# Oxidoreductases and RNA Degradosome Controlling Virulence-Associated Traits of *Salmonella enterica* serovar Typhimurium
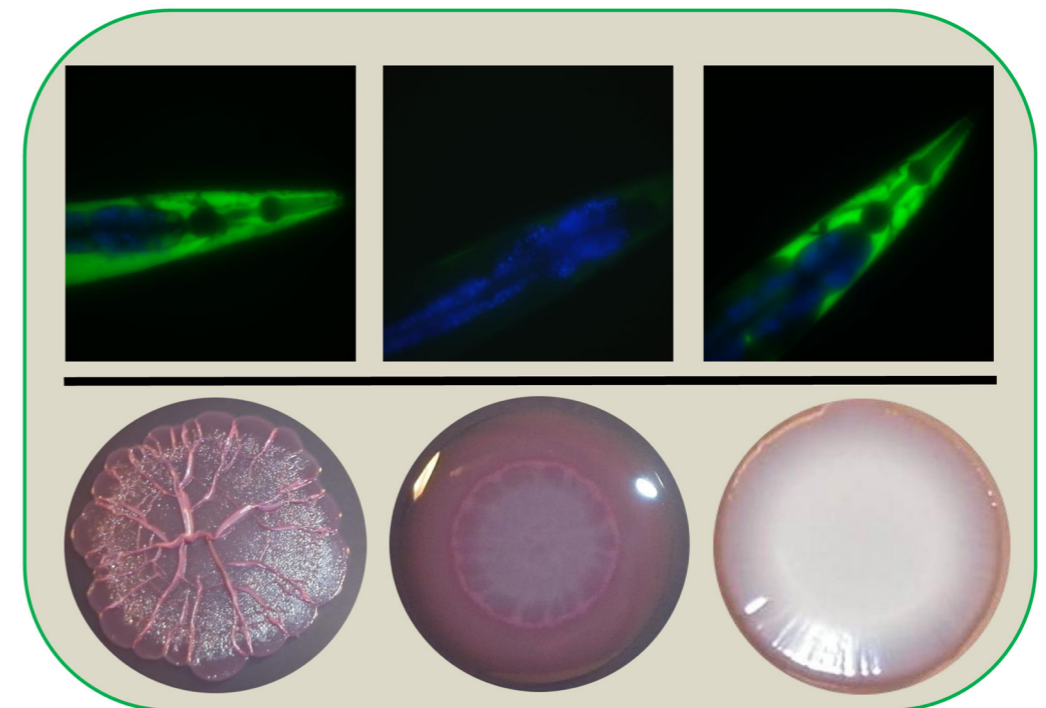


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# OXIDOREDUCTASES AND RNA DEGRADOSOME CONTROLLING VIRULENCE-ASSOCIATED TRAITS IN SALMONELLA ENTERICA SEROVAR TYPHIMURIUM

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Stockholm 2013

**Cover Illustrations:** Upper panel: ROS staining with fluorescent dye $H_2DCFDA$ in the intestine of nematode infection model, *C. elgans*. From left to right; wildtype, *ΔtrxA*, *ΔtrxA/ΔscsABCD*. Lower panel: *Rdar*-morphotype of *S.* Typhimurium on CR plates for *Δdsb* mutants. From left to right; *ΔdsbA*, *ΔdsbB* under reductive stress*, ΔdsbA* under oxidative stress.

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*To my dearest parents*
                              *&*
*beloved soulmate……*

# ABSTRACT

The oxidative stress response is a fundamental and primitive mode of innate immune defense in nearly all forms of life. The host oxidative stress response becomes truly important and effective especially against intracellular pathogens such as *Salmonella*. Pathogens have evolved diverse mechanisms to withstand the oxidative responses. These include the use of oxidoreductases to neutralize oxidative products and repair oxidative damages, rapid alteration in their transcriptome and diversion of such oxidative products in cellular signaling, for their survival and successful infection.

In the first study in this thesis, we have introduced the putative ScsABCD oxidoreductases of the thioredoxin superfamily for their role in oxidative stress tolerance and in virulence of *S.* Typhimurium. We demonstrated that ScsABCD proteins are dispensable for invasion in cultured epithelial cells under normal invasive conditions, although ScsABCD acts as a suppressor of SPI-1 mediated invasion upon oxidative stress. Our results have further shown a functional association between ScsABCD and thiroredoxin 1 (TrxA) oxidoreductase of *S.* Typhimurium. In this, we demonstrated that absence of ScsABCD restored the invasiveness of a *trxA* mutant in epithelial cells and its virulence in *C. elegans*. **(Paper I).**

Next, we present the analyses on the role of periplasmic Dsb oxidoreductase system in *S.* Typhimurium's biofilm-development, specifically under redox stress. In this, we show that DsbA and DsbB act as suppressors of *rdar*-morphotype development and affect biofilm-regulation using either Csg-dependent or -independent mechanism, respectively. Our results further reveal that oxidative stress abrogates *rdar*-morphotype of *S.* Typhimurium, whereas reductive stress reduces *rdar*-morphotype with concomitant plentiful release of extracellular slimy material containing, notably, the extracellular DNA (eDNA). Furthermore, we have demonstrated the oxidative recovery of swimming motility defects of a *dsbA* mutant. **(Paper II).**

Finally, we have demonstrated that exoribonuclease; PNPase and its genetic associate membrane lipoprotein NlpI constitute an operon and are functionally connected **(Papers III and IV).** PNPase was required for *rdar*-morphotype development whereas, NlpI suppresses the biofilm formation. In addition, we established the association of PNPase with c-di-GMP metabolism in biofilm regulation. Moreover, we showed that both PNPase and NlpI are required, independently, for cold adaptation of *S.* Typhimurium.

# LIST OF PUBLICATIONS

I. <u>NAEEM ANWAR</u>**,** Xiao Hui Sem and Mikael Rhen. **Oxidoreductases that act as conditional virulence suppressors in *Salmonella enterica* serovar Typhimurium**. *PLoS One* 2013 Jun; 8(6):e64948

II. <u>NAEEM ANWAR</u>, Ute Römling and Mikael Rhen. **Redox-sensitivity of biofilm-formation in *Salmonella enterica* serovar Typhimurium – A particular impact of the DsbA/DsbB redox system**. *Manuscript.*

III. Syed Fazle Rouf, Irfan Ahmad, <u>NAEEM ANWAR</u>, Suman Kumar Vodnala, Abdul Kader, Ute Römling and Mikael Rhen. **Opposing contributions of polynucleotide phosphorylase and the membrane protein NlpI to biofilm formation by *Salmonella enterica* serovar Typhimurium.** *Journal of Bacteriology* 2011 Jan; 193(2):580-2.

IV. Syed Fazle Rouf, <u>NAEEM ANWAR</u>, Mark O Clements and Mikael Rhen. **Genetic analysis of the pnp-deaD genetic region reveals membrane lipoprotein NlpI as an independent participant in cold acclimatization of *Salmonella enterica* serovar Typhimurium**. *FEMS Microbiology Letters* 2011 Dec; 325(1):56-63.

# PUBLICATIONS NOT INCLUDED IN THE THESIS

I.  Sanna Koskiniemi, Henry S. Gibbons, Linus Sandegren, <u>NAEEM ANWAR</u>, Gary Ouellette, Stacey Broomall, Mark Karavis, Paul McGregor, Alvin Liem, Ed Fochler, Lauren McNew, C. Nicole Rosenzweig, Mikael Rhen, Evan W. Skowronski and Dan I. Andersson. **Pathoadaptive mutations in *Salmonella enterica* isolated after serial passage in mice.** *PLoS One* 2013 Jul; 8(7): e70147.

II. Irfan Ahmad, Edvard Wigren, Soazig Le Guyon, Santtu Vekkeli1, Andra Blanka, <u>NAEEM ANWAR</u>, M. L.Chuah, Heinrich Luensdorf, Mikael Rhen1, Ronald Frank, Zhao-Xun Liang, Ylva Lindqvist, Ute Römling. **Interplay between two retired EAL proteins to regulate motility, virulence and biofilm formation in *Salmonella typhimurium*.** *Manuscript submitted.*

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| c-di-GMP | Cyclic di-guanosine monophosphate |
| *C.elegans* | *Caenorhabditis elegans* |
| CR | Congo red |
| Csg | Curli subunit gene |
| Csr | Carbon storage regulator |
| $CuCl_2$ | Copper Chloride |
| Cys | Cysteine |
| Dsb | Disulfide bond |
| DTT | Dithiothritol |
| eDNA | Extracellular deoxyribonucleic acid |
| GTPase | Guanosine triphosphatases |
| $H_2O_2$ | Hydrogen peroxide |
| HOCL | Hypochlorous acid |
| IL-8 | Interleukin 8 |
| IFN | Interferon gamma |
| iNOS | Inducible nitric oxide synthase |
| NF-κB | Nuclear factor kappa light chain enhancer of activated B-cells |
| NlpI | New lipoprotein I |
| NTS | Non-typhoidal *Salmonella* |
| PNPase | Polynucleotide phosphorylase |
| Rdar | Red dry and rough |
| ROS | Reactive oxygen species |
| RNS | Reactive nitrogen species |
| Scs | Suppressor for copper sensitivity |
| SCV | Salmonella containing vacuole |
| SOD | Superoxide dismutase |
| SPI | *Salmonella* pathogenicity island |
| *S.* Typhimurium | *Salmonella enterica* serovar Typhimurium |
| TNF | Tumor necrosis factor |
| T3SS | Type three secretion system |

# 1   INTRODUCTION

## 1.1   *SALMONELLA* AND EPIDEMIOLOGY

*Salmonellae* are Gram-negative, rod-shaped, motile, facultative anaerobic bacteria belonging to the family *Enterobacteriaceae* and are one of the leading causes of diarrheal infections spread by contaminated food and water. *Salmonella* infection has remained a major burden on the health care system and food industry both in the developed and developing countries (Gast, Guraya *et al.* 2013). In the United States alone, salmonellosis accounts for 1.2 million reported cases with 23,000 hospitalizations and over 500 deaths each year (Bishop, M.Erdman *et al.* 2011; Davis 2012). However, a large proportion of the patients with salmonellosis in countries like the United States do not seek medical care, or otherwise, the cases are not reported by the peripheral surveillance laboratories. Therefore, the actual incidence in the United States is estimated to reach 20 million cases per year (Scallan, Hoekstra *et al.* 2011; Davis 2012).

Developing countries do not always have a well-managed surveillance system for *Salmonella* infections. Therefore, it is hard to estimate the actual incidence of salmonellosis in the developing countries (Pang 2008). Poor sanitation, over-crowded living conditions and poverty allow us to speculate that there is a much higher incidence in the developing countries. Hence, efforts to reduce the burden of *Salmonella* infections must be implemented.

Currently, the United States Centre for Disease Control and Prevention (CDC) divides *Salmonella* genus into two main species; *Salmonella enterica* and *Salmonella bongori,* the former standing as the "*Type Species*". However, in last decade a new species, *Salmonella subterranean*, has been approved by Judicial Commission of the International Committee on the Systematics of Prokaryotes but has not yet been adopted by CDC into *Salmonella* nomenclature system (Shelobolina, Sullivan *et al.* 2004; Bishop, M.Erdman *et al.* 2011).

Humans are infected specifically by *Salmonella enterica. Salmonella enterica* is further divided into six subspecies; I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S.enterica* subsp. *Indica* (Popoff, Bockemuhl *et al.* 2003). More than 2575 serovars of *Salmonella* have been described based on the somatic (O-antigen) and flagellar antigens (H-antigen) although the number is

continuously increasing with the discovery of new serovars. In general, the most common cases of salmonellosis in man are caused by serovars belonging to subspecies I followed by subspecies IV, IIIb, II, and IIIa (Bishop, M.Erdman *et al.* 2011).

## 1.2 SALMONELLOSIS

*Salmonellae* are ubiquitous in their habitat. Usually they reside in the intestinal tract of large number of warm and cold-blooded animals. However, they can also survive outside of the animal host in the dry environment for quite long periods. Due to the diversity in their habitat, *Salmonellae* infect a broad range of hosts. The outcome of infection depends on the host, infecting *Salmonella* serovar, the given strain and immunological status of the host.

In humans, 99% of isolates of *Salmonella* belong to *S. enterica* subspecies *enterica* (Bishop, M.Erdman *et al.* 2011). From disease perspectives, the infection with *Salmonella* - "salmonellosis" - is mainly categorized into invasive and local infection; enteric fever (typhoid fever) and acute gastroenteritis (enterocolitis) respectively. Typhoid and paratyphoid fever are caused by the human specific serovars *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi B and *S.* Paratyphi C whereas acute gastroenteritis can be caused by a number of different serovars collectively known as "Non-Typhoidal *Salmonellae*" (NTS).

*S.* Typhi and *S.* Paratyphi A-C are chiefly transmitted through water contaminated with human excreta from *Salmonella* carriers or with the food irrigated with such contaminated water. The infectious dose ranges between $10^3$-$10^6$ bacteria (Hornick, Greisman *et al.* 1970). After ingestion and survival through gastric acids, at least in infection models for typhoid fever, *Salmonella* goes to the terminal part of small intestine where it attaches to the epithelial surface and transmigrates to the basal laminar layer through specialized epithelial cells of Payer's patches, "the M cells" (Takeuchi and Sprinz 1967; Jones, Ghori *et al.* 1994). This is accomplished with the help of specialized virulence-gene island on the genome of *Salmonella* termed as *Salmonella* pathogenicity island 1 (see below).

Subsequently, *Salmonella* are engulfed by the approaching neutrophils, local tissue macrophages and dendritic cells and are translocated to the regional mesenteric lymph nodes and ultimately to the liver and spleen (Cheminay, Chakravortty *et al.* 2004; Johansson, Ingman *et al.* 2006; Salazar-Gonzalez, Niess *et al.* 2006). Bacteria can survive and replicate inside these macrophages (House, Bishop *et al.* 2001) with the help of *Salmonella* pathogenicity island 2 and *spv* virulence genes (see below).

