGGCTCAGCACC
AGCACCACGCCCCAGGTCCACAGCAGAAAGCGCACGCGCAGGCTCGC-5'

After annealing reactions, the fragments were cloned into pBAD Myc/His vector (Invitrogen) using NcoI and PstI restriction sites, previously added to 5' and 3' respectively of each oligo, and the plasmids pBAD-A2/I2, pBAD-A3/I3, pBAD-A4/I4, pBAD-200sc, pBAD–NUF and pBAD-STA were generated. Ligation was performed according to manufacturer's (Promega) protocols using T4-ligase (Roche).
Recombinant plasmids were used to transform *E. coli* DH5α. DNA was purified using a NucleoSpin plasmid kit (Macherey-Nagel).
The recombinant vectors were then used to transform the *S*. Typhimurium LT2 wild-type (wt) strain to obtain *Stm*(A2/I2), *Stm*(A3/I3), *Stm*(A4/I4), *Stm*(p200sc), *Stm*(NUF) and *Stm*(STA). A control strain, *Stm*(pBAD), carrying the empty pBAD vector was also constructed.
The pBAD system was chosen for the internal membrane fragments since this promoter has high transcription efficiency particularly with short peptide fragments and can be easily induced by arabinose.
To amplify the PhoQ gene, the genomic DNA of *Salmonella* was extracted according to standard procedures (Sambrook and Russell 2001) and the following primers, containing the recognition sequences for NheI and XhoI recstrition enzymes, were used:
OLIGO ***phoQ*** fw: *NheI* 5'-AATAAATTTGCTCGC-3' T melting 55°C
OLIGO ***phoQ*** rw: *XhoI* 3'-TTCCTCTTTCTGTGT-5' T melting 55°C
A first step of amplification was carried out using the TA Cloning Kits (pCR2.1 Invitrogen). *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. The obtained plasmid was amplificated transforming *E.coli* TOP10 strain. The vector PCR®2.1-TOPO has a portion of the gene lacZ codifying for β-galattosidase enzyme that allows the white/blue selection of cells containing the recombinant plasmid.
Afterwards, the vector pET-21b(+) (Novagen) was used for cloning and expressing phoQ. The purified PCR product and the expression vector were digested with the *NdeI* and *XhoI* restriction enzymes and purified. Ligation was performed according to manufacturer's (Promega) protocols using T4-ligase (Roche). The vector's promoter is induced by IPTG. The expressed recombinant protein has a His-Tag at the C-terminus.
The recombinant plasmid was used to transform *E. coli* DH5α strain. DNA was purified and sequence was determined using a NucleoSpin plasmid kit (Macherey-Nagel). The recombinant vector was then used to transform *E. coli* BL21 strain.

9.5 Bacteria electroporation

Bacteria grown o.n. were diluted 1:100 in LB medium and grown at 37°C. When the culture reached an OD_{600} of 0.5-0.7, it was incubated at 4°C for 30 min, pelleted, and washed with ice-cold 10% (wt/wt) glycerol (5x). After washing steps, the pellet was resuspended in ice-cold 10% (wt/wt) glycerol (125 μl/100 ml of culture). Forty microliters of electrocompetent cells was mixed with 1.0μg of DNA in double-distilled H_2O (ddH_2O), and then the mixture was added to a prechilled 0.2-cm chilled cuvette (Bio-Rad, Richmond, CA). Bacteria were pulsed using a Bio-Rad gene pulser set at 2.5 kV, 25 μF, and 200 Ω. Immediately after the bacteria were pulsed, 500μl of LB broth was added, and bacteria were incubated at 37°C for 30 min. Subsequently, 150-μl aliquots were spread on LB agar plates containing 100μg/μl ampicillin (Amp^{100}) (Sigma-Aldrich) and incubated o.n. at 37°C.

9.6 Growth curves

Stm(pBAD), *Stm*(A2/I2), *Stm*(pA3/I3), *Stm*(A4/I4), *Stm*(p200sc), *Stm*(NUF), *Stm*(STA) and *Stm*(p200) strains were inoculated in minimal medium supplemented with 0,4% glucose at 37°C. Each colture was then diluted for having an OD_{600}=0,1 in minimal medium supplemented with 2% arabinose. Bacterial growth rate was monitored spectrophotometrically for 8 hours.

