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# Zinc uptake in *Streptococcus pyogenes*: characterisation of *adcA*

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# List of abbreviations

| | |
|---|---|
| **ABC** | ATP binding cassette |
| **ARF** | Acute rheumatic fever |
| **CSP** | Competence stimulating peptide |
| **DTT** | Dithiothreitol |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **GAS** | Group A Streptococcus |
| **LB** | Luria Bertani |
| **HRP** | Horse-radish peroxydase |
| **kb** | kilobase |
| **MBS** | Metal binding receptor |
| **MES** | 2-(N-morpholino)ethanesulfonic acid |
| **MHC** | Major histocompatibility complex |
| **NF** | Necrotizing fasciitis |
| **O/N** | Overnight |
| **$O.D._{600}$** | Optical density at 600 nm |
| **ORF** | Open reading frame |
| **PBS** | Phosphate buffer saline |
| **PBS-T** | Phosphate buffer saline – Tween 20 |
| **rpm** | Revolutions per minute |
| **RT** | Room temperature |
| **PCR** | Polymerase chain reaction |
| **PSGN** | Post streptococcal glomerulonephritis |
| **RBS** | Ribosome binding site |
| **SAGs** | Superantigens |
| **SBP** | Substrate-binding proteins |
| **SDS** | Sodium dodecyl sulfate |
| **SDS-PAGE** | Sodium dodecyl sulfate – Polyacrilamide gel electrophoresis |
| **SIC** | Streptococcal Inhibitor of Complement |
| **SOEing** | Splicing by overlapping extension |
| **STSS** | Severe streptococcal toxic shock syndrome |
| **TAE** | Tris acetate EDTA buffer |
| **TCR** | T-Cell receptor |
| **THY** | Todd Hewitt broth Yeast extract |
| **TPEN** | N,N,N′,N′-Tetrakis(2-pyridylmethyl) ethylenediamine |
| **TSA** | Tryptic soy agar |

# Abstract

*Streptococcus pyogenes* (also known as Group A Streptococcus, GAS) is a capsulated Gram-positive, human-adapted pathogen. GAS strains express different virulence factors exposed on the bacterial surface or secreted outside the cell. Among the secreted virulence factors, superantigens (SAGs) are certainly the most toxic factors. A recent study demonstrates that, *in vitro*, the streptococcal superantigen SpeI interacts with AdcA and Lmb, the substrate binding subunits of the two zinc transporters. In particular, AdcA belongs to the high-affinity zinc transporter Adc, involved in adhesion, competence and zinc uptake. Zinc is an essential micronutrient for all living organisms but cells have to tightly control its intracellular concentration due to its toxicity.

In this thesis the role of *adcA* in zinc uptake in GAS was studied. Three independent Δ*adcA* null mutants were generated in the strain MGAS5005, and their phenotype was characterised. The mutants were obtained by means of the low copy number temperature-sensitive shuttle vector pJRS233. Deletion of the *adcA* gene in *Streptococcus pneumoniae* leads to a dramatic decrease in competence. Thus, complementation using the pMU1328 plasmid was obtained by transforming the intermediate strain MGAS5005::pJRS233-Δ*adcA* that still contains the wild type *adcA* allele.

The absence of a zinc transporter affects the capacity to uptake zinc from the culture medium and the mutant susceptibility to zinc starvation. The Δ*adca* null mutants clearly displayed a higher sensitivity to zinc starvation compared to the wild type strain. The complementation of one of the mutants with the wild type gene restored the phenotype. When the Δ*adcA* mutant is grown in the presence of the zinc chelator TPEN, growth is rescued both by zinc or manganese. This probably means that the import of these two metals is carried out by the other zinc transporter Lmb, coded within the operon *lmb-htpA*. Hence, the expression of Lmb and HtpA was analysed by Western blot in different growth conditions. In wild type cells AdcA is always expressed at a high level, whereas Lmb and HtpA are highly expressed only in zinc-depleted medium or in Δ*adcA* mutants. This finding supports the notion that AdcA is functionally homologous to ZnuA, the major high affinity zinc transporter in many bacteria, and like ZnuA is responsible for the efficient recruitment of zinc in most conditions.

# Riassunto

*Streptococcus pyogenes* (detto anche Streptococco di gruppo A, GAS) è un batterio Gram-positivo capsulato, adattato ad infettare l'uomo. I ceppi di GAS esprimono diversi fattori di virulenza che possono essere esposti sulla superficie esterna o secreti fuori dalla cellula. Tra i fattori di virulenza, i Superantigeni (SAGs) sono sicuramente tra i più nocivi per l'ospite. Uno studio recente ha dimostrato che *in vitro* SpeI, un superantigene secreto da GAS, interagisce con AdcA a Lmb, due proteine che trasportano lo zinco. In particolare, AdcA appartiene al trasportatore ad alta affinità Adc, che è coinvolto nell'adesione, nella competenza e nel trasporto dello zinco. Questo metallo è un microelemento essenziale per tutti gli organismi viventi, ma poiché possiede una elevata tossicità, le cellule devono regolare finemente la sua concentrazione intracellulare.

In questo lavoro di tesi è stato studiato il ruolo di AdcA nel trasporto dello zinco in GAS. Per questo studio sono stati generati tre mutanti indipendenti nel ceppo MGAS5005, caratterizzandone il loro fenotipo.

I mutanti sono stati ottenuti mediante l'uso del vettore termosensibile pJRS233. La delezione del gene *adcA* in *Streptococcus pneumoniae* comporta una notevole diminuzione della competenza, quindi la complementazione è stata ottenuta trasformando il mutante parziale, cioè l' "eterozigote" intermedio che contiene sia l'allele *wild type* che quello mutato. L'assenza del trasportatore dello zinco influisce sulla capacità di importare lo zinco dal terreno di coltura e sulla suscettibilità alla mancanza di zinco. I mutanti Δ*adcA* mostrano chiaramente una maggiore sensibilità alla deprivazione di zinco se comparati con il ceppo *wild type*. La complementazione di uno dei mutanti riporta il fenotipo del mutante a quello del ceppo *wild type*. Quando il mutante è cresciuto in presenza di una concentrazione inibente del chelante TPEN, la crescita è ristabilita dalla aggiunta di zinco o di manganese. Questo probabilmente significa che il trasporto di questi due metalli può avvenire tramite l'altro trasportatore Lmb, codificato all'interno dell'operone *lmb-htpA*. Di conseguenza, l'espressione di Lmb e HtpA è stata analizzata tramite Western blot in condizioni di crescita diverse. Nelle cellule *wild type* AdcA è sempre espresso ad alti livelli, invece Lmb e HtpA sono espresse ad alti livelli solo nel terreno di coltura depleto di zinco. Questo risultato avvalora il concetto che AdcA è omologo da un punto di vista funzionale a ZnuA, il maggior trasportatore ad alta affinità dello zinco in molti batteri, e come ZnuA ha il compito del reclutamento dello zinco in molte condizioni.