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In human typhoid fever, after a typical 7-14 day incubation period, *Salmonella* can leave macrophages and a widespread bacteremia occurs with possible secondary infections of liver, spleen, bone marrow, gallbladder and Peyer's patches of the terminal ileum (Parry, Hien *et al.* 2002). The first week is asymptomatic followed by typical low-grade fever that rises progressively to 39°C to 40°C by the end of second week. Besides fever, patients experience influenza-like symptoms i.e. chills, a dull frontal headache, malaise, anorexia, nausea, poorly localized abdominal discomfort, a dry cough, and myalgia. Furthermore, in 5% – 30% of cases, rose spots, blanching erythematous maculopapular lesions approximately 2 to 4 mm in diameter can be found on the chest, abdomen and rarely on back arms and legs (Stuart Bm 1946; Parry, Hien *et al.* 2002).

Non-typhoidal *Salmonellae* (NTS) that cause self-limiting gastroenteritis in humans are generally called food-borne serovars. These strains are not human specific and are acquired as zoonotic infections. According to the European Centre for Disease Prevention and Control (ECDC), the most common isolated NTS serovars for diarrheal diseases are *S.* enteric serovar Enteritidis (*S.* Enteritidis) and *S.enterica* serovar Typhimurium (*S.* Typhimurium) (Jansen, Lahuerta-Marin *et al.* 2011). NTS can grow in broad range of temperatures ($7^{o}C$ - $40^{o}C$) and substrates. The most important source of NTS outbreaks are associated with the use of contaminated food animals or their products such as infected chicken, eggs from infected hens, contaminated meat and meat products, unpasteurized milk and cheese.

The infectious dose for NTS is $10^6$-$10^8$ but can vary with different risk factors (Wray and Sojka 1978). Risk factors for NTS diarrheal disease include age, endogenous bowel normal flora, gastrectomy and presence of other infections in the host (Hohmann 2001; Graham 2010). NTS have also been associated with more severe and invasive infections in HIV as well as in malaria infected individuals (Feasey, Dougan *et al.* 2012; MacLennan 2012). Such co-infections are more likely to progress to focal infections, including meningitis, septic arthritis, osteomyelitis, cholangitis, and pneumonia (Fabrega and Vila 2013).

Among NTS, along with gastroenteritis, *S.* Typhimurium also represents a typhoid-like invasive disease in mice (Monack, Hersh *et al.* 2000; Mastroeni and Sheppard 2004). Therefore, *S.* Typhimurium is a very suitable model laboratory organism to study both the invasive and local aspects of *Salmonella* pathogenesis and hence is the model organism used in this thesis work.

## 1.3 *SALMONELLA* VIRULENCE ARMAMENTARIUM

The marvelous ability of *Salmonella* to colonize and succeed within the host makes it an excellent and successful pathogen. *Salmonella* is equipped with an arsenal of virulence factors with extremely versatile functions that are activated during different stages of the pathogenic process from the intestinal stage to systemic dissemination. For instance, the *Salmonella* genome contains genes required for motility and chemotaxis, adhesion, invasion, survival and intracellular replication within host cells, as well as for biofilm-formation (McClelland, Sanderson *et al.* 2001; Fabrega and Vila 2013).

The genes facilitating different virulence steps are mostly distributed on specific genetic continuums that are termed as *Salmonella* Pathogenicity Islands (SPIs). SPIs are believed to have been acquired as genetic blocks during the evolutionary process (Marcus, Brumell *et al.* 2000). In certain strains of *Salmonella*, the virulence genes are located in a highly conserved 8kb region of a 50 to 95 kb virulence plasmid, known as pSLT in *S.* Typhimurium (Rotger and Casadesus 1999; Matsui, Bacot *et al.* 2001). A recent report by Herrero *et al.* stated that complex resistance islands have also been acquired by pSLT during natural recombinational events in certain *S.* Typhimurium isolates (Herrero, Mendoza *et al.* 2008). Furthermore, a number of genes involved in the adaptation, bacterial defense again host bactericidal responses and virulence are found on prophage elements scattered on the bacterial chromosome (Stanley, Ellermeier *et al.* 2000; Ho, Figueroa-Bossi *et al.* 2002; Zou, Li *et al.* 2010; Boyd 2012; Switt, den Bakker *et al.* 2012).

### 1.3.1 *Salmonella* Pathogenicity Islands (SPIs)

SPIs are horizontally acquired genetic elements regarded as 'quantum leaps' in the evolution of *Salmonella* (Groisman and Ochman 1996). To date, more than 21 different SPIs have been identified (Sabbagh, Forest *et al.* 2010; Suez, Porwollik *et al.* 2013). Among the different SPIs, the SPI-1 and SPI-2 have been most extensively studied (Mills, Bajaj *et al.* 1995; Ochman, Soncini *et al.* 1996; Lostroh and Lee 2001; Kuhle and Hensel 2004), SPI-3, SPI-4 and SPI-5 are beginning to be characterized (Blanc-Potard, Solomon *et al.* 1999; Knodler, Celli *et al.* 2002; Dorsey, Laarakker *et al.* 2005; Gerlach, Jackel *et al.* 2007) whereas analyses on rest of the SPIs are still in their infancy (Suez, Porwollik *et al.* 2013).

SPI-1 and SPI-2, each encodes a specific Type Three Secretion System (T3SS). A T3SS is multi-component needle like structure that spans the inner and outer

bacterial membrane and projects from the bacterial surface (Galan 2001; Galan and Wolf-Watz 2006; Mueller, Broz *et al.* 2008). The T3SS are used to translocate virulence-associated effector proteins into the host cell. Such effector proteins are encoded by the SPIs or by genes elsewhere located on the chromosome (Lostroh and Lee 2001; Ellermeier and Slauch 2007).

### 1.3.2 SPI-1

SPI-1 is an approximately 40 kb long genetic region that contains a number of different operons dedicated to specific functions of T3SS-1. Generally, the genes on SPI-1 can be divided into regulators, structural genes, effectors and the chaperons. The structural genes *prg/org, inv/spa* make the needle-like T3SS structure whereas the effectors, encoded by the *sic/sip* operons, are translocated into the host cell. The chaperones on SPI-1 stabilize and protect the effector proteins from degradation and prevent premature interactions of these effector proteins (Ehrbar, Friebel *et al.* 2003; Ehrbar, Hapfelmeier *et al.* 2004). The SPI-1 T3SS (T3SS-1) is mainly activated during the intestinal phase of *Salmonella* invasion into the host cell epithelium (Ly and Casanova 2007). However, the effector proteins encoded on SPI-1 have also been recovered during late stages of infection (Brawn, Hayward *et al.* 2007; Pavlova, Volf *et al.* 2011).

After reaching the terminal ileum, bacteria attach to the intestinal epithelial surface with the help of different fimbriae and adhesins, while T3SS-1 assembles to deliver the effector proteins into the host cell (Baumler, Tsolis *et al.* 1997; Galan 2007; Gerlach, Jackel *et al.* 2007; Chessa, Winter *et al.* 2009). Upon delivery into the host cell, the bacterial effector proteins SipC and SipA respectively nucleate and stabilize the F-actin filaments (Zhou, Mooseker *et al.* 1999; McGhie, Hayward *et al.* 2001; Hayward and Koronakis 2002; Chang, Chen *et al.* 2005). Later on, rearrangement of these filaments for membrane ruffling is achieved with the activated Rho family GTPases; Cdc42, Rac1 and RhoG.

The activation of these GTPases is under the effect of another set of SPI-1 effector proteins; SopE, SopE2 and SopB (Hardt, Chen *et al.* 1998; Stender, Friebel *et al.* 2000). Along with the membrane ruffling, Rho GTPases also elicit mucosal inflammation by activating NF-κB expression that, in turn, enhances the production of pro-inflammatory cytokines such as IL-8 and TNF-α (Hobbie, Chen *et al.* 1997; Patel and Galan 2006). After bacterial uptake, the membrane ruffling returns to normal and the inflammatory response is down-regulated by SPI-1 effector protein SptP. The SptP

inactivates Rac-1 and Cdc42 through their intrinsic GTPases activation (Fu and Galan 1999). In short, with the cooperative activity of different SPI-1 effector proteins, *Salmonella* is taken up by non-phagocytic epithelial cells of intestine.

SPI-1 is an essential tool of *Salmonella* to enter into the host cell, yet through its intrinsic activities it also promotes inflammation. Therefore, the expression of SPI-1 genes should be tightly regulated. The conditions prevailing in the infection site in terminal ileum such as high osmolarity, low oxygen, near-neutral to alkaline pH and bile, act as signals for the regulation of SPI-1 expression (Lostroh and Lee 2001). The signals from the environment are converged to the SPI-1 main regulator HilA, which in concert with HilC, HilD, HilE, and InvF regulators controls the T3SS-1 activity (Bajaj, Lucas *et al.* 1996; Altier 2005; Ellermeier and Slauch 2007).

HilA, encoded by *hilA* within SPI-1, activates other operons on SPI-1 either by direct binding to their promoters (*prg/org* and *inv/spa*) or through the activation of InvF (*sic/sip* operon) (Bajaj, Hwang *et al.* 1995; Darwin and Miller 1999). In addition, direct effects of HilA on expression of the T3SS-1 effector genes located outside of SPI-1 have also been reported (Thijs, De Keersmaecker *et al.* 2007). HilA expression is positively controlled by the combined action of two other forth-mentioned SPI-1 encoded regulators; HilC and HilD, and by RtsA encoded on a distant chromosomal region (Schechter, Damrauer *et al.* 1999; Ellermeier, Ellermeier *et al.* 2005). However, HilC and HilD have also been shown to activate virulence genes expression independently of HilA by direct binding to the HilA-independent promoter of InvF (Akbar, Schechter *et al.* 2003).

HilA, either negatively regulates its own expression or through HilE, encoded outside of SPI-1, that negatively controls the expression of HilA. The negative regulation of HilA by HilE is supposed to be due to post-transcriptional interaction of HilE with HilD (Baxter, Fahlen *et al.* 2003; De Keersmaecker, Marchal *et al.* 2005). The overall regulatory network is illustrated in figure1.

### 1.3.3 SPI-2

SPI-2 was the second SPI to be identified in *S.* Typhimurium. Like SPI-1 it is also of approximately 40kb length and is required for intracellular replication in both macrophages and epithelial cells (Shea, Hensel *et al.* 1996; Cirillo, Valdivia *et al.* 1998). As described in section 1.2, after breaching the intestinal barrier, *Salmonellae* become engulfed by approaching neutrophils, dendritic cells and macrophages.
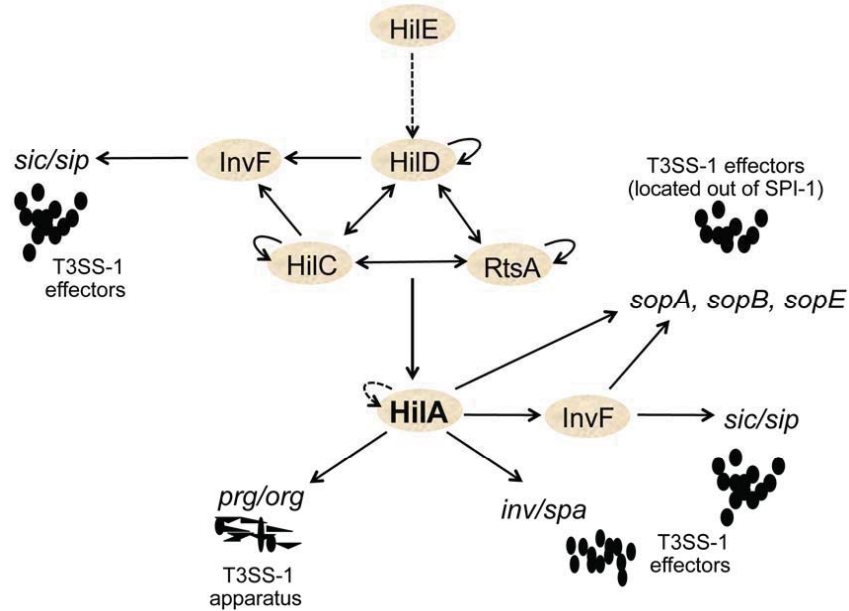
**Figure 1: SPI-1 regulation main players.** Solid lanes indicate activation and dashed lanes indicate negative regulation. Adapted from (Akbar, Schechter *et al.* 2003; Ellermeier, Ellermeier *et al.* 2005; Thijs, De Keersmaecker *et al.* 2007; Ellermeier and Slauch 2008).

Subsequently, the bacteria are transported by the phagocytes to mesenteric lymph nodes and ultimately to the spleen and liver (Cheminay, Chakravortty *et al.* 2004; Johansson, Ingman *et al.* 2006; Salazar-Gonzalez, Niess *et al.* 2006).

The macrophages of reticulo-endothelial systems of these infected organs serve as the niche for *Salmonella* survival and replication within specialized endocytic compartments resembling late endosomes but devoid of lysosomal hydrolases (Steele-Mortimer, Meresse *et al.* 1999). This endocytic vacuole is known as the *Salmonella* containing vacuoles (SCV) (House, Bishop *et al.* 2001). Recently, it has been reported that SCV also divides along with *Salmonella.* The net result is an increase in systemic *Salmonella* load with a substantial requirement for the host cell to clear the infection (Eswarappa, Negi *et al.* 2010).

SPI-2 genes are induced by the acidic pH and poor nutritional status in the developing SCV (Beuzon, Banks *et al.* 1999; Garmendia, Beuzon *et al.* 2003; Lober, Jackel *et al.* 2006). The SPI-2 encoded T3SS (T3SS-2) operates at the membrane of SCV to deliver approximately 30 different effector proteins with largely unknown

functions across the membrane to host cell cytosol (Chakravortty, Rohde *et al.* 2005; Mills, Baruch *et al.* 2008; McGhie, Brawn *et al.* 2009). The concerted action of these T3SS-2 effector proteins is to manipulate host cell endosomal degradation pathway, to restrict the fusion of SCV with lysosomal hydrolases (e.g. SsaB(SpiC), SseJ, SseI, SspH2), to position the SCV in perinuclear position and to allow the replication of *Salmonella* inside SCV (SseF, SseJ, SifA, SseG, PipB2, SigD) (Uchiya, Barbieri *et al.* 1999; Freeman, Ohl *et al.* 2003; Miao, Brittnacher *et al.* 2003; Hernandez, Hueffer *et al.* 2004; Brawn, Hayward *et al.* 2007; Steele-Mortimer 2008; McGhie, Brawn *et al.* 2009). In addition, SPI-2 also contributes to the protection of *Salmonella* in SCV from macrophage-derived oxidative and nitrosative responses (Vazquez-Torres, Xu *et al.* 2000; Chakravortty, Hansen-Wester *et al.* 2002).