9.7 Infection of murine macrophages with *Salmonella*

Entry and intracellular persistence into Mϕ of *Stm*(pBAD), *Stm*(p200), *Stm*(A2/I2), *Stm*(A3/I3), *Stm*(A4/I4), *Stm*(p200sc), *Stm*(NUF), *Stm*(STA) were carried out in 96-well tissue cultures plates (Corning) in triplicate. J774A.1 cells were seeded at a density of 10^5 cells/well 24h prior to infection. *Salmonella* strains were grown overnight on minimal medium (MM9) supplemented with 0,4% glucose at 37°C and after 24h were diluted in minimal medium supplemented with 2% arabinose for having an OD_{600}=0,01 at 37°C. During the log phase (0,1 OD_{600}) the cells were added to the Mϕ monolayer, to which previously the medium was replaced with DMEM low glucose and without antibiotics, to achieve a multiplicity of infection (MOI) of 10:1. Plates were centrifuged for 2 min to synchronize Mϕ infections, which proceeded for 30 min at 37°C. Cultures were washed with phosphate-buffered saline (PBS) to eliminate all noninternalized bacteria. Macrophages were lysed at different time points with 0.1% Triton-X (Sigma-Aldrich, St. Louis,MO) to recover bacteria. Serial dilutions were plated on LB agar containing Amp^{100} and incubated o.n. at 37°C to determine the number of CFU (colony forming units). Survival is expressed as percentage of CFU at each time point (*Tx*) compared to the number of CFU present at time zero (*T*0).

9.8 RNA extraction

Stm(pBAD) and *Stm*(NUF) strains were grown o.n. in MM9 containing 0.4% glucose at 37°C. Each colture was then diluted for having an OD_{600}=0.1 in MM9 with 2% arabinose, using two different concentrations of magnesium chloride ($MgCl_2$), 10μM (-Mg^{2+}) and 10mM (+Mg^{2+}). When the coltures reached an OD=0.5-0.6, total RNA was extracted using the *"RNeasy ® Protect Bacteria Mini Kit" (Qiagen)* according to the manufacturer's instruction.

The quantity and the quality of the extracted RNA were determined by an ND-1000 spectrophotometer (NanoDrop Technologies). To diminish any genomic DNA contamination, RNA was secondarily treated with an RNase-free DNase I of the *RNase-Free DNase Set* kit *(Qiagen).*
1 µl of every sample was loaded on 1,5% agarose gel and PCR reaction was carried out to check the presence of genomic DNA.

9.9 Quantitative real-time PCR

0,5 µg of DNase I-treated RNA was subjected to a first strand cDNA synthesis using *GeneAmp Gold RNA PCR Core Kit* (*Biosystems)*.
Real time PCR reactions were performed in a *LightCycler®480* machine (*Roche*). Each reactions was carried out in a total volume of 20 µl on a 96-well optical reaction plate (Roche) containing 10µl *Maxima® SYBR Green/Fluorescein qPCR Master Mix (2X) (Fermentas),* 5µl cDNA and gene specific primers.

16S fw: CAGGCTTGAGTCTTGTAGAG (TM 53°C)
16S rw: CACAACCTCCAAGTAGACAT
rstA fw: 5' AGGATCTTATCCTCACCACTCAGC 3'
rstA rw: 5' CCATCTCCAGCGCCAGAATATGAT 3'

Real-time cycling conditions were as follows:

Pre-incubation	95°C	5min	
Amplification	95°C	10sec	45 cycles
	60°C	10sec	
	72°C	10sec	
Melting curve	95°C	5sec	1 cycle
	65°C	1min	
	97°C		
Cooling	4°C		

Relative quantification of transcripts was evaluated using the comparative C_t method. The housekeeping gene, 16S, was used as the endogenous normalized control. The ΔC_t values were calculated by determining the difference between the values of genes *rst* and *pag* vs. the values of the housekeeping gene 16S for each sample. Calculation of $\Delta\Delta C_t$ involved the subtraction of the normalized pBAD ($+Mg^{2+}$) ΔC_t value from the normalized ΔC_t value of the other samples. Fold-differences in gene expression were calculated as $2^{-\Delta\Delta Ct}$.