# Introduction

## *Streptococcus pyogenes*: general features

*Streptococcus pyogenes* (also known as Group A Streptococcus, GAS) is a capsulated Gram-positive, facultative anaerobe, non-motile and non-spore forming bacterium. GAS cells possess a β-haemolytic activity. In fact, if grown on a blood-containing agar plate, the area surrounding the colonies shows a clear haemolysis due to the expression of Streptolysin S and O, which cause the lysis of erythrocytes. This microorganism is a human-adapted pathogen and there are no animals or environmental reservoirs that contribute to its life cycle (Musser and Shelburne, III, 2009a). GAS infects each year approximately 600-700 millions of children and adults with a mortality rate lower than 0.1% (Carapetis *et al.*, 2005a; Musser and Shelburne, III, 2009b), while it is commonly present in the respiratory tract of up to 15 % of normal individuals without evident clinical manifestations.

From a clinical viewpoint, GAS leads to several different acute infections ranging from mild pharyngitis and/or tonsillitis (strep-throat) to severe streptococcal toxic shock syndrome (STSS), acute rheumatic fever (ARF), post streptococcal glomerulonephritis (PSGN), invasive necrotizing fasciitis (NF) or skin infections (impetigo, erysipelas and cellulitis)(Musser and Shelburne, III, 2009b; Olsen *et al.*, 2009). However, the incidence of severe invasive GAS diseases is approximately 600,000 cases each year (Carapetis *et al.*, 2005b; Cole *et al.*, 2011), indicating that the most common forms of streptococcal infection are non-invasive and non-life threatening.

The classification of species belonging to the genus *Streptococcus* is historically based on the presence of a surface exposed polymeric carbohydrate that in the case of Group A is composed of N-acetylglucosamine and rhamnose, also called C substance. Among the GAS species, strains are classified by a serological classification, the *emm*-typing. The M protein is an antigenic and highly variable virulence factor. More than 200 *emm* sequence types have been identified to date (Cole *et al.*, 2011) and this variability is one of the causes of the high antigenic variability of the pathogen. The M1 strains are the most common serotypes from an epidemiological viewpoint, because they are usually isolated from patients with streptococcal pharyngitis and invasive infections.

GAS strains express different virulence factors in order to evade the immune system of the host and to allow the colonization and spread of the pathogen. These factors are exposed on the bacterial surface or secreted outside the cell. A schematic representation of the main virulence factors is indicated in Fig. 1 (Mitchell, 2003).

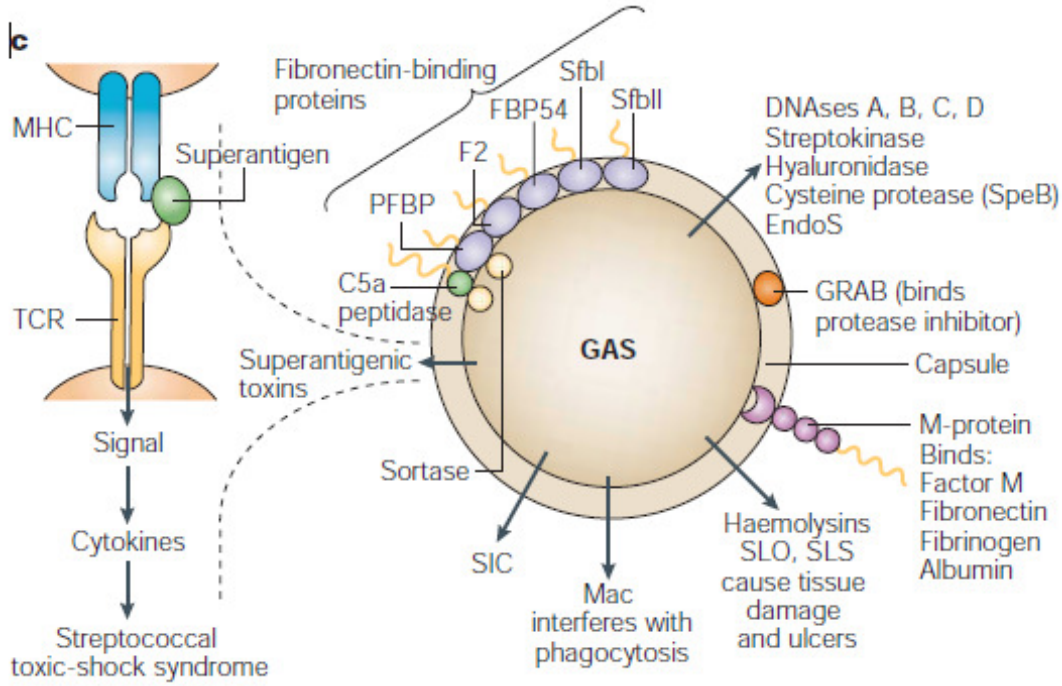



**Figure 1. The main *S. pyogenes* virulence factors.** Group A Streptococcus is often termed as the most versatile of the streptococcal pathogens and this is reflected in the huge array of virulence factors that it produces: These allow evasion of the host immune response and cause tissue damage. Toxins and tissue-degrading enzymes may have an important role in the severe diseases that are associated with GAS infection, such as necrotizing fasciitis. GAS also secretes superantigen toxins which stimulate T cells proliferation and cytokine production, and are associated with streptococcal toxic-shock-syndrome, and the cytotoxins streptolysin O (SLO) and streptolysin S (SLS) (Mitchell *et al.*, 2003 modified**).**

Although not all the *emm*-types produce the same virulence factors, most of them are shared by the all the serotypes. The Streptococcal Inhibitor of Complement (SIC), an exotoxin that is involved in immune evasion and inhibits the lysis of the bacterium, is expressed only in the M1 strains (Bisno *et al.*, 2003).

Among the secreted virulence factors, superantigens (SAGs) are certainly the most toxic factors in addition to being widespread in all serotypes. They are secreted pyrogenic toxins that increase the host susceptibility to the endotoxic shock and, in particular, can cause a mitogenic activation of specific T-cells subsets (Commons *et al.*, 2008). The pathogen secrets these proteins in order to cause a hyperactivation of the immune

system and evade it. Superantigens bind simultaneously to the major histocompatibility complex (MHC) class II molecules and to the Vβ chain of the T-Cell Receptor (TCR). This results in proliferation of the T cells and the production of cytokines. Several of the bacterial SAGs are dependent on zinc ions to be able to properly bind MHC class II. Crystal structures of some of these SAGs in complex with MHC class II show that zinc is important for the SAG to interact with MHC class II with high affinity. In addition, the zinc ion is important for the three-dimensional stability of the SAG itself (Petersson *et al.*, 2004). A recent study demonstrates that, *in vitro*, the streptococcal superantigen SpeI interacts with the substrate binding subunit of the two zinc transporters of *S. pyogenes*. Binding occurs in the presence of zinc ions and is disrupted by the addition of EDTA (Galeotti *et al.*, 2011). This finding is of great interest since acquisition of zinc ions is a key step for dimer formation and activity of superantigens.

