Transcriptional response of Streptococcus pneumoniae to varying concentrations of carbohydrates and metal ions
Manzoor, Irfan

Publication date: 2015

Citation for published version (APA):
Chapter 7

Summary and general discussion
In the last decade, the development of many techniques in the field of molecular biology enabled scientists to explore the mechanisms of gene expression and its regulation in more depth. Although, various techniques are available to study the expression profiling of the genes, but most of them have certain limitations to provide a global overview of the effect on the transcriptome. DNA microarray technology gives a complete picture of large-scale quantitative experiments [316] and can be applied to many situations, like disease diagnosis, drug discovery, or toxicology [317–321]. A microarray denotes a two-dimensional array on a solid substrate, which consists of microscopic features, and is probed with target molecules to study the gene expression (e.g. for diagnostic purposes). In Chapter 2, we have optimized the conditions for bacterial transcriptome analysis, from cell culture treatment to DNA microarray analysis. Time, costs and accuracy of the experiments are important factors to be considered by researchers. To study bacterial transcriptomes, DNA microarray technology has certain advantages over conventional techniques such as qRT-PCR [322]. Here, we developed a user-friendly protocol for DNA microarray analysis of *S. pneumoniae* as a case-study, by comparing the transcriptional responses of *S. pneumoniae* grown in the presence of varying L-serine concentrations in the medium. Total RNA can be isolated by various methods such as phenol-chloroform and TRIzol [323,324]. However, for downstream processes, a good quality RNA sample is always necessary to minimize the inhibitory effects of carry-over impurities. For this purpose, we combined the Macaloid method with a RNA isolation kit to isolate the high quality RNA. Since, the full length cDNA precisely represents the length of the input of RNA, a high yield and quality of cDNA depends on the selection of the suitable reverse transcriptase (RT) for cDNA synthesis [325]. Here, we used SuperScript® III Reverse Transcriptase to prepare cDNA samples and labelled them with one of the two amine-reactive fluorescent dyes. Homemade DNA microarray slides were used for hybridization of the labelled cDNA samples. The techniques used to prepare microarray slides, hybridization and scanning are well-developed and represent no longer a limiting factor. Researchers sometime face problems in the reproducibility of data due to the fact that there are no standards at the level of filtering, which is done according to the researcher's experience [326]. The massive amount of data needs normalization, so one encounters statistical bottlenecks at this point [327]. However, continuous progress is being made in normalization issues [328,329]. Keeping this in mind, we used a commonly accepted MicroPrep package (PrePreP, PreP and PostPreP) [330]. Cyber-T was used to analyze the data generated using *Microprep* for the identification of statistically significant differentially expressed genes [331]. Consequently, the use of these pre-processing frameworks not only reduces the time for normalization of
data but also the amount of discarded data [287]. Image analysis is an important step in data normalization. MicroPrep software package takes only a couple of minutes to convert the raw signal data from image into high-quality data for further processing [330]. For further analysis of the differentially expressed genes in microarray, an in-house software package was used. These software packages include PePPER, FIVA, DISCLOSE, PROSECUTOR and Genome2D [201–203,235]. The use of these windows-based tools and software packages are user-friendly and identifies all genes that are statistically significantly differentially expressed. These software packages make it very convenient for researchers to utilize this technology, as data becomes much more meaningful and relevant.

Carbohydrates and metal ions are important environmental factors that S. pneumoniae might encounter in its natural habitat. A number of studies have been conducted to assess the impact of different carbohydrates and metal ions on the gene expression of S. pneumoniae and the role of many transcriptional regulators that are involved in the regulation of sugar- or metal -responsive systems has been characterized [152,162,170,171,179,188,190,332]. In this thesis, we have further extended the role of carbohydrates and metal ions in the regulation of gene expression in S. pneumoniae and demonstrated the impact of fucose, a carbon source, and the metal ions Mn$^{2+}$, Zn$^{2+}$, Co$^{2+}$, and Ni$^{2+}$ in the regulation of pneumococcal gene expression and characterized the transcriptional regulators FcsR, PsaR and AdcR.