9.10 Expression of recombinant PhoQ in *E. coli* BL21 (DE3)

Transformed *E. coli* BL21 (DE3) strain was grown in LB medium with ampicillin (100 µg mL^{-1}). Preculture was incubated at 37°C on a rotary shaker. As the OD_{600} reached 0.5-0.6, the cells were induced by addition of 1 mM IPTG. The culture was stopped after 4 h of cultivation. Sample (1 mL) was first centrifuged (13,000 rpm, 2 min, RT), and the cell pellet was resuspended in Laemmli buffer (200 µl).

Sample was centrifuged 20' at 13000rpm RT. Cell suspension was loaded on 12% polyacrilamide gel, together with non-induced control, to check protein induction. Both cell suspensions and pellets were stored at –20°C.

9.11 Purification of PhoQ protein

For protein purification, cell pellet was resuspended in 5 ml sonication buffer (Tris/HCl pH 8 1M, NaCl 5M, EDTA 0.5M, PMSF 100mM,Triton X-100, Deossicolate sodium 0.4%, MgCl2 1M, Lisozyme 1mg/ml, DNase 1mg/ml) and disrupted by sonication (5-10 minutes 10" ON/10"OFF (30% power-amplitude). The crude extract was centrifuged (10,000 x *g*, 15 min, 4°C), and the resultant supernatant represents the soluble proteic fraction.
The pellet was washed with 5ml Sonication buffer and centrifuged (15min, 10,000g, 4°C). Then the pellet was washed again 2 times with Sonication buffer without PMSF and Triton X-100 and resuspended in the followed buffer: 25ml of NaH_2PO_4 0.34gr, Tris 0.03gr, Urea 12gr, pH8. Finally, the sample was left 1h at 4°C shaking and centrifuged (13,000rpm, 25min, 4°C). The suspension obtained represents the proteic extract isolated from inclusion bodies. The fractions were quantified using Bradford method.
For purification of the $PhoQ_{His}$ protein, the proteic extract was loaded on to a 500 µl Ni Sepharose High Performance column (GE Healthcare).
The resine was washed three times with H_2O (5 volumes), then 10 volumes of Binding buffer (20mM Na_2HPO_4, 500mM NaCl, 20mM Imidazole pH 7.4) were added and the solutions was centrifuged (2000 rpm, 2 min) (2X).
The proteic extract (4ml) was then loaded (with imidazole 20mM) and stored 3h at 4°C shaking. The sample was centrifuged (13,000rpm, 10 min, 4°C) and the first fraction was collected.
The rest was washed with 10 volumes of Wash buffer (the same composition of Binding buffer but having pH 6.5, with Imidazole 20mM) (3X). The second fraction was then collected. The sample was then eluted with 500µl Elution Buffer (20mM Na_2HPO_4, 500mM NaCl, 0.8% Triton) containing a gradient of different Imidazole concentrations, from 50mM to 200mM, this step was repeated three times for each concentration.
After centrifugation (13,000rpm, 10min) all the last fractions was collected and loaded on 12% acrilamide gel.
The scale-up was performed using HITRAP (AMERSHAM) column on Fplc system (Akta purifier pH/C 900) and with 1L of colture.

9.12 *In situ* digestion

Bands excision: The acrilamide gel was washed with water to remove the excess of reagents and sovents (2x). The bands of interest were excised and placed in microtubes.
Decoloration: To each micro tube 120µl of acetonitryil (ACN) were added. After gel dehydration (about 10 minutes) ACN was removed. The gel was then rehydrated with ammonium bicarbonate (AMBIC) 0.1M. ACN was added again.
Reduction and alchilation: A solution DTT 10mM in 0.1M AMBIC was added and samples were incubated at 56°C for 45 minutes. The solution was removed and ACN was added. After removing ACN, a solution 55mM of Iodoacetamide in 0.1M AMBIC was added and followed by incubation for 30 minutes in the dark at room temperature.

Enzymatic hydrolysis: A cold solution of 12.5ng/µl Trypsin in 10mM AMBIC was added to the gel and incubated at 4°C 2h. The solution not adsorbed was removed and a solution 10mM AMBIC was added and incubated at 37°C overnight.
Extraction and MALDI analysis: The surnatant was recovered and analyzed by MALDI together with one more extraction made with ACN.