# The bacterial ABC transporters

The ATP Binding Cassette (ABC) transporters belong to one of the largest superfamilies of proteins known (Davidson *et al.*, 2008a; Davidson and Chen, 2004) and are present in organisms from all kingdoms of life. In *E. coli*, for instance, approximately 80 different systems (5 % of the genome), have been described while approximately 50 ABC transporters hve been identified in humans (Rees *et al.*, 2009).

ABC transporters set the translocation of different solutes across membranes against a concentration gradient to the hydrolysis of ATP and are involved in many essential processes, such as uptake of metals or pathogenesis. They are divided into three main functional categories: a) importers, b) exporters and c) involved in mRNA translation and DNA repair, although the latter is not clearly involved in transport (Davidson *et al.*, 2008b).

The ABC transporter system is a common strategy used by bacteria for the acquisition of transition metals. Their genetic organisation is minimally comprised of three subunits encoded by different genes. Very commonly, the three genes are co-transcribed as an operon (Klein and Lewinson, 2011; Mitchell, 2003). A bacterial ABC transporter is composed of distinct domains (see Fig. 2):

- two transmembrane domains (TMD);
- two nucleotide-binding domains (NBD or ABC).

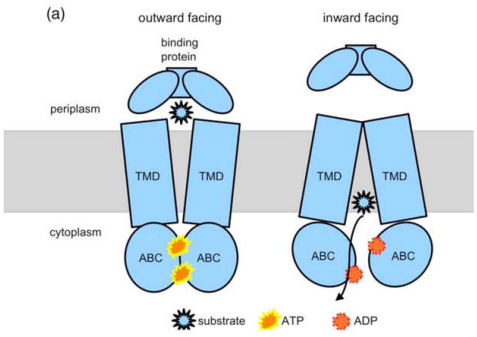



**Figure 2: Molecular architecture of ABC transporters.** A cartoon of the modular organisation of the ATP binding cassette (ABC) transporters (Rees *et al.*, 2003, modified).

In addition to these conserved domains, most prokaryotic ABC transporters involved in solute uptake employ a specific ligand-binding protein to capture the substrate (van der Heide and Poolman, 2002), named Substrate-Binding Proteins (SBPs) or Metal Binding Receptor (MBR) if this protein is involved in metal trafficking. In Gram-negative organisms, the SBPs are soluble proteins located in the periplasmic space. In Gram-positive bacteria, which lack the periplasm, the SBPs are often lipoprotein anchored to

the outer surface of the cell membrane via an N-terminal anchor moiety (van der Heide and Poolman, 2002). The SBPs are the components which confer the metal or the substrate specificity to the entire ABC transporter. This is the case in particular for the essential transition metals (zinc, manganese, iron, nickel and cobalt).

To date, many ABC transporters of bacteria, in particular of Gram-negative species, have been identified and characterised. Nearly all the systems studied are importers for peptides, metals or sugars, such as mannose. Although the main structures are similar, the specificities are different. The specificity and functional properties of a particular transporter are usually elucidated by generating the corresponding mutants. In addition, for pathogenic bacteria often the characterisation of a tranporter includes an analysis of virulence in the absence of the transporter. In *Pseudomonas.aeruginosa*, it has been reported that mutations in the *cueA* gene (coding for a copper exporter) results in a 20-fold attenuation of virulence (Schwan *et al.*, 2005). In *Mycobacterium tuberculosis*, the Δ*ctpV* (copper efflux) mutant showed a significantly reduced ability to infect the lungs in mice (Ward *et al.*, 2010). The deletion of genes whose products are components of ABC importers, in several cases has a dramatic effect and generates almost avirulent strains, such as *sfaABC* for iron uptake in *Salmonella enterica* (Pattery *et al.*, 1999) or *yfeABCD* for iron/manganese uptake in *Yersinia pestis*.

Bioinformatic analysis has revealed that the genome of S. *pyogenes* strain SF370 codes for 36 ABC transporters (Ferretti *et al.*, 2001). However, only a few have been characterised in the literature. These are involved in trafficking of metals, peptides and sugars. The crystal structure of the transporter Lbp, for example, revealed a binding site specific for zinc (Linke *et al.*, 2009) suggesting the involvement of this protein in zinc acquisition and homeostasis.

# The *adc* operon in the genus *Streptococcus*

The *adc* operon was first identified in *Streptococcus pneumoniae* in 1997 by Dintilhac and Claverys and proposed to be involved in competence acquisition for the uptake of exogenous DNA. The operon was discovered due to its genetic organisation, typical of the bacterial ATP binding cassette transport operons and to its homology to other ABC transporters, such as FimA or PsaA of pneumococcus (Dintilhac and Claverys, 1997). The name *adc* was assigned because of the similarity with several streptococcal adhesins and its involvement in the competence process (*ad*hesin *c*ompetence).
In the genus *Streptococcus* the operon is organised into at least 3 ORFs, *adcR*, *adcC* and *adcB*. In some species, such as *S. pneumoniae* and *Streptococcus gordonii,* another ORF, *adcA,* is located downstream of *adcB*. In contrast, in other species, such as *S. pyogenes* (Fig. 3) or *Streptococcus mutans,* this ORF is present at a distant genomic locus.

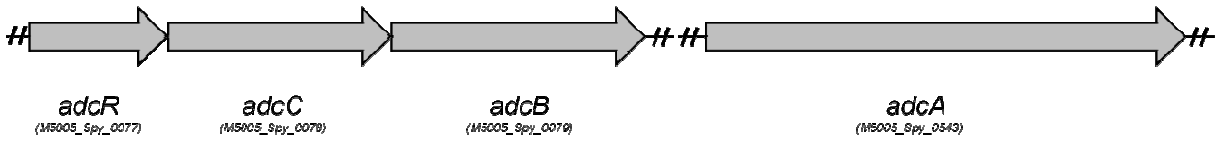



**Figure 3**

**Genetic organisation of the *adc* operon in *S. pyogenes* strain MGAS5005.**

The three shared ORFs of all the *adc* streptococcal loci are *adcR*, *adcC* and *adcB* whose products are, respectively, the regulator (repressor), the ATP binding protein and the permease, consistent with a typical genetic organisation of a bacterial ABC transporter. The *adcA* ORF, not present in all the streptococcal species downstream of the operon, codes for the substrate binding protein. At present, only AdcR has been studied in GAS and there are no reports in the literature on the characterisation of the other components of the operon. However, several studies have focused on the involvement of the Adc proteins in metal trafficking or in adherence in other streptococci. The *adc* operon has been analysed in different streptococcal species. In *S. gordonii* it has been associated with manganese homeostasis and biofilm formation. It was shown by Loo *et al.*, that a high level of extracellular manganese (10 mM) leads to a higher expression of AdcR (as a fusion *adcR*::*lacZ*) (Loo *et al.*, 2003).