**Chapter 3** aims to explore the global gene expression of S. pneumoniae D39 in the presence of fucose. The expression of various genes and operons, including the fucose uptake PTS and utilization operon (fcs operon, type-2 operon), was highly upregulated in our microarray analysis. Pneumococcal strains encode for two types of fsc operons (fucose utilization), *i.e.* the type-1 and type-2 operons [218]. The type-1 encodes ABC (ATP-binding cassette) transporters, while the type-2 operon encodes a PTS (phosphotransferase systems). Despite the fact that S. pneumoniae is unable to utilize fucose as a sole carbon source, the fsc operon is also found to be involved in virulence [189,219]. The deletion of the complete fcs operon, or the disruption of four genes (fcsK, eIIA, eIIC and gh98) of the fcs operon, compromises the bacterium's ability to cause acute respiratory disease in the mouse model [219,220]. The genome of Escherichia coli encodes for two fucose utilization operon, *i.e.* fucPIK and fucAO, which are positively regulated by the DeoR-family transcriptional regulator FucR [234]. Usually the DeoR-family transcriptional regulators are common in bacteria and they often act as transcriptional activators or repressors of sugar or nucleotide metabolism [230–232]. Moreover, DeoR-family transcriptional regulators have been shown to regulate the catabolic pathways of different sugars including lactose, fructose, ascorbate,
mannitol, glycerol and xylitol [230,233]. *S. pneumoniae* also encodes for a DeoR-family transcriptional regulator, FcsR, that has 31% sequence similarity with FucR of *E.coli*. The presence of *fcsR*, directly upstream of the *fcs* operon, suggests a putative role in the regulation of the *fcs* operon. The deletion of *fcsR* led to loss in expression directed by the *fcs* promoter, suggesting the role of FcsR as an activator of *fcs* operon. We have also predicted a 19 bps putative FcsR regulatory site in the promoter region of the *fcs* operon. The functionality of this predicted FcsR regulatory site was further confirmed by promoter truncation experiments, where deletion of full or half of the FscR regulatory site led to the abolition of expression of the *fcs* operon.

Trace metal ions are important cofactors and structural components of many proteins. They also play a vital role in the virulence of pathogenic bacteria [92,333–336]. *S. pneumoniae* encodes various metal ion-uptake and -efflux systems. Dedicated metal-dependent transcriptional regulators tightly regulate the expression of these systems. Usually, these transcriptional regulators respond to a specific metal ion, but some of them can respond to more than one. The role of Zn$^{2+}$, Mn$^{2+}$, Cd$^{2+}$ and Cu$^{2+}$ on the gene expression of *S. pneumoniae* is already studied. In this thesis, we have explored the impact of Co$^{2+}$ (Chapter 4) and Ni$^{2+}$ (Chapter 5 and 6) on the gene expression of *S. pneumoniae*.

In Chapter 4, we have investigated the transcriptional response of *S. pneumoniae* to Co$^{2+}$. The expression of several virulence genes belonging to the PsaR regulon (*psaBCA*, *pcpA* and *prtA*), the *cbi* operon (putative Co$^{2+}$ transport operon), the *nrd* operon and *czcD* (Zn$^{2+}$-efflux system), was upregulated under Co$^{2+}$ stress. The upregulation of the *cbi* genes and *czcD* in the presence of Co$^{2+}$ might indicate their function in Co$^{2+}$ homeostasis. Previously, SczA mediated expression of *PczcD* was shown to increase in the presence of Co$^{2+}$ in the undefined rich growth medium GM17 [109]. In addition, CzcD was found to contribute to resistance of cells to high concentrations of Co$^{2+}$. Our results confirm the previous findings that the Zn$^{2+}$-efflux system CzcD responds to Co$^{2+}$ and has a putative role in Co$^{2+}$-efflux (Chapter 4).

Interestingly, the genes belonging to the PsaR regulon was also highly upregulated in the presence of Co$^{2+}$ in chemically defined medium (CDM). Previously, the regulation of the PsaR regulon was studied in undefined rich growth medium (GM17). It was shown that Mn$^{2+}$/Zn$^{2+}$-dependent transcriptional regulator PsaR mediates the regulation of the PsaR regulon in the presence of Mn$^{2+}$ and Zn$^{2+}$ and no effect of Co$^{2+}$ concentrations on the expression of the PsaR regulon was observed [259]. Possibly, the presence of Co$^{2+}$-chelating compounds in the GM17 medium obscured the Co$^{2+}$-dependent de-repressive effect on the
Chapter 7

PsaR regulon. PsaR belongs to the DtxR family of proteins and has 15% sequence homology with MntR [259] that represses the expression of a Mn$^{2+}$-uptake system in *B. subtilis* [279]. MntR has ability to bind with Cd$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, Cu$^{2+}$ or Co$^{2+}$ [280–282]. Moreover, structural studies of MntR have shown that Co$^{2+}$ prevents the binding of Mn$^{2+}$ to MntR [283]. Interestingly, the metal ion binding residues of MntR (D8, E99, E102 and H103) are conserved in PsaR (D7, E99, E102, and H103) [100,259] as well.