SPI-2 gene expression is primarily regulated by a two-component regulatory system SsrA/SsrB that is encoded inside the SPI-2 locus (Cirillo, Valdivia *et al.* 1998; Garmendia, Beuzon *et al.* 2003). SsrA is the membrane located sensor kinase that can sense the acidic pH, low osmolarity, low $Mg^{2+}$ and $Ca^{2+}$ in the SCV and transmits the signals to SsrB (Garmendia, Beuzon *et al.* 2003). SsrB is a transcriptional activator that induces the expression of SPI-2 effector proteins located inside SPI-2 or elsewhere on the chromosome (Worley, Ching *et al.* 2000; Feng, Walthers *et al.* 2004). With respect to the complex regulation pattern of SPI-1, it is not surprising to note that SsrA/SsrB is also further regulated by multiple factors, which is discussed in section 1.3.4.

### 1.3.4   Crosstalk between SPI-1 and SPI-2

According to the information given in section 1.3.2 and 1.3.3, SPI-1 is activated during the intestinal phase of *Salmonella* infection whereas SPI-2 is required for the growth and survival of bacteria inside macrophages of reticulo-endothelial system of deeper tissues like liver and spleen. However, in recent years many reports claim a role for SPI-1 effector proteins in the intracellular survival and replication within SCV (McGhie, Brawn *et al.* 2009). For instance, the SPI-1 effector SipA appears exposed on the outer surface of SCV and stimulates bacterial replication. SipA furthermore helps in perinuclear positioning of SCV in cooperation with SPI-2 effector protein SifA (Brawn, Hayward *et al.* 2007). Another SPI-1 effector, SopB, delays phagosomal-lysosomal fusion and contributes to SCV maturation at early stages through phosphatidylinositol-3-phosphate (PI3P) accumulation on the SCV membrane (Kuijl, Savage *et al.* 2007; Mallo, Espina *et al.* 2008; Wasylnka, Bakowski *et al.* 2008).

Until now, a role for SPI-2 effectors has not been associated directly with the intestinal phase of infection, albeit both SPI-1 and SPI-2 have been shown to induce the intestinal inflammation that can help in *Salmonella* invasion (Tsolis, Adams *et al.* 1999; Zhang, Santos *et al.* 2002; Zhang, Adams *et al.* 2003; Stecher, Macpherson *et al.* 2005). This being said, reactive oxygen species (ROS), produced as a consequence of inflammation react with intestinal thiosulfate to form a new respiratory electron acceptor, tetrathionate. The *ttr* genes located in SPI-2 allow utilization of tetrathionate as an electron acceptor and provides *Salmonella* with the opportunity to outgrow the normal flora of intestine and increase their success of transmission (Winter, Thiennimitr *et al.* 2010). Thus, continued functional interplay between the two SPIs effector proteins and their role in facilitating the same steps in pathogenesis would imply that they share similar regulatory pathways.

### 1.3.5 Regulation of SPI-1 and SPI-2

A number of different two-component regulatory systems maintain a fine balance of SPI-1 and SPI-2 expression at different stages of the infection. The Phop/PhoQ two-component system is activated under low divalent cation concentration and with low pH. PhoQ is a sensor kinase that transmits the signals to PhoP regulator (Vescovi, Soncini *et al.* 1996). PhoP controls SsrA post-transcriptionally whereas it activates SsrB expression by binding directly to the promotor of SsrB. The activated SsrA/SsrB then controls the expression of SPI-2 genes (Deiwick, Nikolaus *et al.* 1999; Worley, Ching *et al.* 2000; Bijlsma and Groisman 2005). Similarly, PhoP directly activates the SPI-1 *orgBC* operon in the macrophages, which is believed to play a role in the later stages of the disease (Aguirre, Cabeza *et al.* 2006). However, generally, PhoP represses *hilA* mediated SPI-1 expression through HilD. This, in turn, is achieved by activating *pag* genes that are required for survival and replication inside the macrophages (Belden and Miller 1994; Boddicker, Knosp *et al.* 2003).

SirA/BarA is another important two-component regulatory system that is essential for activation of SPI-1 mediated invasion. BarA is a sensor kinase and SirA is the corresponding regulator (Teplitski, Goodier *et al.* 2003; Ellermeier and Slauch 2007). SirA can directly activate *hilA* and *hilC* by binding to their promoters (Teplitski, Goodier *et al.* 2003). Otherwise, SirA mediated activation of invasion genes occurs through the carbon storage regulator (Csr) system. SirA enhances expression of the CsrB and CsrC small regulatory RNAs that bind to CsrA (Fortune,

Suyemoto *et al.* 2006). CsrA, an RNA binding protein, inhibits SPI-1 gene expression by binding to *hilD* mRNA. CsrA in its bound condition with CsrB and CsrC, is unable to bind further to *hilD* mRNA and hence cannot exert its inhibitory effect on SPI-1 mediated invasion (Altier, Suyemoto *et al.* 2000).

The EnvZ/OmpR two-component system is also important for controlling the expression of SPI-1 and SPI-2 genes. EnvZ, as sensor kinase, activates the response regulator OmpR (Lee, Detweiler *et al.* 2000; Lucas, Lostroh *et al.* 2000; Garmendia, Beuzon *et al.* 2003). OmpR has been reported to induce expression of *hilC* and post-translational activation of HilD (Lucas and Lee 2001; Ellermeier, Ellermeier *et al.* 2005). Similarly, OmpR can activate *ssrA/ssrB* genes by direct binding to their promoters and hence increasing SPI-2 expression (Lee, Detweiler *et al.* 2000; Feng, Oropeza *et al.* 2003).

Besides the aforementioned regulatory systems, SPI-1 and SPI-2 gene expression is also under the control of less well described regulatory circuits. For instance, the RcsCDB phosphorelay system represses the invasion-associated genes of SPI-1 whereas it activates SPI-2 genes (Garcia-Calderon, Casadesus *et al.* 2007; Wang, Zhao *et al.* 2007). RtsA activates HilA expression whereas the gene *rtsB* in the same operon is believed to represses SPI-1 genes by suppressing FliZ (Ellermeier and Slauch 2003; Saini, Slauch *et al.* 2010). QseC/QsecB, yet another two-component system, activates the expression of both the SPI-1 and SPI-2 *in vitro* and *in vivo* (Moreira, Weinshenker *et al.* 2010)

Taken together, the extremely complex regulatory network ensures the proper and timely expression of virulence genes during infection pathogenesis and still a lot of unidentified players remain to be described.

## 1.4 PROFESSIONAL PHAGOCYTES AND OXIDATIVE STRESS

After success against the multiple host barriers; such as low gastric pH, peristalsis, intestinal commensal flora, antimicrobial peptides, mucin layer on intestinal epithelium and escape from being captured into a phagolysosome, *Salmonella* ultimately establishes its own niche (SCV) within the professional phagocytes of the reticuloendothelial system (Eswarappa, Negi *et al.* 2010; Alvarez-Ordonez, Begley *et al.* 2011). Yet, *Salmonella* is not out of danger, as it has to combat with a deadly bactericidal weapon of the macrophages known as "Respiratory Burst or Oxidative Burst".

Oxidative burst is a generic term used to describe a coordinated series of metabolic events with increased consumption of oxygen and net production of reactive oxygen species (ROS) (Babior 1978). Phagocytes rely on NADPH-dependent oxidase (NOX2) for production of ROS (Nauseef 2004). NOX2 is a multimeric enzyme complex that assembles at the membrane of phagosome and transfers electron to the molecular oxygen into the phagosome (Petry, Weitnauer *et al.* 2010). The oxygen is reduced to superoxide radical ($O_2^-$). This radical, in later steps, is dismutated to hydrogen peroxide ($H_2O_2$) either spontaneously or by enzymatic catalysis. Furthermore, $H_2O_2$ is either converted to hydroxyl radicals (HO·) in the presence of $Fe^{2+}$ during the Fenton reaction or converted to hypochlorous acid (HOCl) by enzymatic action of myeloperoxidase (MPO). HOCl is further converted to singlet oxygen ($^1O_2$) by reacting with a second molecule of $H_2O_2$ (Hampton, Kettle *et al.* 1998; Janssen, van der Straaten *et al.* 2003; Fang 2011; Paustian 2013). The overall series of reactions for production of different ROS species is shown in figure 2.
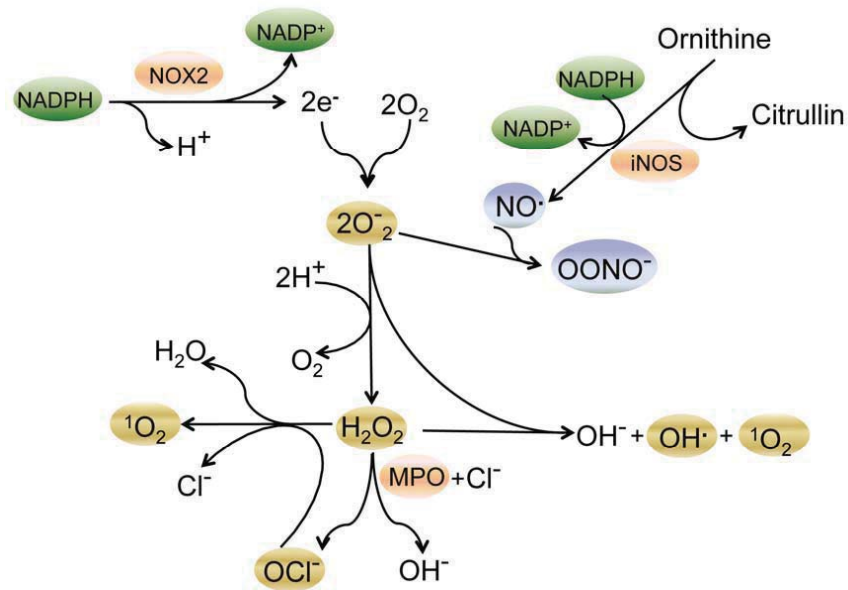


**Figure 2: Respiratory burst and bactericidal or bacteriostatic ROS and RNS production.** Shading legends: orange = enzyme; golden = ROS and blue = RNS. ROS = reactive oxygen species; RNS = reactive nitrogen species. Adapted from (Babior 1978; Janssen, van der Straaten *et al.* 2003; Winterbourn 2008; Flannagan, Jaumouille *et al.* 2012)

ROS mediated damage to bacterial cells varies depending on the particular ROS and the target. The target might be any macro-molecule within bacterial cell i.e. cell-membrane, DNA or certain proteins. In the cytoplasm, ROS target DNA and either oxidize purines particularly guanine or cause strand breaks. These DNA interferences may result in mutations or may be lethal in the absence of a repair system (Imlay and Linn 1988; Lloyd, Carmichael et al. 1998). Another important target for ROS is the iron-sulfur centre in a number of proteins especially enzymes that are involved in metabolic pathways (Janssen, van der Straaten et al. 2003; Salmeen and Barford 2005; Imlay 2006). The thiol groups in side chains of cysteine in these iron-sulfur centers can be oxidized to form a disulfide bond resulting in conformational changes and ultimately reversible inactivation of enzymes (Feng and Forgac 1994; Biswas, Chida et al. 2006; Suto, Iuchi et al. 2007). The reaction with iron-sulfur center also releases iron ($Fe^{+2}$) from these centers. This released iron can produce more ROS particularly $HO^{\cdot}$ through the Fenton reaction that can amplify the deleterious effects of ROS (Janssen, van der Straaten et al. 2003).

Thus, indeed, coping with ROS is a big challenge for ingested bacteria. However, Salmonella does not reside in a typical phagolysosome, rather it has created its own niche; "the SCV" (Eswarappa, Negi et al. 2010; Alvarez-Ordonez, Begley et al. 2011). In fact, Salmonella infection is much pronounced in mice deficient in NOX2 compared to wild type littermates (Mastroeni, Vazquez-Torres et al. 2000). Previously, Vazques-Torres and Gallios demonstrated that NOX2 assembly is excluded from the SCV membrane in a T3SS-2-dependant manner (Vazquez-Torres, Xu et al. 2000; Gallois, Klein et al. 2001). In contrast, Grant et al. and other groups have proposed that NOX2 assembly can also take place on SCVs even in the presence of a functional T3SS-2 (Aussel, Zhao et al. 2011; Grant, Morgan et al. 2012). This being said, these facts strongly suggest that ROS-mediated control of Salmonella takes place even in the SCV that relies on NOX2 presence.

To augment the activity of ROS, phagocytes have developed another system that results in the production of reactive nitrogen species (RNS) (Chakravortty, Hansen-Wester et al. 2002; Chakravortty and Hensel 2003). The first RNS is nitric oxide (NO) which is produced by the inducible nitric oxide synthase (iNOS)-mediated oxidation of L-arginine in the presence of NADPH in the phagocyte cytoplasm (Fang 1997). NO diffuses across the phagosome/SCV membrane and reacts immediately with $O_2^-$ to produce a more potent antimicrobial compound peroxynitrite ($ONOO-$) that can be converted to other RNS in later reactions (Fang 1997; Aktan 2004). The antimicrobial

role for RNS usually becomes evident only in the later stages of infection as iNOS is not expressed under the basal metabolic state of cell (Vazquez-Torres, Jones-Carson *et al.* 2000; Pautz, Art *et al.* 2010). The interaction of RNS production with ROS is summarized in figure 2.

## 1.5   *SALMONELLA* DEFENSE AGAINST ROS DAMAGE

To counteract the ROS-mediated damage, *Salmonella* has developed two distinct mechanisms; 1) Detoxification of ROS by induction of stress induced detoxifying enzymes 2) Reparation of the damage and scavenging the ROS.