AdcR belongs to the MarR family of transcriptional regulators. It binds to the genes containing the AdcR-binding motif. In GAS the motif is TTAACYRGTTAA and, interestingly, this palindrome occurs twice in the region upstream of *adcA* (Panina *et al.*, 2003). Guerra et al., showed that the binding of $Zn^{2+}$ stabilised the dimeric form of AdcR, making it suitable for DNA binding (Guerra *et al.*, 2011). Several reports have focused on the involvement of AdcR in zinc uptake. In *S.pneumoniae* the Δ*adcR* mutant shows, in the presence of external high zinc concentration, a higher capability to uptake zinc from the external milieu with respect to the wild type., Thus, in this environmental condition the import of zinc through the Adc proteins is partially repressed (Jacobsen *et al.*, 2011). Deletion of *adcR* in *S. pyogenes* has been very helpful for the identification of its activity as a repressor. In fact, the Δ*adcR* mutant displays a higher relative abundance of transcripts of six genes that possess an AdcR-binding motif with respect to the wild type strain (Brenot *et al.*, 2007).

Although there are no specific reports on AdcC and AdcB function and regulation, Bayle et al., have shown that the pneumococcus mutant of *adcB* is more susceptible to zinc starvation than the wild type. Their work indicates that the permease AdcB is shared by the two zinc transporters AdcA and AdcAII (the homologue of Lmb in GAS), since the double mutant Δ*adcA*/*adcAII* shows the same sensitivity to zinc starvation (Bayle *et al.*, 2011).

AdcA proteins belong to the cluster IX family of ABC transporters, a family of external solute-binding transporters, as these proteins do not clearly belong to any of the other eight families (Claverys, 2001). The contribution of AdcA in metal import is evident also in the annotation of this protein in *S. pyogenes* strain MGAS5005, where it is named as the ZnuA precursor, by analogy with the periplasmic ZnuA protein, a subunit of the high affinity zinc transporter described in Gram-negative bacteria such as *E. coli* (Patzer and Hantke, 1998) or *Campylobacter jejuni* (Davis *et al.*, 2009).

# Zinc acquisition and homeostasis in bacteria

Zinc is an essential micronutrient for all living organisms as it is the cofactor of many enzymes, of some ribosomal proteins and of DNA and RNA polymerases. It has been estimated that in *E. coli* nearly 100 proteins bind zinc (Ma *et al.*, 2009). Moreover, cells have to tightly control the intracellular zinc concentration due to its toxicity. This metal in fact can bind to the thiol groups of proteins, generating a misfolded product, unable to carry out its biological function (Hantke, 2005). The storage of zinc and the regulation of a correct import/export are components responsible for the surveillance of the cytoplasmic concentration of zinc. In *E. coli,* for example, the concentration sufficient to stimulate *in vitro* transcription of genes coding for the proteins involved in zinc trafficking is femtomolar. This observation leads to the hypothesis that intracellular proteins have a considerable zinc binding capacity and there is stringent control of its import/export (Outten and O'Halloran, 2001). An example of how bacteria could store zinc ions was studied in *Bacillus subtilis*. This microorganism expresses two paralogues of a 50S ribosomal protein, RpmE and YtiA. Zinc homeostasis in *B. subtilis* is regulated by Zur (Zinc Uptake Regulator), a transcriptional factor that after binding zinc represses the expression of several genes involved in zinc homeostasis. In the presence of a physiological zinc concentration, the expression of YtiA is repressed by Zur and, at the same time, the binding of zinc to RpmE stabilises this ribosomal protein. Conversely, under zinc limiting conditions Zur is inactive and allows the expression of YtiA, while RpmE becomes unstable due to the loss of zinc (Nanamiya *et al.*, 2004). This mechanism makes available a low amount of zinc to other metalloproteins when there is no free zinc in the cell.

Among the streptococcal species, pneumococcus is the best characterised for zinc homeostasis (see Fig 4). In *S. pneumoniae* zinc homeostasis is regulated by two proteins, AdcR and SczA, which control, respectively, the import and the export of zinc ions. The regulator protein AdcR binds to the regulatory regions that possess the specific binding motif and the dimerisation (necessary for the binding) is mediated by zinc. The recognition between AdcR and the regulatory regions leads to the repression of the genes regulated downstream, generally involved in zinc uptake such as AdcCBA or AdcAII-PhtD or the activation of the zinc-dependent alcohol dehydrogenase (Adh). Indeed, a transcriptome analysis in the presence of 0.2 mM $Zn^{2+}$ of the *adcR* mutant revealed a higher abundance of the transcripts of the *adc* operon and a lower abundance of the *adh* transcript compared to the wild type strain (Shafeeq *et al.*, 2011).