In Chapter 4, we also investigated the transcriptional response of *nrdD* to the different concentrations of Co$^{2+}$ and Zn$^{2+}$ in CDM. The β-galactosidase activity of *PnrdD* showed that the expression of *PnrdD* is increased with the increasing concentrations of Co$^{2+}$ and Zn$^{2+}$. Ribonucleotide reductase (Nrd) is responsible for the conversion of ribonucleotides to 2'-deoxyribonucleotides, and therefore is important for DNA synthesis and DNA repair in almost all living organisms [268]. The catalytic activity of RNR enzymes has been shown to depend on different metal cofactors [337]. *S. pneumoniae* encodes two functional dNTP biosynthesis pathways, one aerobic and one anaerobic. The *nrd* operon (anaerobic ribonucleotide reductases) was highly upregulated in our transcriptome performed in the presence of Co$^{2+}$. This was shown in previous studies under Zn$^{2+}$ and Cu$^{2+}$ stress and thus suggested the inhibition of the aerobic dNTP biosynthetic pathway [259,275].

Another important trace metal ion, nickel (Ni$^{2+}$), is also considered to be an essential element for bacteria [128–130]. Very little is known about the role of Ni$^{2+}$ in *S. pneumoniae*. Ni$^{2+}$-binding proteins and motifs in *S. pneumoniae* were identified by Immobilized Metal Affinity Column (IMAC) and LTQ-Orbitrap mass spectrometry (MS) [127]. Furthermore, the Zn$^{2+}$-efflux protein CzcD has also been shown to respond to Ni$^{2+}$ availability [259]. In Chapters 5 and 6, we explore the transcriptional response of *S. pneumoniae* to high Ni$^{2+}$ concentrations. A number of genes were highly upregulated in the presence of elevated extracellular concentrations of Ni$^{2+}$. These included the AdcR regulon (*adcRCBA, adcAII* (*lmB*), *phtA, phtB, phtD, phtE*), the PsaR regulon (*psaBCA, pcpA, prtA*) and the Zn$^{2+}$/ Co$^{2+}$-efflux system czcD. The *adcRCBA* operon and *adcAII* are directly involved in Zn$^{2+}$ acquisition in *S. pneumoniae* [134,289,290]. In *S. pneumoniae* and other streptococci, inactivation of the *adc* operon leads to both a reduction in adhesion and virulence [338]. The gene *adcB* is important for virulence in STM lung infection models and for adhesion to the human lung epithelial cells [219,339]. The genes belong to the Pht protein family (*phtA, phtB, phtD* and *phtE*) are putatively involved in the virulence, and are also potential vaccine candidates [340]. The AdcR regulon was previously shown to be repressed by the transcriptional regulator AdcR in the presence of Zn$^{2+}$ [341]. In *B. subtilis*, the expression of
cadA and czcD-trkA is regulated by Zn\(^{2+}\)/ Cu\(^{2+}\)-responsive transcriptional regulator CzrA, where Cu\(^{2+}\) represses, and Zn\(^{2+}\) derepresses the expression of these genes [278]. Similarly, in *S. pneumoniae*, the expression of the *cop* operon (Cu\(^{2+}\) efflux system) is activated by Cu\(^{2+}\)-responsive regulator CopY in the presence of Cu\(^{2+}\), and repressed in the presence of Zn\(^{2+}\) [117]. On the other side, the Zn\(^{2+}\)-efflux systems have also been shown to play a role in virulence of bacteria. For example, in *Helicobacter pylori*, a human pathogen, the *cznABC* operon is involved in metal ion homeostasis and responds to Ni\(^{2+}\), Cd\(^{2+}\) and Zn\(^{2+}\) [342]. The *cznA* and *cznC* mutant strains showed higher activity of urease, a Ni\(^{2+}\)-dependent enzyme, and accumulate high intracellular levels of Ni\(^{2+}\) [342]. The β-galactosidase assays and EMSAs experiments suggested that the expression of the AdcR regulon is de-repressed by the addition of Ni\(^{2+}\). Recent studies have shown that the expression of the AdcR regulon is also de-repressed in the presence of ascorbic acid [343]. The high expression of the AdcR regulon is directly linked to Zn\(^{2+}\) starvation in treated cells caused by ascorbic acid [343]. However, the ICP-MS analysis performed in the presence of Ni\(^{2+}\) showed that the high expression of the AdcR regulon is not linked to Zn\(^{2+}\) starvation but due to the direct interaction of Ni\(^{2+}\) with AdcR.