### 1.5.1   Detoxification

*S. enterica* contains a variety of enzymes for detoxification of ROS. Some of these enzymes are functional in the bacterial periplasm whereas others work in the cytoplasm. *Salmonella* has four superoxide dismutases (SODs), SodCI, SodCII, SodA and SodB that scavenge $O_2^-$ in the periplasm (SodCI, SodCII) as well as in cytoplasm (SodA and SodB) and convert it to $H_2O_2$ (McCord and Fridovich 1969; Fang 1997; Rushing and Slauch 2011). SODs also prevent the formation of peroxynitrite in the periplasm, probably by limiting the available $O_2^-$ to react with $NO^·$ (DeGroote, Ochsner *et al.* 1997). *Salmonella* strains deficient in either SodCI or SodCII are more prone to death by ROS in macrophages (Sly, Guiney *et al.* 2002; Golubeva and Slauch 2006; Kim, Richards *et al.* 2010).

$H_2O_2$ produced by the dismutation of $O_2^-$ in the periplasm readily penetrates the cell membrane and is converted to $HO^·$ by the Fenton reaction (Janssen, van der Straaten *et al.* 2003). *Salmonella* has three catalases (KatE, KatG, KatN) and three peroxidases (AhpC, TsaA, Tpx), that degrade $H_2O_2$ to water and oxygen in the cytoplasm (Loewen 1984; Hebrard, Viala *et al.* 2009; Horst, Jaeger *et al.* 2010). The single mutation of any of these genes usually does not affect the survival of *Salmonella* however, a *Slamonella* mutant deficient in specific single thiol peroxidase, Tpx, was found sensitive to $H_2O_2$ *in vitro* and was restricted for replication in activated macrophages (Pappszabo, Firtel *et al.* 1994; Buchmeier, Libby *et al.* 1995; Horst, Jaeger *et al.* 2010).

### 1.5.2   Damage Repair

Once ROS are out of control from the bacterial scavenging/detoxification systems, they can cause damage to nucleic acids, lipids and proteins (Lloyd,

Carmichael *et al.* 1998; Biswas, Chida *et al.* 2006; Imlay 2006; Suto, Iuchi *et al.* 2007). DNA damage signals for the SOS response and activates the DNA-repair RecA/RecBCD system or RuvAB resolvasome system (Kuzminov 1999). Indeed, the RecA/RecBCD and RuvAB systems have been shown to be important for *Salmonella* survival in macrophages cell lines and *in vivo* (Buchmeier, Lipps *et al.* 1993; Cano, Pucciarelli *et al.* 2002; Craig and Slauch 2009).

Under normal conditions, the sulfur atoms in the cysteine and methionine of bacterial proteins are mostly in thiol/thiolate state due to the reducing cytoplasmic environment. ROS attacks these thiol groups resulting in disturbance of cellular redox balance (the balance of oxidized and reduced molecules in cell), hence perturbing the biological functions such as inactivation of enzymes or modulation of redox-sensitive signal transduction (Feng and Forgac 1994; Biswas, Chida *et al.* 2006; Suto, Iuchi *et al.* 2007). Nevertheless, the oxidations at thiol groups are usually reversible and bacteria have evolved a number of different anti-oxidant oxidoreductase mechanisms that can repair the oxidative modifications (Ritz, Patel *et al.* 2000). The change of methionine to methionine sulfoxide is seen more like a ROS scavenging system in the normal physiology of the cell (Levine, Berlett *et al.* 1999). The sulfoxide groups are readily recovered to the reduced thiolated from by methionine sulfoxide reductases (Msrs) (Sharov, Ferrington *et al.* 1999; Grimaud, Ezraty *et al.* 2001; Ezraty, Aussel *et al.* 2005).

The three well-studied and most important repair systems for maintaining redox dynamics at thiol groups of cysteine residues are thioredoxin, glutathione/glutaredoxin and the Dsb systems in Gram-negative bacteria. The former two operate in the cytoplasm whereas the latter is functional in the bacterial periplasm (Holmgren, Johansson *et al.* 2005; Inaba 2009). The members in these systems are the oxidoreductases that belong to "Thioredoxin Superfamily" which are characterized by a "*Cys-X-X-Cys*" motifs catalyzing reversible reduction of disulfides (Sjoberg and Holmgren 1972; Hoog, Jornvall *et al.* 1983; Wunderlich and Glockshuber 1993; Aslund and Beckwith 1999).

The prime thioredoxin system consists of thioredoxin I (TrxA), thioredoxin reductase (TrxR) and NADPH. TrxA, a small protein with a molecular mass of 12 kDa, is the main effector that is capable of reducing a wide range of proteins by making temporary thiol-disulfides with the proteins (Laurent, Moore *et al.* 1964; Holmgren 1995; Lu and Holmgren 2013). TrxA itself is reduced by TrxR that, in turn, gets its reducing equivalents from NADPH (Holmgren 1995; Aslund and Beckwith 1999).

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Besides reducing the unwanted oxidized proteins in the cytosol, TrxA also act as a communication point to transfer the reducing equivalents to the periplasmic Dsb oxidoreductase system (Rietsch, Belin *et al.* 1996; Rietsch, Bessette *et al.* 1997; Krupp, Chan *et al.* 2001).

The glutathione/glutaredoxin system comprises of glutathione reductase (GR), glutathione (GSSG/GSH), glutaredoxins (Grx) and NADPH (Holmgren, Johansson *et al.* 2005; Meyer, Belin *et al.* 2012). In this system, glutaredoxins (GrxA, GrxB, GrxC) are the main effectors that are reduced by NADPH via GR and GSH (Holmgren and Aslund 1995; Lillig, Berndt *et al.* 2008). Grx can reduce protein disulfides by dithiol or monothiol mechanism (Bushweller, Aslund *et al.* 1992; Mesecke, Mittler *et al.* 2008). The monothiol mechanism involves the mixed Grx-GSH-protein$^{ox}$ complex and is the specificity of glutaredoxin system that can't be carried out by the thioredoxin system (Bushweller, Aslund *et al.* 1992; Mesecke, Mittler *et al.* 2008).

The periplasmic disulfide bond (Dsb) system ensures the proper disulfide configuration of the proteins that are either functional in the periplasm or are exported out of the cell. The Dsb system has two distinct arms; DsbA/DsbB responsible for introducing disulfide bonds in the newly transported proteins in periplasm, and DsbC/DsbD that isomerizes the incorrectly introduced disulfide bonds (Berkmen, Boyd *et al.* 2005; Kim, Lee *et al.* 2006; Ito 2010). In the DsbA/DsbB pair, DsbA is the one that introduce disulfide bonds to the proteins by taking reducing equivalents from the thiol groups of the native proteins (Bardwell, McGovern *et al.* 1991). For efficient and prompt function, DsbA is recycled to the oxidized form by the membrane located DsbB protein (Regeimbal and Bardwell 2002; Tapley, Eichner *et al.* 2007). Being a strong oxidant, DsbA can also introduce disulfide bonds that are not required; hence, the DsbC/DsbD system comes into play (Kim, Lee *et al.* 2006; Ito 2010). DsbC is the isomerase to correct wrongly folded proteins reducing disulfide bonds and getting itself oxidized (Rietsch, Belin *et al.* 1996; Welk, Rudolph *et al.* 2011). DsbC is recycled to its reduced state by the membrane counterpart DsbD (Rietsch, Bessette *et al.* 1997).

## 1.6   OXIDOREDUCTASES AND VIRULENCE

Bacterial oxidoreductases have also been directly associated with disease progression and pathogenicity. In *Salmonella*, Msrs have been implicated in $H_2O_2$ tolerance, replication in macrophages and survival in mice (Denkel, Horst *et al.* 2011; Denkel, Horst *et al.* 2011). Similarly, TrxA of *Salmonella* has been shown to promote intracellular replication and survival in mice by activating the expression of T3SS-2

(Bjur, Eriksson-Ygberg *et al.* 2006; Negrea, Bjur *et al.* 2009). Moreover, TrxA induces inflammation in mice and causes increased ROS production in *C. elegans* (Peters, Paterson *et al.* 2010; Sem and Rhen 2012). DsbA of the periplasmic redox system has been implicated in a number of different pathogenic processes. For instance, DsbA is essential for P fimbrie assembly in uropathogenic *E. coli* (UPEC) (Jacobdubuisson, Pinkner *et al.* 1994), required for forming the twitching motility component type 4 fimbriae in UPEC, *V. cholera,* and *N. meningitidis*, (Peek and Taylor 1992; Zhang and Donnenberg 1996; Tinsley, Voulhoux *et al.* 2004) and is needed for maturation of intimin, an outer membrane adhesion of enteropathogenic *E. coli* (Bodelon, Marin *et al.* 2009). Furthermore, DsbA has also been shown as an essential component for flagellar motor function, certain toxins and virulence factor maturation and secretion, as well as proper functioning of T3SSs (Peek and Taylor 1992; Dailey and Berg 1993; Jackson and Plano 1999; Agudo, Mendoza *et al.* 2004; Miki, Okada *et al.* 2004; Shouldice, Heras *et al.* 2011).

Besides the protective role of oxidoreductases, *Salmonella* has evolved the strategy to divert the ROS and integrate it in the control of virulence genes. For instance, the oxidative stress sensor OxyR is activated by ROS that introduces disulfide bonds between its two cysteine residues (Zheng, Aslund *et al.* 1998). The oxidized OxyR can bind to the DNA and activate a number of different oxido-protective enzymes (Doyle 1989; Imlay 2008). Similarly, SsrB has an RNS responsive $Cys^{203}$ residue and the oxidation of $Cys^{203}$ by RNS increases the fitness of *Salmonella* in murine model of acute oral infection (Husain, Jones-Carson *et al.* 2010).

Taken together, there exist a large number of different genes belonging to oxidoreductase systems as well as regulators with active cysteine residues in their proteins sequences and their involvement in pathogen fitness still remains to be elucidated.

## 1.7    BIOFILMS

Biofilms, an antique but poorly understood mode of growth, are complex communities of microorganisms embedded in self-produced extracellular matrix in which they attach to each other and grow on either biotic or abiotic surfaces (Costerton, Stewart *et al.* 1999; Hall-Stoodley, Costerton *et al.* 2004; Bridier, Briandet *et al.* 2011). Biofilm embedded bacteria constitute around 10% of biofilm total dry mass and have the advantage of protection from harsh environmental conditions, such as dehydration, phagocytosis, UV exposure and resistance to antibiotics and antimicrobial agents (Le Magrex-Debar, Lemoine *et al.* 2000; Espeland and Wetzel 2001; Leid, Shirtliff *et al.* 2002; Smith and Hunter 2008; Flemming and Wingender 2010; Wong, Townsend *et al.* 2010).

Biofilms are implicated in the development and complications of a number of diseases (Costerton, Stewart *et al.* 1999). Recurrent urinary tract infections with indwelling catheters, prosthetic heart valve-associated endocardtitis, wound infections with implants, dental caries and lung infection with underlying genetic diseases are some of the biofilm-associated disease examples (Ge, Kitten *et al.* 2008; Giacaman, Araneda *et al.* 2010; Bonkat, Widmer *et al.* 2013; Gross and Welch 2013; Molina-Manso, del Prado *et al.* 2013). Moreover, due to the close proximity of bacteria in a biofilm, higher genetic exchange events can happen that lead to the development and spread of antibiotic resistance strains (Nguyen, Piastro *et al.* 2010; Bridier, Briandet *et al.* 2011; Marks, Reddinger *et al.* 2012).

The nature, structure and development of biofilms are determined by the growth conditions, temperature and the genetic background of microorganism (Mika and Hengge 2013). Nevertheless, typical biofilm development follows five sequential steps: initial attachment, irreversible attachment, microcolony formation, maturation and finally dispersion. In the initial reversible attachment, swimming bacteria migrate over the surface and establish pili- or fimbriae-mediated contact with the surface. Subsequently, extracellular matrix production is triggered leading to irreversible attachment and hence cell division starts to form a microcolony (clusters of up to 50 cells). This matrix embedded microcolony leads to the mature three-dimensional complex biofilm architecture with inter-communicating channels, live and dead bacteria plus massive amount of extracellular material ($\geq$ 90% of dry mass). The last stage is the dispersal to ensure remodeling of mature biofilm and seeding of new site for biofilm development (O'Toole, Kaplan *et al.* 2000; Gjermansen, Ragas *et al.* 2006; Flemming and Wingender 2010; Li, Brown *et al.* 2012; Römling 2013).

*Salmonella enterica* has three different growth behaviors: swimming, swarming and sessility in a complex multicellular biofilm formation. Under laboratory conditions *Salmonella* biofilms can be studied in liquid cultures (pellicle formation and flow cell based system) and on a low-osmolarity solid agar surface (Branda, Vik *et al.* 2005). Biofilm of *S.* Typhimurium on low-osmolarity solid medium and at low temperature is characterized by a flat spreading colony with a rough and dry surface, netlike warping and an undulate margin (Römling 2005). The extracellular matrix components described so far in *Salmonella* biofilms are cellulose, surface associated proteinacious curli fimbriae, large surface protein BapA, capsular polysaccharide and others including the LPS like polysaccharide (Collinson, Clouthier *et al.* 1996; Zogaj, Bokranz *et al.* 2003; Latasa, Roux *et al.* 2005; Anriany, Sahu *et al.* 2006). However, another very important biofilm-matrix component, extracellular DNA (eDNA), of many biofilms has not yet been described in *Salmonella* (Whitchurch, Tolker-Nielsen *et al.* 2002; Allesen-Holm, Barken *et al.* 2006; Bockelmann, Janke *et al.* 2006; Izano, Amarante *et al.* 2008; Ma, Conover *et al.* 2009).