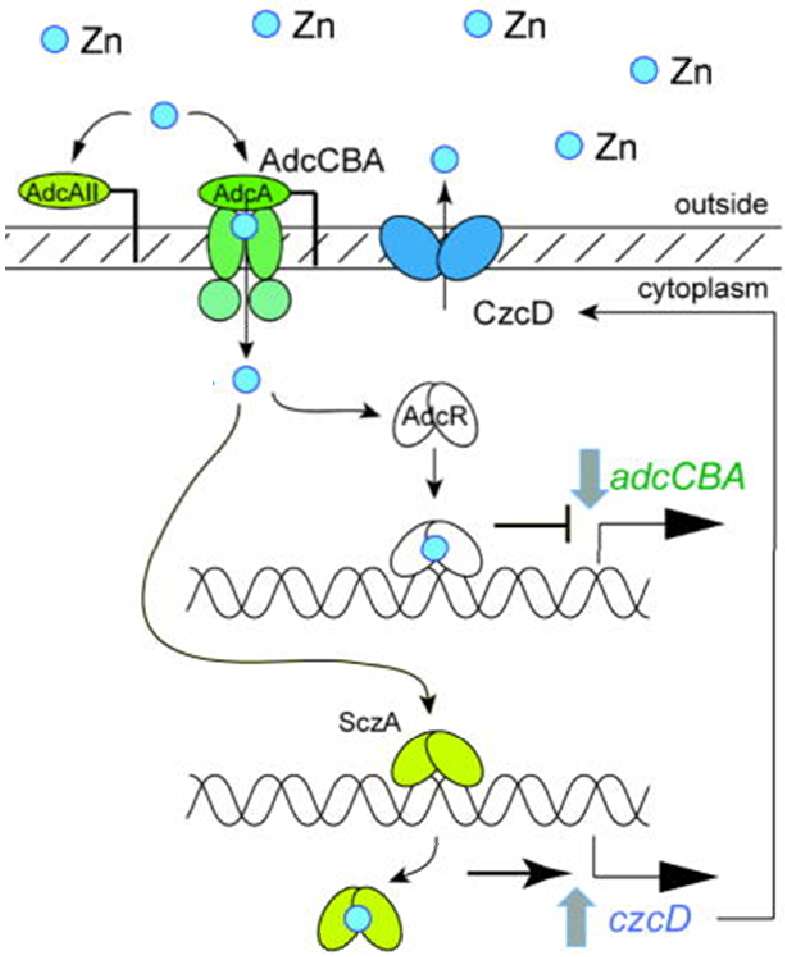



**Figure 4: Schematic representation of Zn homeostasis in *S.pneumoniae*.** (Jacobsen *et al.*, 2011 modified).

To date, the export of zinc in *S. pneumoniae* is known to be mediated only by CzcD, a cation efflux transporter which contributes to the heavy metals resistance of several bacteria. In the Gram-negative bacterium *Ralstonia metallidurans*, a microorganism which can survive at toxic concentrations of heavy metal ions, a higher expression of the heavy metal resistance genes *czcCBA* results from the deletion of *czcD* (Grosse *et al.*, 2004). The expression of *czcD* in *S.pneumoniae* protects the bacterium from the toxicity of a high intracellular zinc concentration. The gene product CzcD is positively regulated by SczA (Streptococcal czcD Activator), as shown by the binding of the regulator to the operator sequence of the *czcD* gene promoter. Its nucleotide sequence is conserved among several streptococcal species, including GAS strain MGAS5005. It was demonstrated also that the binding of SczA to the *czcD* operator is $Zn^{2+}$-dependent and binding of zinc is required for activating transcription of *czcD* (Kloosterman *et al.*, 2007).

# Aim of the project and experimental approaches

The ability of bacteria to adapt to the different host tissues depends also on their capacities to regulate the import and then the intracellular homeostasis of metal ions. The uptake, the first step to control the intracellular concentration, relies mainly on the availability of metals in the extracellular milieu. The concentration of metals in fact varies throughout the human anatomical districts. The concentration of zinc, for instance, ranges from 5 µM in the nasopharynx to 300 µM in the lung. Thus, bacteria should have different mechanisms to acquire zinc ions.

The most studied zinc-uptake system is ZnuABC of the Gram-negative bacteria, such as *E. coli* or *Salmonella spp*. However, less is known on the Gram-positive orthologue protein complexes. In streptococcal species, for example, the most studied is the Adc import system of pneumococcus, although only a few reports are present in the literature on the import of zinc in *Streptococcus pyogenes*. In this thesis the role of *adcA* in zinc uptake in GAS was studied. In particular, the aim of this work is: 1) the characterisation of zinc uptake in knock-out mutants of *adcA*, the substrate binding subunit of the high-affinity zinc transporter Adc, and 2) the analysis of the expression of proteins involved in zinc homeostasis.

In order to achieve these objectives, three independent null mutants of *adcA* were constructed in the GAS strain MGAS5005 and their phenotype was characterised. In particular, this work is focused on the analysis of their susceptibility to zinc starvation. Furthermore, the level of expression of the other zinc transporter Lmb and of the histidine triad protein HtpA were assayed in the Δ*adcA* mutant.

# Materials and Methods

## 1) Bacterial strains and growth conditions

GAS strain MGAS5005 (serotype *emm1*, ATCC cod. BAA-947) was used for all the experiments of this work. The strain was grown in liquid Todd Hewitt Broth (Difco Laboratories) supplemented with 5 % (w/v) Yeast extract (Difco Laboratories) (THY) or onto plates containing Tryptic Soy Agar (Difco Laboratories) (TSA) with 5% (v/v) of defibrinated ram blood in a water-jacketed incubator at 30°C or 37°C with 5% of $CO_2$. Chemically competent *E. coli* strains DH10B (Invitrogen) carrying pJRS233-Δ*adcA* and HB101 (Promega) carrying pMU1328-*adcA* were grown in Luria Bertani (LB) broth containing 200 µg/ml or 100 µg/ml of erythromycin (Sigma Aldrich) respectively, in a water-jacketed incubator at 37°C with 5 % of $CO_2$.

## 2) DNA techniques

### 2.1) Cloning of Δ*adcA* into pJRS233

In order to obtain a null mutant of *adcA*, 1 kb of the two flanking regions of *adcA*, using the genomic DNA of GAS strain SF370 as a template, were amplified by means of two sets of primers: P1for*Xho*I_*adcA* + P2rev_*adcA* to amplify 1 kb upstream of *adcA* (using the program A for amplification, see *Addendum*) and P3for_*adcA* + P4rev*BamH*I_*adcA* (program A) to amplify 1 kb downstream to *adcA*. The two amplicons were then joined through a SOEing PCR by using the set of primers P1for*Xho*I_*adcA* + P4rev*Bam*HI_*adcA* (program B) to obtain the Δ*adcA* PCR fragment. All the primers are used at 50 µM concentration and the amplification was performed with 1.25 U of the Pwo DNA polymerase (Roche) in a final volume of 100 µ. The 2 kb product as well as pJRS233 was digested with 20 U of XhoI (New England Biolabs) and 20 U of BamHI (New England Biolabs) in the presence of TA buffer (33 mM Tris-acetate pH 7.8, 66 mM potassium acetate 10 mM magnesium acetate, 0.5 mM DTT)(O'Farrell *et al.*, 1980) and 1 mM spermidine.

The ligation reaction between pJRS233/*BamH*I/*Xho*I and Δ*adcA/BamH*I/*Xho*I was performed at 16°C O/N using 20 U of the T4 DNA ligase (New England Biolabs), with a fragment:insertion vector molar ratio of 5:1, in a final volume of 20 µl (100 ng of total DNA). The ligation solution was then transformed into the chemically competent cells of *E. coli* strain DH10B (Invitrogen) following the manufacturer's instructions and the

bacteria plated onto LB + 200 µg/ml erythromycin and incubated at 37°C for selection of positive clones. The crude lysates were obtained by boiling a 1 µl-loop full of bacteria in 50 µl of $H_2O$ for 10 min at 99°C. One microliter of crude lysate was added as template to 14 µl of the PCR mastermix containing the primers P5for_*adcA* and P6rev_*adcA*, (both at 50 µM) and 0.37 U of ExTaq DNA polymerase (Takara) (program C).