9.13 Mice

C57Bl/6j mice were purchased from Harlan Laboratories (Udine, Italy) and maintained in specific pathogen-free conditions. This study was carried out in strict accordance with the recommendations in the Institutional animal care guidelines, Italian D.L. no. 116 of 27 January 1992 and European Communities Council Directive of 24 November 1986 (86/609/ECC). The ethics committee of Pharmaceutical and Biomedical Department of the University of Salerno approved this study.

9.13.1 Animals survival

Cells of *Stm*(pBAD), *Stm*(p200), *Stm*(p212) and *Stm*(NUF) strains grown in arabinose-containing medium and stopped in logarithmic-phase, were diluted in sterile Phosphate buffer saline ($5x10^3$ cells/ml) and injected intravenously (i.v) into C57/B6 female mice 4 weeks old (6/group). One more group, representing the control group, was injected with sterile Phosphate buffer saline. Survival of infected mice was monitored daily.
The group of mice infected with *Stm*(p200) strain, that survived the infection up to 40 days, and the control group, were rechallenged with *Salmonella* wild type strain ($5x10^3$ cells/ml), and their survival was monitored.

9.13.2 Spleen weighing and enumeration of bacteria

Mice (5/group) infected with *Stm*(p200) strain were sacrificed at established time points after infection (0,3,7,14 days). Saline injected mice were also used and sacrificed 21 days after injection.
The spleens were harvested from mice and weighted.
The spleens were digested by collagenase A (1 U/ml) (Sigma-Aldrich, Milan, Italy) and red blood cells were lysed. The supernatants were plated on Salmonella-Shigella (SS) agar plates and the CFU counts were determined after overnight incubation.

9.13.3 Cytokine analysis

Erythrocyte-depleted spleen cells were resuspended in RPMI supplemented with 5% FCS, L-glutamine, sodium pyruvate, essential amino acids, non-essential amino acids, pen/strep (all reagents from Gibco BRL). The cytokines content was measured in supernatants of spleen cells as well as in serum samples 3,7,14,21 days post-infection. Spleens and sera of saline-injected mice sacrificed 21 days after injection were used as control. IL-12p40 levels were quantitated using specific kits from RD System, UK. TNF-α and INF-γ levels were determined by kits from eBioscience (San Diego, California, USA) according to the manufacturer's instructions.

9.13.4 *In vitro* recall immune response

Salmonella cells from overnight culture were pelletted and resuspended in PBS. The cells were disrupted by sonication (30 minutes; 9" ON/9"OFF; 30% power-amplitude). Mice rechallenged with *Stm* wt strain were sacrificed 5 weeks after infection. Spleen cells obtained from harvested spleens were seeded at a density of $1x10^6$ cells/ml in 24-well plates (Nunc) without further stimuli, stimulated with sonicated *Salmonella* antigens (1μg/ml) or with LPS (1μg/ml). Cell-free culture supernatants were collected at 48h for INF-γ determinations. Cytokine analysis was conducted using a specific ELISA kit from eBioscience (San Diego, California, USA) according to the manufacturer's instructions.

9.13.5 Measurement of anti-*Salmonella* antibodies in serum

Serum samples were obtained from mice rechallenged with *Stm* wt strain (5 weeks) and from saline-injected mice. The presence of *Salmonella*-specific antibodies of IgG2b isotype was determined by ELISA protocol. Overnight culture of *Salmonella* was washed in PBS (3X). Aliquots of $1x10^7$cells/100μl were heated at 75°C for 1h. Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated with a preparation of heat-killed *Salmonella* organisms ($2x10^7$ cells/well) and incubated overnight at 4°C. Plates were washed with washing buffer (3X) (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) and blocked for 30min at room temperature (RT) with blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0). Serum samples were diluted (1:20; 1:200) in conjugate buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20), added to the plates and incubated 1h/RT. Plates were washed with washing buffer (3X). Antibody anti-IgG2b HRP (Horseradish peroxidase)-conjugated (Bethyl Laboratories, Texas, USA) was diluted (1:25,000) in conjugate buffer and added to plates (1h/RT). After washing (3X), enzyme substrate TMB (3,3',5,5'-Tetramethylbenzidine) was added and the reaction was stopped with 0.18 M H2SO4. Plates were readed at 450nm using a microplate reader.

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