The addition of a diazo Congo red (CR) dye to the medium gives purple color to the developing biofilm due to the binding of CR with cellulose and curli fimbriae, hence the *S.* Typhimurium biofilm is called red dry and rough (*rdar*) morphotype (Ross, Weinhouse *et al.* 1987; Römling, Bokranz *et al.* 2003). Variants of the *rdar*-mophotype are produced on CR supplemented Luria Agar plates without salt in the presence or absence of the aforementioned extracellular biofilm components and are given in figure 3.
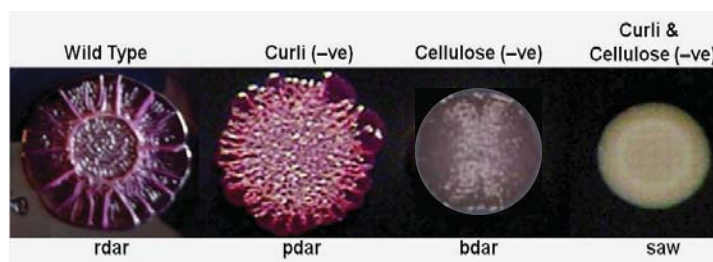


**Figure 3: *Salmonella* Typhimurium biofilm formation on LA without salt agar plate with CR dye.**
Rdar = red dry and rough; pdar = pink dry and rough; bdar = brown dry and rough; saw = smooth and white. (-ve) = absence of extracellular matrix component. Adapted from (Simm, Lusch *et al.* 2007)

### 1.7.1 CsgD -The Master Regulator of Biofilm Formation

The regulatory signals for *Salmonella rdar*-morphotype development are converged at the CsgD regulatory protein (curli subunit gene D) that inhibits planktonic growth and promotes sessility (Ross, Weinhouse *et al.* 1987; Carpousis 2007; Schild, Tamayo *et al.* 2007; Ogasawara, Yamamoto *et al.* 2011). CsgD is a member of FixJ/LuxR/UhpA family of transcriptional regulators and is the product of *csgD* gene located on *csgDEFG* operon (Hammar, Arnqvist *et al.* 1995; Gerstel and Römling 2003).

CsgD regulates the promoter activity of *csgDEFG-csgBAC* operons (Carpousis 2007; Zakikhany, Harrington *et al.* 2010). The products of *csgEFG-csgBAC* are involved in the synthesis, secretion and assembly of curli fimbrie (Das, Bhutia *et al.* 2011). CsgA is the major structural protein subunit of curli fibers that can bind fibronectin and Congo red. CsgB is the cognate nucleator for insoluble CsgA polymers formation at the bacterial cell surface (Das, Bhutia *et al.* 2011). Other members of the *csgDEFG-csgBAC* operon are accessory proteins that facilitate transport and assembly of CsgA (Barnhart and Chapman 2006; Robinson, Ashman *et al.* 2006; Gibson, White *et al.* 2007). Besides CsgD mediated control of *csgBAC* operon, *csgBAC* is also regulated by diguanylate cyclases and phosphodiesterase which are otherwise involved in cyclic-3'-5'-diguanylic acid (cyclic di-GMP or c-di-GMP) metabolism as described in section 1.7.2. (Sommerfeldt, Possling *et al.* 2009; Tagliabue, Maciag *et al.* 2010).

CsgD indirectly activates cellulose biosynthesis by binding to the *adrA* promoter (Bokranz, Wang *et al.* 2005). AdrA enhances production of c-di-GMP by its diguanylate cyclase synthase activity (Zogaj, Nimtz *et al.* 2001). Subsequently, c-di-GMP activates the synthesis of cellulose by binding to the main cellulose synthase BcsA (Römling, Rohde *et al.* 2000). However, as is true for CsgD-independent control of curli fimbriae, cellulose production can also occur without CsgD-mediated *adrA* activation. This is achieved by the direct increase in the c-di-GMP level independent of AdrA (Garcia, Latasa *et al.* 2004; Da Re and Ghigo 2006). In addition to the aforementioned two main biofilm matrix components, CsgD also induces the expression of BapA and lipopolysaccharide (Latasa, Roux *et al.* 2005; Gibson, White *et al.* 2006).

CsgD expression is influenced directly or indirectly by multiple environmental signals such as nutrient limitation, low temperature, oxygen tension, alkaline pH and high cell density (Gerstel and Römling 2001; Brombacher, Dorel *et al.* 2003; Gerstel and Römling 2003; Römling 2005). The signals from varying environments are

transmitted to certain global regulators that influence CsgD expression (Gerstel and Römling 2003; Ishihama 2010; Ogasawara, Yamada *et al.* 2010; Ogasawara, Yamamoto *et al.* 2010). Among many regulators, the stress/stationary sigma factor RpoS and osmolarity response regulator OmpR are the most important for CsgD positive transcription (Römling, Bian *et al.* 1998; Gualdi, Tagliabue *et al.* 2007). Along with the global regulators, evidence for small regulatory RNA mediated regulation of CsgD are increasing rapidly (Udekwu, Darfeuille *et al.* 2005; Holmqvist, Reimegard *et al.* 2010; Mika, Busse *et al.* 2012; Monteiro, Papenfort *et al.* 2012; Thomason, Fontaine *et al.* 2012). Furthermore, the expression of CsgD is strongly influenced by the c-di-GMP concentration in the cell (Kader, Simm *et al.* 2006; Weber, Pesavento *et al.* 2006; Krasteva, Fong *et al.* 2010).

### 1.7.2   Cyclic-di-GMP Control of Biofilm Formation

Cyclic-di-GMP is a small and well known bacterial secondary messenger that has been implicated in biofilm formation and dispersion, motility, virulence, regulation of the cell cycle, differentiation and in a growing number of other properties (Jenal 2004; Simm, Morr *et al.* 2004; Wolfe and Visick 2008; Lamprokostopoulou, Monteiro *et al.* 2010; Ahmad, Lamprokostopoulou *et al.* 2011; Römling, Galperin *et al.* 2013). Interestingly, a recent report has claimed to identify the presence of c-di-GMP in eukaryotes as well (Chen and Schaap 2012). The cellular concentration of c-di-GMP is maintained by GGDEF and EAL or HD-GYP domain proteins, which act as diguanylate cyclases and phosphodiesterases respectively (Römling, Rohde *et al.* 2000; Römling 2005; Scher, Römling *et al.* 2005). The overall metabolism of c-di-GMP is summarized in figure 4.

Besides its classical function of activating cellulose synthesis that constitute a major component of biofilm matrix of many organisms (Ross, Weinhouse *et al.* 1987; Römling, Rohde *et al.* 2000; Bokranz, Wang *et al.* 2005; Da Re and Ghigo 2006), c-di-GMP inhibits motility and flagellar gene expression which aids in the establishment of biofilms (Simm, Morr *et al.* 2004; Kader, Simm *et al.* 2006; Ryjenkov, Simm *et al.* 2006; Meissner, Wild *et al.* 2007). The motility is repressed by binding of c-di-GMP with the PilZ domain of YcgR and BcsA, that inhibits FliG-meditated flagellar rotation and imparts steric hindrance to flagellar rotation in dense cellulose matrix respectively (Ryjenkov, Simm *et al.* 2006; Fang and Gomelsky 2010; Zorraquino, Garcia *et al.* 2013).
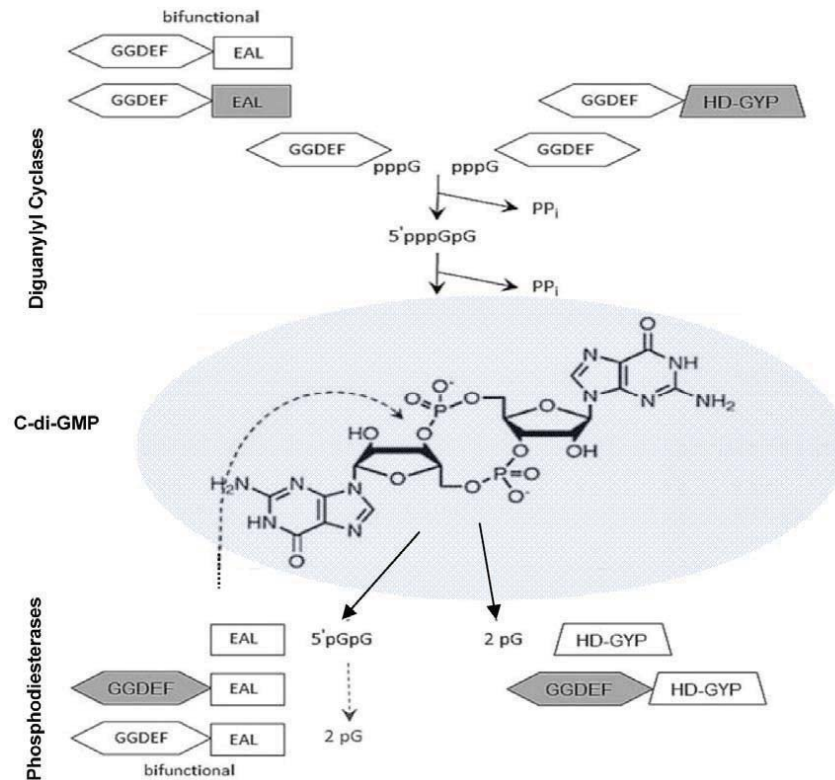
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**Figure 4: Synthesis and degradation of c-di-GMP.** Synthesis is carried out by proteins with GGDEF domains and degradation is achieved by EAL and HD-GYP domain proteins. White shading indicates the active domains and grey background represents inactive domains. Adapted from (Römling, Galperin *et al.* 2013).

The transition from motility to sessility is analogous to the change from the acute virulent infection state to a less virulent but persistent chronic stage of infection (Tamayo, Pratt *et al.* 2007; Römling, Galperin *et al.* 2013). Indeed, a number of reports have shown the involvement of a high c-di-GMP concentration in promoting the persistence of *Pseudomonas aeruginosa* in airways of cystic fibrosis patients and in animal infection models (Starkey, Hickman *et al.* 2009; Malone, Jaeger *et al.* 2010; Byrd, Pang *et al.* 2011). Similarly, elevated intracellular levels of c-di-GMP suppress acute stages of infection and promote chronic infection in *S.* Typhimurium (Lamprokostopoulou, Monteiro *et al.* 2010; Ahmad, Lamprokostopoulou *et al.* 2011). However, motility is also regarded as the initiation step of biofilm formation and is essential for colonization of new habitats (Danhorn and Fuqua 2007).

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This contradiction can't yet be resolved due to our limited understanding of c-di-GMP signaling, complexity of regulatory switches, multiple targeting at transcriptional, post-transcriptional and post-translational levels and intracellular spatial restriction of c-di-GMP pool (Römling, Galperin *et al.* 2013). Altogether, the orchestrated control of c-di-GMP is ultimately decisive for choosing either planktonic life style (acute infection mode) or multicellular sessile growth behavior (chronic infection mode). However, further efforts to solve the issues regarding c-di-GMP-mediated controls are still needed.

## 1.8 BIOFILMS, REDOX STRESS AND OXIDOREDUCTASES

Bacterial adaptation and transition from a planktonic lifestyle to sessile biofilm growth result in substantial changes in the bacterial metabolome. Biofilms are regarded as oxidizing environments and the expression of a significant number of oxidative stress tolerant genes is affected by biofilm-forming conditions (Römling and Tummler 2000; Bokranz, Wang *et al.* 2005; Resch, Rosenstein *et al.* 2005; Da Re and Ghigo 2006; Suo, Huang *et al.* 2012; Yeom, Shin *et al.* 2013).

In the last decade, continuous efforts have been focused on understanding the biofilm environment and the role of redox stress in modulation of biofilm (Bitoun, Nguyen *et al.* 2011; Bitoun, Liao *et al.* 2012; Liu, Sun *et al.* 2013). In addition to this, several reports connect oxidoreductases with biofilm regulation (Ryjenkov, Simm *et al.* 2006; Tarutina, Ryjenkov *et al.* 2006; Lee, Kim *et al.* 2008; Lee, Oh *et al.* 2009; Suo, Huang *et al.* 2012). The periplasmic superoxide dismutase (SOD) is known to be essential for biofilm formation in *E. coli* and *Listeria monocytogenes* (Kim, Lee *et al.* 2006; Tarutina, Ryjenkov *et al.* 2006; Suo, Huang *et al.* 2012). Kim *et. al.* has further shown that thiol peroxidase, Tpx, is also a requirement for the stability of biofilms in *E. coli* (Kim, Lee *et al.* 2006). The protein disulfide isomerase DsbA suppresses biofilm formation in *Pseudomonas putida* (Lee, Oh *et al.* 2009; Lee, Seo *et al.* 2011) whereas in *E. coli* DsbA is essential for static growth of bacteria (Lee, Kim *et al.* 2008). In *Streptococcus mutans,* another newly defined oxidoreductase, SMu0629, has also been demonstrated to affect biofilm formation probably through its effect on autolysin AltA that is essential for biofilm development (Ahn and Burne 2007). However, in the presence of certain metabolite oxidoreductases can act as antimicrobials and contribute to the reshaping and development of biofilms (Welk, Rudolph *et al.* 2011; Zhu and Kreth 2012).

Furthermore, as discussed in section 1.6, bacteria can divert the product of oxidative stress to regulate and coordinate their metabolism and virulence. Recently, in *Staphylococcus epidermidis,* a redox sensing regulator, AbfR (aggregation and biofilm-formation regulator) has been defined to repress biofilm. AbfR can negatively regulate its own expression by binding to its promoter in reduced form and oxidation at its Cys[13] and Cys[116] results in dissociation from promoter, hence suppression of biofilm-formation (Liu, Sun *et al.* 2013). By *in silico* analysis, we also found a pair of Cys residues in CsgD as well (unpublished data). The presence of such a Cys pair invites the speculations of ROS mediated changes at active Cys residues of CsgD and hence the regulation of biofilm in *Salmonella* and *E.coli.*

In short, the complex biology of biofilms seems to be influenced in part by redox stress and oxidoreductases. However, to clearly define the mechanisms underlying the involvement of oxidoreductases and redox stress in biofilm control still awaits further efforts.

## 1.9 RNA DEGRADOSOME

The central dogma of molecular biology states that information passes from DNA to proteins through the intermediate messenger RNA (mRNA) (Crick 1970). Gene regulation at post-transcriptional levels largely involves mRNA and relies on the stability and half-life of mRNA. The half-lives of bacterial transcripts in model organisms such as *E. coli* are less than 10 minutes on average, suggesting their rapid but selective turnover for a successful adaptation to changing environmental conditions (Bernstein, Khodursky *et al.* 2002; Selinger, Saxena *et al.* 2003; Dressaire, Picard *et al.* 2013).