## 2.2) Cloning of *adcA* into pMU1328

The plasmid vector pMU1328 was used in order to complement the null-mutant MGAS5005 Δ*adcA*. The genomic region was amplified from the position -110 downstream to the *adcA* ribosome binding site (RBS) to the *adcA* terminator from the genomic DNA of MGAS5005 by using the set of primers 5'*adcA*_Bam_for and 3'*adcA*_Sal_rev at 50 µM. The amplification was performed with 1.25 U of the Pwo DNA polymerase (Roche) in a final volume of 100 µl (program D). This DNA polymerase generates a PCR product blunt. A SmaI (New England Biolabs) blunt digestion of the plasmid DNA pMU1328 was carried out to linearise the vector. The ligation reaction at 16°C O/N using 20 U of the T4 DNA ligase (New England Biolabs), was performed with a fragment:vector molar ratio of 5:1, (1 µg of total DNA) in a final volume of 20 µl. The ligation solution was then transformed into the chemically competent cells of *E. coli* strain HB101 (Promega) following the manufacturer's instructions and the bacteria plated onto LB + 100 µg/ml erythromycin for selection of positive clones. For colony sceening, crude lysates were obtained by boiling a small loopfull of bacteria in 50 µl of $H_2O$ for 10 min at 99 °C. One microliter of crude lysate was added as template to 14 µl of the PCR mastermix containing the primers pMU1328-for and pMU1328-rev, (both at 50 µM) and 0.37 U of ExTaq DNA polymerase (Takara) (program E).

## 2.3) Extraction of DNA

Genomic DNA from GAS strains was isolated using a NucleoBond AX-G kit (Macherey-Nagel) following the manufacturer's instructions from an O/N bacterial culture of 4 ml.
The plasmid pJRS233 DNA from *E.coli* was extracted with a standard phenol/chloroform method as follows. 100 ml of an O/N culture of HB101/pJRS233 grown in LB + 200 µg/ml erythromycin was harvested by centrifugation at 3,000 x *g* for 10 min at 4°C. The pellet was then resuspended with Solution A (50 mM glucose, 100 mM EDTA, 25 mM Tris-HCl pH 8, 4 mg/ml lysozyme) and incubated at RT for 5 min. After adding 500 µl of Solution B (0.2 N NaOH, 1 % SDS), the suspension was gently mixed by inversion (to avoid the sharing of chromosomal DNA) 3 times and incubated on ice for 5 min. 375 µl of cold

Solution C (3 M $CH_3COO^-K^+$, 5 M $CH_3COOH$) were added to precipitate lipids, membranes and salts and the solution was vortexed and incubated on ice for 5-30 min. After a centrifugation step (13,000 rpm, 4°C 5 mintues in a benchtop centrifuge), the supernatant was transferred to a new Eppendorf tube to which was added the same volume of phenol (Sigma Aldrich), vortexed for 30 seconds and centrifuged at RT for 5 min at 13,000 rpm. The upper phase was transferred to a new Eppendorf tube and a same volume of chloroform:isoamylalchol (24:1, Sigma Aldrich) was added, vortexed for 30 seconds and centrifuged at RT for 5 min at 13,000 rpm. The upper phase was transferred to a new Eppendorf tube and at least 2 volumes of cold ethanol were added and the solution mixed by vortexing. The solution was then stored at -20°C for at least 30 min to allow the precipitation of DNA. After a centrifugation step (13,000 rpm, 15 min 4°C) the pellet was incubated with 50 µl of mQ containing 50 µg/ml of RNAse A for 30 min at 37°C in a water bath. The plasmid solution was then checked and quantified onto a 0.8 % agarose/TAE gel.

Two different kits were used for the other plasmids extraction, Wizard®Plus MaxiPreps kit (Promega) for pJRS233-Δ*adcA* and ChargeSwitch®-Pro Filter Midiprep kit (Invitrogen, based on positively charged membranes) for pMU1328 and pMU1328-*adcA*. All these procedures were performed accordingly to manufacturer's instructions.

## 3) Preparation of GAS competent cells and electroporation

Two procedures from the literature were used to induce competence in *Streptococcus pyogenes* strain MGAS5005: 1) (Sitkiewicz and Musser, 2006) for transformation with plasmid pJRS233-Δ*adcA* and 2) procedure adapted from (Kimoto and Taketo, 2003) for transformation with plasmids pMU1328 or pMU1328-*adcA*.

### 3.1) Procedure 1 (Sitkiewicz and Musser, 2006)

A glycerol stock of MGAS5005 (500µl) was inoculated into 10 ml of THY containing 250 mM sucrose and 40 mM threonine and incubated O/N at 37°C. The overnight culture was diluted into 100 ml (final volume) of THY containing 250 mM Sucrose (Sigma Aldrich) and 40 mM Threonine (Sigma Aldrich) and incubated at 37°C until $O.D._{600}$ reached 0.2. The culture was then centrifuged at 3,000 x *g* at 4°C. The pellet was then washed twice with 0.5 M sucrose and resuspended with 0.5 M sucrose in 20 % glycerol. After adding the DNA sample to 100 µl of culture suspension, the bacteria-DNA mix was incubated on ice for 30 min into a 0.1 cm electroporation cuvette and then subjected to

electroporation (1.8 kV, 400 Ω, 25 µF). The cuvette was then incubated 5 min on ice and, after adding 900 µl of THY containing 250 mM sucrose and 20 % glycerol, the bacteria were incubated at 30 °C for 2 hours, then plated onto TSA + 5 % sheep blood + 0.5 µg/ml erythromycin and incubated at 30 °C.

### 3.2) Procedure 2 (Kimoto and Taketo, 2003)

A glycerol stock of MGAS5005 (500µl) was inoculated into 10 ml of THY and incubated O/N at 37°C. Three ml of the overnight culture was diluted into 100 ml (final volume) of THY and incubated at 37°C until $O.D._{600}$ reached 0.25. The culture was then centrifuged at 4,500 x *g* at 4°C. The pellet was washed 4 times with 10 % glycerol and the bacteria were resuspended with 1 ml of a 10 % glycerol solution. After adding the DNA to 50 µl of bacterial suspension, the bacteria-DNA mix was incubated on ice for 30 min into a 0.2 cm electroporation cuvette and then subjected to electroporation (2.5 kV, 600 Ω, 25 µF). The cuvette was then incubated 5 min on ice and, after adding 950 µl of THY, the bacteria were incubated at 37 °C for 2 hours, plated onto TSA + 5 % sheep blood + 1 µg/ml erythromycin and incubated at 30 °C.