Manganese (Mn\(^{2+}\)) is an important transition metal ion that is critical for colonization and invasive disease in *S. pneumoniae* [214,344]. Moreover, Mn\(^{2+}\) is an essential cofactor for many pneumococcal proteins that are essential for the resistance to oxidative stress, which can result from the production of hydrogen peroxide during pneumococcal metabolism [57]. Therefore, proper homeostasis of Mn\(^{2+}\) must be maintained. In *S. pneumoniae*, the PsaBCA (ATP-binding cassette transporter) operon is involved in Mn\(^{2+}\) import from the environment to the cytosol [75]. The deletion of the Mn\(^{2+}\) transporter genes *psaB* or *psaC* can lead to deficiency in pneumococcal adherence [345]. In Chapter 6, we show that the accumulation of intracellular Ni\(^{2+}\) dysregulates Mn\(^{2+}\) homeostasis. ICP-MS analysis revealed that the cell-associated accumulation of Mn\(^{2+}\) was decreased by the addition of extracellular Ni\(^{2+}\). Moreover, the expression of all genes belonging to the PsaR regulon was highly upregulated in the presence of Ni\(^{2+}\). The cell-surface lipoprotein PsaA is the only target protein that is involved in Mn\(^{2+}\)-deficiency [57,248]. PsaA is not only involved in the accumulation of cell-associated Mn\(^{2+}\), but it is also capable of binding to Mn\(^{2+}\) and Zn\(^{2+}\) [96,309]. Later, it was discovered that extracellular Zn\(^{2+}\) competitively inhibits the Mn\(^{2+}\) uptake via PsaA and compromises the oxidative stress management in *S. pneumoniae* [248,277]. Recently, the role of Cd\(^{2+}\) in the regulation of the AdcR and the PsaR regulons was described [57]. Cd\(^{2+}\) competes with Mn\(^{2+}\) to bind to PsaA, leading to the dysregulation of Mn\(^{2+}\) accumulation [57].
Chapter 7

We have proposed that high extracellular concentrations of Ni\(^{2+}\) also inhibit Mn\(^{2+}\) uptake, possibly via PsaA. **Chapter 6** also sheds light on the regulatory mechanism of the PsaR regulon. High expression of the PsaR regulon was not only linked to Mn\(^{2+}\) starvation, but the direct role of Ni\(^{2+}\) in the regulation of the PsaR regulon was also established. EMSAs results showed that the Mn\(^{2+}\)-PsaR interaction to the promoter regions of *psaB*, *pcpA* and *prtA* is diminished by Ni\(^{2+}\). Therefore, we have concluded that Ni\(^{2+}\) mimics Zn\(^{2+}\) in the regulation of the PsaR regulon and Mn\(^{2+}\) homeostasis.

The role of metal ions (Mn\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\), Fe\(^{2+}\) and Cd\(^{2+}\)) is well-understood in the regulation of metal ion uptake- and efflux-systems, which are in turn involved in virulence. Therefore, these systems could be used as potential targets for anti-infectious therapies. This could be done by developing vaccines against these systems, or by generating compounds that can inhibit them.

![Figure 1](image.png)

**Figure 1:** An overview of the work done in this study

**Concluding statements**

In conclusion, this thesis contributes to a better understanding of the metal- and carbohydrate-dependent gene regulation in *S. pneumoniae*. Thereby, it will open new avenues for further understanding the role of metal ions and carbohydrates in the pathogenesis of the pneumococcus, which eventually could lead to new strategies to combat *S. pneumoniae* for medical purposes.