The well organized and controlled RNA turnover process is accomplished with the activity of a highly conserved 500kDa cytoplasmic membrane associated multiprotein complex known as RNA degradosome (Carpousis, Van Houwe *et al.* 1994; Py, Causton *et al.* 1994; Carpousis 2007; Burger, Whiteley *et al.* 2011; Gorna, Carpousis *et al.* 2012; Bandyra, Bouvier *et al.* 2013). The four major components of the RNA degradosome are endoribonuclease (RNase E), exoribonuclease (PNPase), RNA helicase (mainly RhlB) and a metabolic enzyme enolase (Py, Higgins *et al.* 1996; Chandran, Poljak *et al.* 2007; Zhou, Zhang *et al.* 2013). In addition, a number of other components are being isolated that add to the selectivity and specificity of the RNA degradosome (Butland, Peregrin-Alvarez *et al.* 2005; Kaberdin and Lin-Chao 2009; Burger, Whiteley *et al.* 2011). RNase E is the core of the RNA degradosome that

anchors itself to the cytoplasmic membrane and acts as a scaffold for the attachment of other components of the degradosome (Callaghan, Aurikko *et al.* 2004; Taghbalout and Rothfield 2007; Khemici, Poljak *et al.* 2008). RNase E is important in initiation of mRNA decay by endonuclease activity, especially for mRNAs with PNPase-resistant-signal at their 3' ends, and is also involved in the maturation of mRNAs (Carpousis 2007; Mackie 2013). Helicases facilitate RNAse E- and PNPase-mediated decay by unwinding the complex RNA structures, although the functions of enolase are not yet fully resolved (Carpousis 2007). The focus here is on the PNPase and will be discussed further along with some aspects of cold-shock associated helicase, CsdA or DeaD helicase, in the following section.

### 1.9.1 Polynucleotide phosphorylase (PNPase)

PNPase is a 3′-5′ exoribonuclease encoded by *pnp* that catalyzes the reversible addition of inorganic phosphate ($P_i$) across the 5′–3′ phosphodiester bond of single stranded RNA with the concomitant release of a nucleoside diphosphate from the 3′-end (Grunberg-Manago and Ochoa 1955). PNPase is an ubiquitous enzyme with a conserved domain structure (Leszczyniecka, DeSalle *et al.* 2004). PNPase has two RNase PH domains at N-terminus, which are separated by an α-helix. The second RNase PH domain from N-terminus is catalytically active and is called the PNPase domain (Jarrige, Brechemier-Baey *et al.* 2002). The C-terminus contains two additional domains, KH and S1 that bind and target senescent RNA molecules to RNA degradosome (Symmons, Williams *et al.* 2002; Leszczyniecka, DeSalle *et al.* 2004). PNPase activity is decreased by approximately 90% - 95% by deleting either KH or S1 domain respectively whereas deletion of both domains results in a 99% loss of enzymatic activity (Stickney, Hankins *et al.* 2005).

#### 1.9.1.1 *PNPase and Virulence*

The importance of PNPase is exemplified by its roles in virulence fitness and adaptation to stress environments (Sukupolvi, Edelstein *et al.* 1997; Clements, Eriksson *et al.* 2002; Rosenzweig, Weltman *et al.* 2005; Rosenzweig and Schesser 2007). In *S.* Typhimurium mutations in the PNPase locus result in enhanced virulence marked with spleenomegaly and persistency of infection in mouse infection model (Sukupolvi, Edelstein *et al.* 1997; Clements, Eriksson *et al.* 2002). Clements *et. al.* have further shown that mutational inactivation of the PNPase gene remarkably affects the mRNA level of virulence-associated genes, the majority belonging to SPI-1 and SPI-2

activities. This effect on mRNA levels occurred either by altering the expression or the stabilization of mRNA (Clements, Eriksson *et al.* 2002). Moreover, PNPase represses *spv* virulence genes that are required for intracellular survival and replication of *S.* Typhimurium (Ygberg, Clements *et al.* 2006). We have added to the list of PNPase controlled virulence traits in *S.* Typhimurium where PNPase is required for biofilm formation at low temperature and integrates with the c-di-GMP metabolism (Rouf, Ahmad *et al.* 2011). Interestingly, in a recent work, Wang *et al.* have shown that c-di-GMP can directly bind to the PNPase (Wang, Chin *et al.* 2012). Such findings allow for the speculation that acute infections in PNPase proficient strains may function through sequestration of c-di-GMP, the latter otherwise would promote biofilm formation and chronic infection.

### 1.9.1.2  *PNPase and Environmental Adaptation*

The classical function of PNPase is its role in bacterial adaptation to cold shock (temperature shift from $37^{\circ}$C to $15^{\circ}$C). In *E. coli*, a shift to low temperature induces cold shock associated proteins (CSPs) including PNPase (Jones, VanBogelen *et al.* 1987; Yamanaka and Inouye 2001). For growth resumption to pre-stress condition during the acclimatization phase, PNPase specifically degrades the CSP transcripts with concomitant induction of non-CSP mRNAs. The degradation of the CSP transcript also releases captive ribosomes that are recycled in the translation of other non-CSP transcripts (Thieringer, Jones *et al.* 1998; Neuhaus, Rapposch *et al.* 2000; Polissi, De Laurentis *et al.* 2003). Hence, an *E. coli* strain lacking PNPase is compromised in cold adaptation and half-life of CSP transcripts are prolonged (Yamanaka and Inouye 2001). Furthermore, in *Yersinia*, the catalytic activity of PNPase and its S1 domain have also been associated with cold adaptation (Rosenzweig, Weltman *et al.* 2005).

Interestingly, PNPase has recently been demonstrated to be required for efficient adaptation to oxidative stress environments in many organisms (Wu, Jiang *et al.* 2009; Xiao, Xu *et al.* 2011; Henry, Shanks *et al.* 2012; Liu, Gong *et al.* 2012). As discussed in section 1.4, one of the preferred targets for ROS damage is nucleic acid. Indeed, PNPase-deficient strains are compromised in their growth in an oxidative stress environment due to accumulation of toxic oxidized RNAs in the cell (Wu, Jiang *et al.* 2009). PNPase has been shown to bind with a higher affinity to the oxidized RNAs compared to undamaged RNAs and hence is essential for clearance of these toxic damaged RNAs (Xiao, Xu *et al.* 2011). In *E. coli*, the PNPase response to oxidative stress adaptation is believed to be degradosome-independent (Wu, Jiang *et al.* 2009)

whereas in *Yersinia pseudotuberculosis*, this function of PNPase is also degradosome assembly dependent (Henry, Shanks *et al.* 2012).

### 1.9.2 DEAD Box RNA Helicase – CsdA/DeaD

In enterobacteria, the *deaD* gene downstream to *pnp* encodes a 70kDa DEAD box (Asp-Glu-Ala-Asp) RNA helicase, CsdA (cold-shock DEAD box protein A) which is now commonly referred to as DeaD RNA helicase to avoid confusion with *c*ysteine *s*ulfinate *d*esulfinase (*csd*) (Jones and Inouye 1994; Blattner, Plunkett *et al.* 1997; McClelland, Sanderson *et al.* 2001; Iost, Bizebard *et al.* 2013). DeaD helicase expression is induced under cold shock and deletion of the *deaD* gene results in retarded growth at low temperatures (Yamanaka and Inouye 2001; Prud'homme-Genereux, Beran *et al.* 2004). DeaD helicase can replace the 37°C functional counterpart RhlB helicase on RNA degradosome during the cold shock response and hence produces a "cold shock degradosome" in association with other essential components of RNA degradosome (Khemici, Toesca *et al.* 2004). The association of DeaD helicase with PNPase facilitates the degradation of complex mRNAs (Jones, Mitta *et al.* 1996; Yamanaka and Inouye 2001; Prud'homme-Genereux, Beran *et al.* 2004). Furthermore, DeaD helicase has also been reported to be involved in the maturation of 50S ribosomal subunit in *E. coli* (Charollais, Dreyfus *et al.* 2004; Peil, Virumae *et al.* 2008).

The fact that DeaD helicase enhances stability of CSP transcripts and delays the maturation of ribosomes (Awano, Xu *et al.* 2007; Peil, Virumae *et al.* 2008), still leaves an open question, that whether longevity of CSP transcripts or maturation-defect of ribosomes is responsible for compromised growth of *deaD* mutant at low temperature?

### 1.10 NEW LIPOPROTEIN I (NLPI)

NlpI is a 32kDa outer membrane-associated globular lipoprotein encoded by the corresponding gene *nlpI,* located between *pnp* and *deaD* on the chromosome (Blattner, Plunkett *et al.* 1997; McClelland and Wilson 1998; McClelland, Sanderson *et al.* 2001). NlpI is highly conserved among *Enterobacteriaceae* and the mature membrane anchored protein is 276 amino acids long after cleavage of 18 residues at the N-terminus (Ohara, Wu *et al.* 1999). However, NlpI is also subject to periplamic Pre-protease (Prc/Tsp) processing at its C-terminus that results in truncated NlpI with possibly different functions to that of its full length membrane associate (Tadokoro,

Hayashi *et al.* 2004). NlpI is the first prokaryotic protein described so far with a tetratricopeptide repeats (TPRs) that may provide large surface area for protein-protein interactions (Wilson, Kajander *et al.* 2005).

NlpI was shown to be involved in *E. coli* cell division. The mutation in the *nlpI* gene rendered bacteria osmosensitive and thermosensitive with growth problems and altered morphology respectively (Ohara, Wu *et al.* 1999). Moreover, at low temperatures, NlpI has been associated with peptidoglycan redistribution with increased septation and cocobacilli formation (Pierce, Gillette *et al.* 2011). Besides a role in cell division, NlpI was also shown to participate in virulence. In *E. coli*, NlpI is required for efficient adhesion and invasion, facilitates binding to the microvasculature of blood brain barrier and promotes bacteremia by evasion from complement-mediated killing (Barnich, Bringer *et al.* 2004; Teng, Tseng *et al.* 2010; Tseng, Wang *et al.* 2012). Furthermore, NlpI facilitates eDNA release in *E. coli* and suppresses biofilm formation in *S.* Typhimurium (Sanchez-Torres, Maeda *et al.* 2010; Rouf, Ahmad *et al.* 2011).

In short, the involvement of NlpI in multiple phenotype regulation raises the possibility that NlpI could act as a relay center for communicating signals from the environment to the metabolic and virulence regulatory cascades. Therefore, more work is needed to answer the remaining questions about NlpI involvement in such regulatory mechanisms.

## 2 AIMS OF THE THESIS

This thesis focuses on establishing the role of redox stress and the oxidoreductase systems in the virulence and environmental adaptation of *Salmonella enterica* serovar Typhimurium.

The specific aims are:

- To investigate the role of the ScsABCD oxidoreductase system in oxidative stress tolerance and virulence of *S.*Typhimurium
- To analyze the role of periplasmic Dsb oxidoreductase system in the development and regulation of biofilm-formation of *S.* Typhimurium.
- To probe the role of membrane protein NlpI and exoribonuclease PNPase in biofilm-development and regulation.
- To establish the functional connection between different members of RNA degradosome and NlpI.

# 3   RESULTS AND DISCUSSIONS

## 3.1   PAPER I

**Oxidoreductases that act as conditional virulence suppressors in *Salmonella enterica* serovar Typhimurium**

Microarray analyses conducted on *S.* Typhimurium growing in murine macrophage-like cells (Eriksson, Lucchini *et al.* 2003) and in environment mimicking intracellular oxidative stress (Bjur *et al.*, unpublished data) have shown an up-regulation of the *scsABCD* gene cluster along with other known virulence genes. Since then, no attempts have been made to define the role of this genetic locus in *Salmonella*. The *scsABCD* gene cluster encodes four individual suppressor for copper sensitivity (Scs) proteins named as ScsA, ScsB, ScsC and ScsD. In the first paper of this thesis we have defined the ScsABCD as a potential new addition to the thioredoxin superfamily of oxidoreductases and have described its virulence-associated role in *S.* Typhimurium (Anwar, Sem *et al.* 2013).

We started by *in silico* analysis of *scsABCD* locus. The *scsABCD* region is ~3.4kb long and is located between the *cbp* and *agp* region of *S.* Typhimurium strains LT2 and 14028s genomes as well as in selected *Enterobacteriaceae* members (paper I; Fig. 1A). The GC content of this particular genetic region is consistent with the overall GC content of *S.* Typhimurium genome i.e. 50% - 52%. Furthermore, we could not detect any inverted repeats encompassing *scsABCD* locus that would be an indication of insertion of this particular genetic locus (unpublished data). These facts gave the impression that the *scsABCD* cluster is not a horizontally acquired genetic islet. Rather, *Salmonella* has retained *scsABCD* locus during evolution for fitness in versatile environments. We further found that each of the Scs proteins contains a *Cys-X-X-Cys* motif (Paper I; Fig.1B, 1C), which is a hallmark of oxidoreductase thioredoxin superfamily (Messens and Collet 2006) and thus defines the Scs proteins as putative new members in the thioredoxin superfamily.

The ScsA and ScsB were predicted as outer membrane proteins based on the presence of lipobox sequence on N-termini whereas ScsD was predicted as integral cytoplasmic membrane protein. The ScsC protein was predicted as periplasmic protein based on classical signal sequence present at its N-terminal. In the hunt for the actual localization of these proteins in the bacterial subcellular compartments, ScsB was detected in the cytoplasmic membrane fraction whereas ScsC was recovered in the

predicted periplasmic fraction by using recombinant plasmid expressing His-tag variants of respective protein (Paper I; Fig. 1E). However, ScsA and ScsD were not detected with such a strategy, perhaps due to the instability of the tagged proteins. To overcome this limitation, we constructed the TrxA-Scs-His fusion protein which would give stability to the protein. By this approach, we were able to detect TrxA-ScsD-His in the predicted cytoplasmic membrane but we still did not recover ScsA (PaperI; Fig. 1E).