## 4) Generation of ΔadcA null mutants and complementation

The generation of MGAS5005Δ*adcA* mutants was performed as described by Perez-Casal, 1993 (Perez-Casal *et al.*, 1993). After transformation with plasmid pJRS233-Δ*adcA*, the GAS erythromycin resistant colonies were plated again onto TSA + 5 % sheep blood + 1 µg/ml erythromycin and incubated at 30°C O/N. Colonies that had grown were checked by colony screening as follows.

A crude lysate was obtained by boiling a small loopfull of bacteria resuspended into 50 µl $H_2O$ for 10 min at 99°C. One microliter of crude lysate was added as a template to 14 µl of the PCR mastermix containing the primers P5for_*adcA* and P6rev_*adcA*, (both at 50 µM) and 0.37 U of ExTaq DNA polymerase (Takara) (program C). Positive clones were then inoculated into 3 ml of THY containing 1 µg/ml erythromycin and incubated at 37 °C O/N for the integration of pJRS233-Δ*adcA* into the chromosome, generating the "intermediate" strain MGAS5005::pJRS233Δ*adcA*. The integrant strains were re-plated 4 consecutive times onto THY-agar with and without the erythromycin selection, in order to allow the excision of the plasmid from the chromosome, resulting in an *adcA* deletion.

The complemented strain MGAS5005ΔadcA/pMU1328-adcA (as well as the mock MGAS5005ΔadcA/pMU1328) was generated by transforming the "intermediate" strain. Excision of the chromosomal integrated plasmid pJRS233-adcA was obtained by re-plating onto THY-agar with and without erythromycin selection, in order to allow the excision of the plasmid from the chromosome, resulting in deletion of *adcA*. The positive clones were screened by PCR. The crude lysates were obtained by boiling a small loopfull of bacteria in 50 µl of $H_2O$ for 10 min at 99°C. One microliter of crude lysate was added as a template to 14 µl of the PCR mastermix containing the primers pMU1328-for and pMU1328-rev, (both at 50 µM) and 0.37 U of ExTaq DNA polymerase (Takara) (program E).

# 5) Proteins analysis

## 5.1) Total cellular proteins extraction

A glycerol stock (0.5 ml) of GAS cells is inoculated into the suitable medium and grown until $O.D._{600}$ reached 0.4. The culture is centrifuged for 5 min at 3,000 x *g* at 4°C in a swinging bucket rotor centrifuge. The bacterial pellet was resuspended in 1 ml of PBS and transferred into a 1.5 ml eppendorf tube. After 5 min at 6,000 rpm in a bench-top centrifuge, the pellet was resuspended with 500 µl of 10 mM Tris-HCl pH 8 containing 200 U/ml of Mutanolysin (Sigma Aldrich) and 2 mg/ml of Lysozyme (Sigma Aldrich) and incubated 1 hour at 37 °C with shaking. After centrifugation for 5 min at 13,000 rpm in a bench-top centrifuge, the pellet was resuspended in 150 µl of 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 2% SDS and vortexed for 30 seconds and added 50 µl of 4X LDS sample buffer (Invitrogen) and 10 µl of 20x Reducing agent (Invitrogen). The samples were then boiled at 99°C for 5 min and stored at -20 °C until needed.

The total cell extracts were checked by SDS-PAGE, by using 1-mm 12-wells 4-12% Novex Bis-Tris NuPAGE pre-casted gels (Invitrogen) in a MES buffer (Invitrogen). Four µl (≈ 10 µg) of each total cell extract were loaded, separated at 200 V for 35 min, washed 3 times (10 min each) with mQ $H_2O$, stained with SimplyBlue SafeStain (Invitrogen) O/N at RT with shacking and destained with mQ $H_2O$.

## 5.2) Proteins expression analysis

The expression of proteins was analysed by Western Blot of total cell extracts using specific antisera. Two µl (≈ 5 µg) of each total cell extracts were loaded onto 1-mm 12-wells 4-12% Novex Bis-Tris NuPAGE pre-casted gels (Invitrogen), separated at 200 V for 35 min and transferred onto nitrocellulose membranes using the dry system iBlot (Invitrogen). The membranes were saturated with 10 % skimmed milk (Difco) in 0.05 % PBS-T and incubated for 1 h at RT under gentle agitation. The blocked membranes were then incubated with the specific antiserum diluted in 1% skimmed milk PBS-T (1:10,000 for rabbit anti-AdcA, 1:3,000 for mouse anti-Lmb and mouse anti-HtpA) and incubated 1 hour at RT. The membranes were washed with PBS-T once for 15 min and twice for 5 min. The specific secondary HRP-labeled antibodies were then added, diluted 1:20,000 in PBS-T and incubated for 40 min under gentle agitation. After one wash of 15 min and 3 washes of 5 min with PBS-T, the membranes were overlaid with the substrate SuperSignal WestPico (Pierce) solution and incubated for 5 min at RT under gentle agitation. The substrate in excess was removed with a paper towel and the membrane exposed to a radiographic film.

## 6) Metal chelation growth yield assays

In order to test the sensitivity of MGAS5005 strains to zinc starvation, the wild type, the mutant and the complemented strains were grown in the presence of the zinc chelating agent TPEN, as described by Weston *et al.*(Weston *et al.*, 2009).

The strains MGAS5005 w.t., MGAS5005 Δ*adcA,* MGAS5005 w.t./pMU1328, MGAS5005 Δ*adcA*/pMU1328 and MGAS5005 Δ*adcA*/pMU1328-*adcA* were plated for single colonies onto TSA + 5 % sheep blood with or without 1 µg/ml erythromycin and incubated O/N at 37°C. A single colony was inoculated into 3 ml of THY and incubated O/N at 37°C. The bacterial culture was then diluted 1:1,000 into THY containing the appropriate concentration of TPEN (Sigma Aldrich) or TPEN and $ZnCl_2$ (Sigma Aldrich) or TPEN and $MnCl_2$ (Sigma Aldrich). The culture was then incubated at 37°C O/N and the final growth was measured by reading the $O.D._{600}$.

# Results

## Isogenic null mutants of *adcA* in MGAS5005

The function of AdcA in zinc-uptake in *S. pyogenes* has not been investigated to date, however the gene products of orthologues of *adcA* (belonging to the *znuA* family) have been described in both Gram-negative and -positive bacteria and identified as part of a high affinity zinc transporter (Ammendola *et al.*, 2007; Davis *et al.*, 2009).