In *E. coli* copper sensitive mutants (Cu$^s$), the tolerance to copper (CuCl$_2$) can be restored by *trans*-complementation with the *scsABCD* genes whereas *the scsABCD* fails to restore tolerance of selected *S.* Typhimurium Cu$^s$ mutants (Gupta, Wu *et al.* 1997). This may imply that such tolerance conferred by *scsABCD* in E. coli is not against copper metal, rather its against the CuCl$_2$-induced non-enzymatic damage to protein disulphide bonds as described previously (Lehrer 1975; Hurme, Namork *et al.* 1994). Therefore, we checked the tolerance of Δ*scsA*, Δ*scsB*, Δ*scsC*, Δ*scsD* individual and Δ*scsABCD* quadruple mutants of *S.* Typhimurium to CuCl$_2$. Every defined mutant was sensitive to CuCl$_2$ except the Δ*scsA* mutant (Paper I; Fig. 2A). However, any such differential sensitivity was not found for Δ*scs* mutants when treated with the transition metal salt, ZnCl$_2$ (Paper I; Fig. 2B).

CuCl$_2$ is also a potential inducer of ROS by the Fenton reaction (Rietsch, Belin *et al.* 1996). Therefore, we tested the viability of Δ*scs* mutants to a prototype ROS, hydrogen peroxide (H$_2$O$_2$). Only the viability of Δ*scsA* mutant was reduced whereas all other mutants paralleled the survival of the wild type upon H$_2$O$_2$ stress (Paper I; Fig. 2D). The stand alone behavior of Δ*scsA* mutant for CuCl$_2$ and H$_2$O$_2$ could be explained by the fact that the ScsA is predicted to have a peroxidase function and that the processing at N-terminal would delete the putative thioredoxin motif (Gupta, Wu *et al.* 1997). Furthermore, an imbalance in the Scs protein content rather than lack of ScsA might explain the selective sensitization to H$_2$O$_2$. Such reports have already been published for the periplasmic protein DsbA, where overexpression of DsbA protein suppresses the motility contrary to the requirement of DsbA for proper motility (Lee, Oh *et al.* 2009).

That the Δ*scsB*, Δ*scsC*, Δ*scsD* and Δ*scsABCD* mutants were as viable as the wild type upon treatment with H$_2$O$_2$, but sensitive to CuCl$_2$ raised the question regarding effects on cellular redox balance in the Δ*scsABCD* mutant. Disturbances in redox status of a cell can, in turn, result in ROS-mediated oxidation of proteins with introduction of carbonyl groups (e.g. ketones, aldehydes, carboxylate) or disulfide

bonds in side chains of amino acids (Stadtman 1993; Biswas, Chida *et al.* 2006). Indeed, the *scsABCD* mutation resulted in pronounced protein carbonylation upon exposure to $H_2O_2$ (Paper I; Fig. 3A), notably in the periplasmic fraction (Paper I; Fig. 3C). Collectively, these findings imply that the *scsABCD* gene cluster is important for the oxidative stress tolerance that interferes with periplasmic disulphide bond formation in *S. enterica* and thus define the ScsABCD as new members in the oxidoreductase thioredoxin superfamily.

Thioredoxin 1 (*trxA*, TrxA), the prototype of thioredoxin superfamily, contributes to copper tolerance in *E. coli* (Rietsch, Belin *et al.* 1996) and is required for efficient invasiveness of *S.* Typhimurium in a mammalian epithelial cell line (Bjur, Eriksson-Ygberg *et al.* 2006). We noted a decreased $CuCl_2$ tolerance of Δ*trxA* mutant in *S.* Typhimurium as well (Paper I; Fig. 4A) and determined that full invasiveness relied on its catalytic motif indicating the role of redox dependent invasion activity of *S.* Typhimurium (Paper I; Fig. 4B). Therefore, we set out to test if there is some connection between Δ*scs* mutants and Δ*trxA* mutant regarding invasiveness as both stand $CuCl_2$ sensitive.

We found that the invasion was not affected by deletion of *scsABCD* (Paper I; Fig. 4B) however the *scsABCD* deletion in the Δ*trxA* mutant enhanced the invasion of mammalian cells (Paper I; Fig. 4C). This implies that attenuation in the invasiveness of the *S.* Typhimurium Δ*trxA* mutant is conditionally relying on the presence of *scsABCD*. Furthermore, we showed that the *scsABCD* mutation can restore the virulence of the Δ*trxA* mutant in a soil nematode *C. elegans* infection model (Paper I; Fig. 7). Upon pre-invasion $CuCl_2$ treatment of bacterial cultures, the invasiveness of the wild type *S.* Typhimurium was lost whereas the invasion index for Δ*trxA* mutants was increased and was much retained in Δ*scsABCD* or Δ*scsABCD/*Δ*trxA* mutants (Paper I; Figs. 4E and 4F). Thus, the $CuCl_2$-associated suppression of invasion in wild type or the Δ*trxA* mutant is also dependent on the presence of ScsABCD oxidoreductases. Our findings are in accord with previously reported conditional phenotypes for oxidoreductase in *E. coli* (Takemoto, Zhang *et al.* 1998; Hiniker, Collet *et al.* 2005)*.* For instance, deletion of DsbA, which is dispensable for $CuCl_2$ stress tolerance, substantially increases $CuCl_2$ sensitivity of a Δ*dsbC* mutant (Hiniker, Collet *et al.* 2005).

Furthermore, we reported that the retained invasiveness of the Δ*scsABCD* mutant upon $CuCl_2$ exposure still relied on SPI1 T3SS activity. However, as assayed by measuring the expression and secretion of a SPI-1 T3SS effector fusion protein SipB-β-

lactamase, the Δ*scsABCD* mutant deviated from the wild type in that the Δ*scsABCD* mutant retained much more SPI-1 effector proteins as compared to wild type (Paper I; Fig. 5). The expression of the genes for the main regulator of the SPI-1 T3SS *hilA* and main structural component *prgH* was much reduced under $CuCl_2$ stress with no remarkable difference between Δ*scsABCD* mutant and wild type. Thus, these observations indicated that SPI-1 T3SS expression is redox sensitive and that the *scsABCD* deletion affects the secretion of a SipB fusion protein.

The conditionally enhanced invasion of the Δ*trxA* mutant or in the presence of $CuCl_2$ can be explained in two ways. First, the presence of $CuCl_2$ or TrxA-deficiency could cause differential Scs-mediated accumulation of oxidized periplasmic proteins, being these Scs proteins themselves or their substrates. These unwanted accumulated proteins could subsequently interfere with the T3SS activity and thus affect invasion. Indeed, in a previous report, the DsbA activity of *S.* Typhimurium has been connected to both SPI1 gene expression and in the SPI1 T3SS apparatus functionality (Lin, Rao *et al.* 2008). Second, assuming that in the absence of $CuCl_2$, the expression of SPI1 T3SS mediated invasion genes in wild type is highly active but leaky allowing secretion of effector proteins even in the absence of host cell contact. In the Δ*scsABCD* mutant the apparatus is either less active or more strictly regulated. Effector proteins accumulating inside mutant bacteria create a secretion competent pool applicable even after copper induced down-regulation of SPI1 gene expression, and notably, only used for translocation.

To conclude, the newly defined ScsABCD oxidoreductase system can be seen more like a fine-tuner for maintaining the redox balance inside the cell and redox-dependent virulence associated traits of *S.* Typhimurium. Their role as conditional virulence suppressor invites to the speculations regarding other oxidoreductases of the thioredoxin superfamily standing as conditional suppressors not restricted to *Salmonella* only rather in more general terms.

## 3.2   PAPER II

**Redox-sensitivity of biofilm-formation in *Salmonella enterica* serovar Typhimurium – A particular impact of the DsbA/DsbB redox system**

Salmonellae are ubiquitous in their habitat and at any given stage of their life they come across with varying environmental conditions such as extreme pH, low temperature, desiccation, low/high osmolarity and oxidative stress (Foster 1995; Gruzdev, Pinto, and Sela 2012). For survival and adaptation under such challenging

conditions *Salmonella* tend to adapt to a sessile growth phase (Costerton, Stewart *et al.* 1999) with a substantial requirement of changes in its transcriptome and proteome. Interestingly, a proportion of genes related to oxidative stress tolerance becomes induced under biofilm-forming conditions (Resch, Rosenstein *et al.* 2005). Therefore, in the second study of this thesis, we assess the role of periplasmic disulfide bond (Dsb) oxidoreductase system in biofilm-development in *S.* Typhimurium.

Among the different members of Dsb oxidoreductase system, we found that the DsbA and DsbB (DsbA/DsbB redox pair) suppressed the red dry and rough (*rdar*) morphotype development in *S.* Typhimurium on a solid low osmolarity growth medium supplemented with Congo red (CR) but were required to make a pellicle at the air liquid interface in low osmolarity liquid cultures (Paper II; Fig. 1) In accordance with the requirement for pellicle formation in broth, both mutants also revealed a decreased transcription of biofilm master regulator *csgD* in liquid cultures (Paper II; Fig. 2C). However, this decrease was much more pronounced for the Δ*dsbA* mutant. Our results for *dsbA* mutation in *S.* Typhimurium corroborate a role for DsbA for liquid-based biofilm formation in *E. coli* O157 (Lee *et al.*, 2008) and for biofilm-development on solid media in *Pseuodomoas putida* (Lee, Oh *et al.* 2009). However, the influence of *dsbA* for pellicle formation in *S.* Typhimurium is contradictory to those observations in *Pseuodomoas putida* where *dsbA* down-regulates pellicle development (Lee, Oh *et al.* 2009). Our findings along with previous reports indicate a versatile role of DsbA in regulation of biofilm morphotypes under selected environmental conditions. Furthermore, we demonstrate a new role of DsbB as a modulator of biofilm-development. Hence the DsbA/DsbB redox pair can be envisioned as a connection between the bacterial redox-shuffling system and biofilm-development and -regulation.

In dissecting the molecular effects of *dsbA* and *dsbB* mutations on biofilm regulation we found that enhanced biofilm development on the agar plates is due to the increased production of surface associated curli fimbriae Csg*A* (Paper II; Fig. 2A). The increased CsgA was accompanied by enhanced expression of its main regulator CsgD in both the Δ*dsbA* and Δ*dsbB* mutants (Paper II; Fig. 2B). These results raised the new question of whether the effects on biofilm-formation by DsbA and DsbB are directly mediated through CsgD or some other mechanism is involved for enhanced *rdar*-morphotype development in *S.* Typhimurium.

Interestingly, while a Δ*dsbA*/Δ*csgD* double mutant failed to generate a *rdar*-morphotype on agar plates, a Δ*dsbB*/Δ*csgD* double mutant still resulted in an increased

biofilm-formation (Paper II; Fig. 3). Furthermore, over-expression of the YhjH phophodiesterase, responsible for breaking down c-di-GMP and hence reducing the concentration of CsgD, resulted in loss of biofilm-formation in both the Δ*dsbA* and Δ*dsbB* mutants (Paper II; Fig. 3). From these results we deduced that DsbA affects the biofilm formation through CsgD-mediated regulation, although at present, we cannot clearly define the regulatory cascade for the DsbB in controlling biofilm-formation in *S.* Typhimurium.

That the oxidative stress promotes biofilm formation (Costerton, Stewart *et al.* 1999) and periplasmic oxidoreductases such as DsbA and DsbB modulate biofilm-regulation led us to test the effects of exogenous redox stress on biofilm development. Under $CuCl_2$ induced oxidative stress *S.* Typhimurium lost its ability to develop a *rdar*-morhotype in a dose dependent manner on CR plates regardless of the genetic background of the strains (Paper II; Fig. S2). Similarly, supplementation of CR low osmolarity growth media with the reductant dithiothritol (DTT) reduced the *rdar*-morphotype in both the Δ*dsbA* and Δ*dsbB* mutants (Paper II; Fig. 4A). This was accompanied by decreased expression of both CsgA and CsgD (Paper II; Figs. 4A and 4B).

For the Δ*dsbA* and Δ*dsbB* mutants the DTT-induced reduction in *rdar*-morphotype was accompanied by the production of an extremely slimy colony morphotype. Sliminess was further enhanced in both the Δ*dsbA* and Δ*dsbB* mutants upon expressing YhjH from the recombinant plasmid or by introducing a *csgD* mutation in the Δ*dsb* mutants. Surprisingly though, the attempt to complement Δ*dsbA* and Δ*dsbB* mutants under reductive stress rather resulted in enhanced *rdar*-morphotype, even more than isogenic wild type, and in suppression of slime production (Paper II; Figs. 4A and 5A). In order to further trace the cause of the slimy colony morphotype, we generated mutants defective in either colanic acid synthesis (*wcaM*) or in their ability to ligate the LPS O-antigen (*waaL*) in the Δ*dsb* mutants. However, under DTT stress on agar plates all double mutants generated a slimy colony morphotype.

In selected bacteria nucleic acids form an essential component of the biofilm (Whitchurch, Tolker-Nielsen *et al.* 2002; Allesen-Holm, Barken *et al.* 2006; Bockelmann, Janke *et al.* 2006; Izano, Amarante *et al.* 2008; Ma, Conover *et al.* 2009). To assess whether the slimy colony morphotype originated from nucleic acid release we suspended colonies of wild type and Δ*dsb* mutants grown under DTT stress in buffer, separated the bacteria by centrifugation and subjected the supernatant to nucleic acid isolation protocols. Upon a final ethanol precipitation the supernatants from the

*Δdsb* mutants, but not those from wild type bacteria, yielded a massive cotton-like precipitate. The precipitate readily dissolved in distilled water into a viscous material. When run on agarose gels, it revealed a "chromosome-like" band that disappeared upon cleavage with restriction endonucleases (Paper II; Fig. 5B).

Motility is regarded as the initiation step of biofilm formation and is essential for colonization of new habitats (Danhorn and Fuqua 2007). Motility is the opposite of sessility yet the two modes of growth are interconnected. Previously, both DsbA and DsbB have been shown to be required for swimming motility in many organisms (Dailey and Berg 1993; Abe and Nakazawa 1996; Hayashi, Abe *et al.* 2000; Lee, Kim *et al.* 2008). As expected, our *ΔdsbA* mutant was defective in motility and this defect was possible to complement by providing a non-specific disulfide oxidant $CuCl_2$. Interestingly, the *ΔdsbB* mutant was dispensable for motility in *S.* Typhimurium (Paper II; Figs. 6A and 6B). These results imply that oxidant function of DsbA for proper folding of flagellar basal body protein FlgI (Dailey and Berg 1993) can work independently of DsbB mediated oxidation and as a result DsbA can get its reducing equivalent from some alternative oxidase in the periplasm. However, the presence of an oxidase with such a high redox potential is not yet described. In contrast to oxidative stress, supplementation of DTT in the motility agar plates did not affect the *ΔdsbA* mutant motility defect, albeit, *ΔdsbB* mutant was lagging in motility too (Paper II; Fig. 6C).

Taken together, the current study describes the contributions of DsbA and DsbB oxidoreductases in the regulation of biofilm-development and motility. Furthermore, under stress conditions, additional biofilm matrix components can be induced conditionally in the absence of DsbA/DsbB. These findings invite the target based designing of novel antibiotics to eradicate biofilm-associated chronic infections.

## 3.3    PAPER III

**Opposing contributions of polynucleotide phosphorylase and the membrane protein NlpI to biofilm formation by *Salmonella enterica* serovar Typhimurium**

*S.* Typhimurium, when subjected to low temperature and osmolarity, tend to restrict motility and start growing as sessile multicellular communities known as the biofilm. The transition from motility to sessility is analogous to the change from the acute virulent infection state to the less virulent but persistent chronic stage of infection (Tamayo, Pratt *et al.* 2007; Römling, Galperin *et al.* 2013). Among the different factors promoting chronic infection, polynucleotide phosphorylase (PNPase encoded by *pnp*),

an evolutionarily conserved 3' - 5' exoribonuclease in eubacteria and eukaryotes (Sarkar and Fisher 2006; Carpousis 2007; Das, Bhutia *et al.* 2011), is involved in the persistence of *S.* Typhimurium infection in mouse infection model (Sukupolvi, Edelstein *et al.* 1997; Clements, Eriksson *et al.* 2002). Sukupolvi *et al.* further demonstrated that PNPase promotes curli fimbriae submunit, CsgA production (Sukupolvi, Edelstein *et al.* 1997).

The *nlpI* gene, encoding an outer membrane lipoprotein NlpI, is conserved among enteric bacteria and is located downstream of *pnp* on the genome (Blattner, Plunkett *et al.* 1997; McClelland and Wilson 1998). Recently, NlpI has been associated with secretion of eDNA component of biofilm in *E.coli* (Sanchez-Torres, Maeda *et al.* 2010). Therefore, we started with the question, if NlpI affects biofilm-formation in *S.* Typhimurium.

The biofilm formation was assayed on Luria Bertani (LB) without salt agar plates supplemented with Congo red dye at 28°C. Under such conditions, *Salmonella* makes biofilm and gives a characteristic rogous growth termed as red dry and rough (*rdar*) morphotype (Römling 2005). We found that the *pnp* mutation resulted in compromised *rdar*-morphotype development whereas Δ*nlpI* mutant was much more proficient in biofilm formation as compared to wild type. Surprisingly though, the biofilm development in *pnp-nlpI* double mutant was intermediate relative to the Δ*pnp* and Δ*nlpI* single mutants and approached to wild type level (Paper III; Fig. 1A).

The rogousness of the biofilm in *S.* Typhimurium is due to the production of surface associated fimbrial subunit CsgA the expression of that in turn is controlled by the biofilm master regulator CsgD (Hammar, Arnqvist *et al.* 1995; Römling 2005). In accordance with the *rdar*-morphotypes on CR plates, the protein expression of CsgA and CsgD was reduced and increased in the Δ*pnp* and Δ*nlpI* mutants respectively (Paper III; Fig. 2). Furthermore, the transcript levels of *csgA* and *csgD* for both mutants followed their protein expression profile relative to wild type (Paper III; Fig. 3).

To find the mechanistic details for the effect of *pnp* and *nlpI* on CsgD we extended our study to check the turnover of the bacterial secondary messenger cyclic-di-guanosine monophosphate (c-di-GMP) that controls the expression of CsgD (Römling 2005). The turnover of C-di-GMP is controlled by synthetases and phosphodiesterases. Hence, we determined the mRNA levels for biofilm-enhancer c-di-GMP synthetase AdrA and biofilm-suppressor phosphodiesterases YciR, YjcC and YhjH in Δ*pnp*, Δ*nlpI* and Δ*pnp-nlpI* double mutants. In the Δ*pnp* and Δ*nlpI* mutants the mRNA levels of *adrA, yciR*, and *yhjH* were slightly but inversely affected whereas *yjcC*

mRNA was markedly increased and decreased in the respective Δ*pnp* and Δ*nlpI* mutants. However, the opposing effects on the expression of all these genes were restored to wild type levels in the Δ*pnp-nlpI* double mutant (Paper III; Fig. 3). The transcript data was in agreement with enhanced biofilm formation on CR plates and increased CsgD and CsgA expression in Δ*pnp-yjcC* mutant when compared to the single Δ*pnp* mutant (Paper III; Figs. 1C and 2).

In conclusion, the PNPase and NlpI have opposite effects on biofilm formation in *S*. Typhimurium. These effects are mediated through the biofilm suppressor YjcC phosphodiesterase that plays major role in CsgD turn over via c-di-GMP (Simm, Lusch *et al.* 2007). Our findings are supported by a recent report where c-di-GMP *was* shown to take part directly in the activation of PNPase for RNA processing (Tuckerman, Gonzalez *et al.* 2011). That the NlpI suppresses biofilm development and is required for proper motility in *S.* Typhimurium (unpublished data) brings in the speculations for some unknown functions for NlpI that need the bacteria to be in the motile state. Furthermore, NlpI location at outer membrane protein might suggest that it can act as a sensor, which mediates the downstream gene regulatory connections with c-di-GMP metabolism.

## 3.4   PAPER IV

**Genetic analysis of the *pnp-deaD* genetic region reveals membrane lipoprotein NlpI as an independent participant in cold acclimatization of *Salmonella enterica* serovar Typhimurium**

*Salmonella* Typhimurium can infect a variety of organisms with remarkable living temperature differences (Charkowski, Barak *et al.* 2002; Tenor, McCormick *et al.* 2004; David, Wandili *et al.* 2009). To be a successful pathogen, *S.* Typhimurium experiences the adaptation pressure in such altering living temperatures. The shift from higher to low temperature (temperature shift from 37$^{o}$C to 15$^{o}$C) results in specific cold-shock responses, associated with cold-shock protein (CSP) induction and modulations in RNA turnover (Phadtare, Alsina *et al.* 1999; Phadtare and Severinov 2010). Two major component of RNA degradosome, the exoribonuclease PNPase and the alternative cold-shock RNA helicase CsdA, are required for cold adaptation in *E. coli*. (Beran and Simons 2001; Yamanaka and Inouye 2001; Prud'homme-Genereux, Beran *et al.* 2004). In the *S.* Typhimurium genome, the *pnp* and *csdA* (termed as *deaD* gene in *Salmonella*) genes are separated by *nlpI* (McClelland, Sanderson *et al.* 2001). The genetic organization of *pnp* and *nlpI* is also connected functionally to the

regulation of biofilm development at decreased growth temperatures in *S.* Typhimurium (Rouf, Ahmad *et al.* 2011). Therefore, we set out this study to define any genetic association between *pnp, nlpI* and *deaD* genes. Furthermore, we questioned if NlpI and Dead along with PNPase contribute to cold adaptation in *S.* Typhimurium.

To define the genetic association between *pnp, nlpI* and *deaD* genes we used a cDNA PCR approach. By using different combination of primer sets we were able to amplify integenic region between *pnp* and *nlpI* by using cDNA as template. However, we could not amplify any fragment from intergenic region between *nlpI* and *deaD* gene (Paper IV; Fig 3). This allowed us to deduce that *pnp* and *nlpI* constitute a genetic operon whereas *deaD* is a transcriptionally independent gene.

We tested Δ*nlpI*, Δ*deaD* and three different ORF mutants of *pnp* for their ability to grow upon a temperature shift from 37°C to 15°C in a broth based culture. All the mutants were compromised in growth upon the temperature shift relative to the wild type *S.* Typhimurium (Paper IV; Fig 4). Cold sensitization of these mutants was also corroborated with the follow up of a serially diluted culture with drop on agar plate-assay at 15°C after shift from 37°C (Paper IV; Fig 5). We also found that a Δ*pnp-nlpI* double mutant is further restricted in growth at low temperature and it can only be restored to wild type upon supplement of both the *pnp* and *nlpI* clones. This implies that *pnp* and *nlpI,* despite of making an operon, contribute individually to the cold adaptation of *S.* Typhimurium. Furthermore, we were unable to complement the growth defect of the *deaD* mutant by providing either *pnp* or *nlpI* clones implying the individual contribution of DeaD along with PNPase *and* NlpI to cold adaptation in *S.* Typhimurium (Paper IV; Fig 5).

We also determined the effect of *nlp*I mutation on the *pnp* transcript and *vice versa*. We found that mutation in *nlpI* apparently did not alter *pnp* expression. However, the *nlpI* transcript was increased in two non-polar *pnp* mutants (Paper IV; Fig 2). Such an increase in the *nlpI* transcript level could be due to the tentative promoter between the *nlpI* and *pnp* region.

To summarize, our observations imply that the *pnp, nlpI* and *deaD* genes contribute individually to cold adaptation in *S.* Typhimurium and that *pnp-nlpI* make a transcriptional unit. Furthermore, an outer membrane protein NlpI can be seen as new addition to the RNA degradosome components for the turnover of cold-shock induced transcripts.

# 4 CONCLUSIONS AND OUTLOOK

In recent years enormous efforts have been made to understand the role of oxidative stress in host-pathogen interactions. From a bacterial pathogenesis point of view an increasing number of reports on oxidoreductases are becoming available, depicting their role in oxidative stress tolerance and virulence in *S.* Typhimurium (Bjur, Eriksson-Ygberg *et al.* 2006; Negrea, Bjur *et al.* 2009; Horst, Jaeger *et al.* 2010; Peters, Paterson *et al.* 2010; Denkel, Horst *et al.* 2011; Sem and Rhen 2012).

A novel finding in one of the studies comprising this thesis was that the ScsABCD proteins function as a new periplasmic oxidoreductase system that is involved in the oxidative stress tolerance and virulence of *S.* Typhimurium. Furthermore, we have shown that ScsABCD in concert with TrxA modulates the oxidative stress-associated virulence properties of *S.*Typhimurium (Anwar, Sem *et al.* 2013). However, there are still many unanswered question that need to be addressed in order to understand the complexity of virulence-associated roles of different oxidoreductases. We are currently not able to define the exact roles of individual Scs proteins, especially regarding the ScsA protein that appeared to contribute differently under various oxidative stresses. The ScsA contains a peroxidase motif in addition to Cys-X-X-Cys motif at its N-teminus (Gupta, Wu *et al.* 1997) suggesting a dual role in handling the redox stress. This raises the questions whether ScsA is a regulator of *scsBCD* operon or if it is a connector between *scsBCD* operon and other oxidoreductase systems such as Dsb system or thioredoxin/glutaredoxin system. In fact, the *ΔscsABCD* mutant was sensitive to $CuCl_2$ stress (Anwar, Sem *et al.* 2013), as is true for the *ΔdsbC* mutant belonging to periplasmic Dsb oxidoreductase system (Hiniker, Collet *et al.* 2005). Hence, a possible interaction between the two systems cannot be ignored. Furthermore, the *scsABCD* mutation increased the in vivo fitness of *S.* Typhimurium in mouse infection models (Anwar, Sem *et al.* 2013) but how it happens still requires further research.

In the second study, we demonstrated that the DsbA and DsbB proteins of periplasmic Dsb oxidoreductase system differentially control the biofilm formation and that the control is differentially regulated for solid surface-associated biofilms and liquid culture biofilms. In addition, we showed that DsbA-mediated biofilm control goes through the biofilm master regulator CsgD while this is not the same for DsbB (Paper II). These results suggest the role of DsbA in biofilm-development that dissociates its dependency on DsbB oxidase function. Furthermore, in a recent report

on *Staphylococcus epidermidis,* the aggregation and biofilm formation regulator AbfR has been shown to be oxidatively modified at its two Cys residues and this results in the negative control of biofilm-formation (Liu, Sun *et al.* 2013). Interestingly, CsgD also contains only two Cys residues which are apart from each other mirroring AbfR (unpublished data). Hence, it is very interesting to determine whether the effects of DsbA- and DsbB-mediated oxidation of Cys residues of CsgD affect the three dimensional structure of CsgD and hence result in alteration in its regulatory potential. Moreover, reductive stress induced the production of extracellular slime in DsbA and DsbB deficient *S.* Typhimurium (Paper II). Currently, we were able to identify nucleic acids (eDNA) as one of the components of that slime. However, relatively large amount of unidentified materials such as surface polysaccharides exists and need further investigation. In addition, the mechanism of extracellular slime release under reductive stress is still an interesting topic to investigate.

Lastly we showed that PNPase and NlpI affect the biofilm regulation and that PNPase associates with the c-di-GMP metabolism (Rouf, Ahmad *et al.* 2011). PNPase is an exoribonuclease and is responsible for the turnover of small regulatory RNAs of Csr system (Viegas, Pfeiffer *et al.* 2007). Furthermore, CsrA, the main small regulatory RNA (sRNA) binding protein of Csr system, is directly involved in the c-di-GMP metabolism and hence controls the biofilm formation and motility (Jonas, Edwards *et al.* 2010). The genetic and functional association of NlpI with PNPase would invite the speculation as to whether NlpI is also involved in the c-di-GMP metabolism and sRNAs turnover and hence plays a global regulatory role instead of just targeted phenotype control.

In conclusion, the current study presents new aspects of the regulatory control of *S.* Tyhpimurium virulence and environmental adaptations and opens up new horizons to understand the underlying regulatory mechanism not only in *Salmonella* but rather in more general terms.

# 5 ACKNOWLEDGEMENTS

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