In order to characterise the orthologue of *adcA* in GAS, three independent isogenic null mutants of *adcA* were generated in strain MGAS5005, an isolate from cerebrospinal fluid of a patient in a clinical study carried out in Ontario in 1995. It belongs to the *emm1* type and its genome has been completely sequenced and annotated. The strain has 3 prophages integrated in the genome (Φ5005.1, Φ5005.2 and Φ5005.3) (Sumby *et al.*, 2005), while the other sequenced M1 strain SF370 contains 4 prophages (Φ370.1, Φ370.2, Φ370.3 and 370.4) integrated in the genome (Fig. 5).

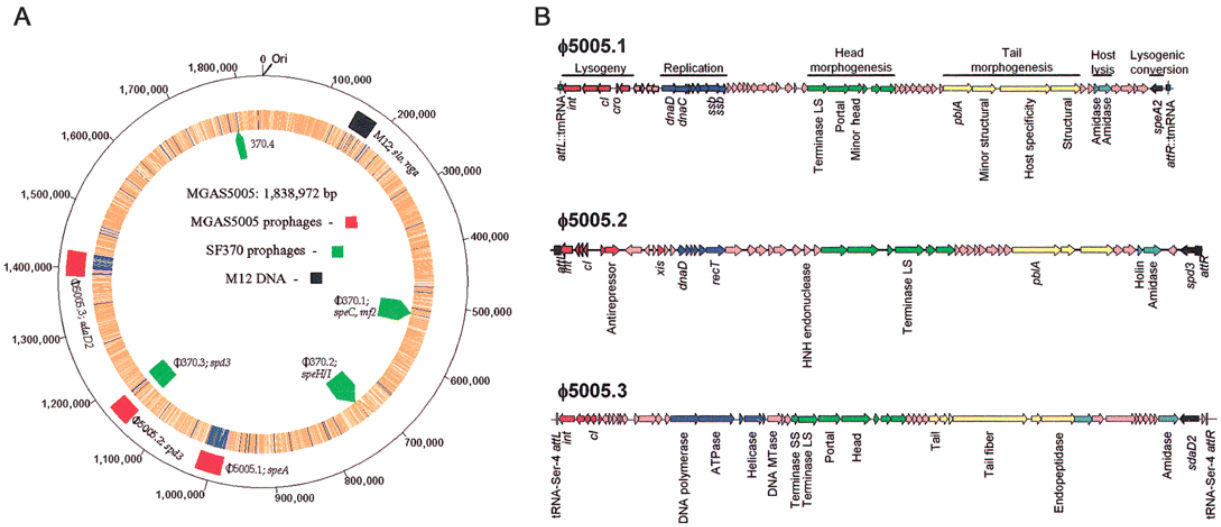



**Figure 5:Schematic representation of the MGAS5005 genome and integrated prophages.**

**A: Red blocks outside the circle indicate MGAS5005 prophage content, green arrows indicates SF370 prophage content. B: Organization and ORF maps of the 3 prophages integrated into the MGAS5005 genome (Sumby *et al.*, 2005).**

In addition, the MGAS5005 strain differs from the SF370 strain as it lacks several genes coding for superantigens (SAgs) (Table 1). The absence of these genes in the strain used in this work should be helpful to determine whether AdcA is involved in the

secretion and/or maturation of superantigens. In fact, it has been reported recently by Protein Chip technology (Galeotti *et al.*, 2011) that AdcA interacts *in vitro* with a streptococcal superantigen, and the interaction was then confirmed by Biacore. This interaction requires the presence of zinc, and the binding is disrupted in the presence of EDTA. The interaction between these two proteins could be significant from a biological point of view because zinc is essential for the binding of the superantigen to the MCH II receptor.

| SAgs | SF370 | MGAS5005 |
|---|---|---|
| *speG* | Spy0212 | M5005_Spy_0182 |
| *speJ* | Spy0436 | M5005_Spy_0356 |
| *smeZ* | Spy1998 | M5005_Spy_1702 |
| | | M5005_Spy_0667 |
| *speC* | Spy0711 | (fragment) |
| *speA2* | absent | M5005_Spy_0996 |
| *speH* | Spy1008 | absent |
| *speI* | Spy1007 | absent |

**Table 1:Superantigen genes present in the SF370 and MGAS5005 genomes.**

## Cloning of Δ*adcA*

The mutants were obtained by means of the low copy number temperature-sensitive shuttle vector pJRS233 (Fig. 6), as described by Perez-Casal in 1993 (Perez-Casal *et al.*, 1993).

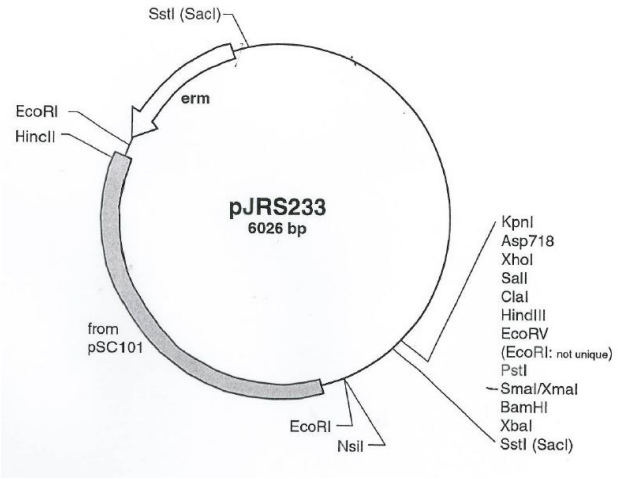



**Figure 6: Restriction map of the thermosensitive shuttle vector pJRS233.**

The plasmid possesses two distinct replication origins, one for *Escherichia coli* (from pSC101) and one for Gram-positive bacteria that is known to be functional in *S. pyogenes* (Perez-Casal *et al.*, 1993).

In Gram-positive bacteria the replication origin of this plasmid is temperature-sensitive and it allows the episomal replication of the vector only at 30°C. Indeed at 37°C the plasmid does not replicate but integrates into the genome. For the purpose of this work, *circa* 1 kilobase (kb) of each of the two flanking regions of *adcA* were PCR amplified from *S. pyogenes* strain SF370 genomic DNA. The nucleotide sequences of the restriction sites *Xho*I and *Bam*HI were added at the 5' and 3' ends, respectively. The two flanking amplicons, 5'-*Xho*I-*pepD* and 3'-*Spy0715*-*Bam*HI were then joined together by SOEing (Splicing by Overlapping Extension) PCR and cloned into the pJRS233/*Bam*HI-*Xho*I linearised vector. The pJRS233-Δ*adcA* construct obtained (Fig. 7) was used to transform the chemically competent cells of *E.coli* strain DH10B for plasmid preparation and its sequence verified by DNA sequencing.

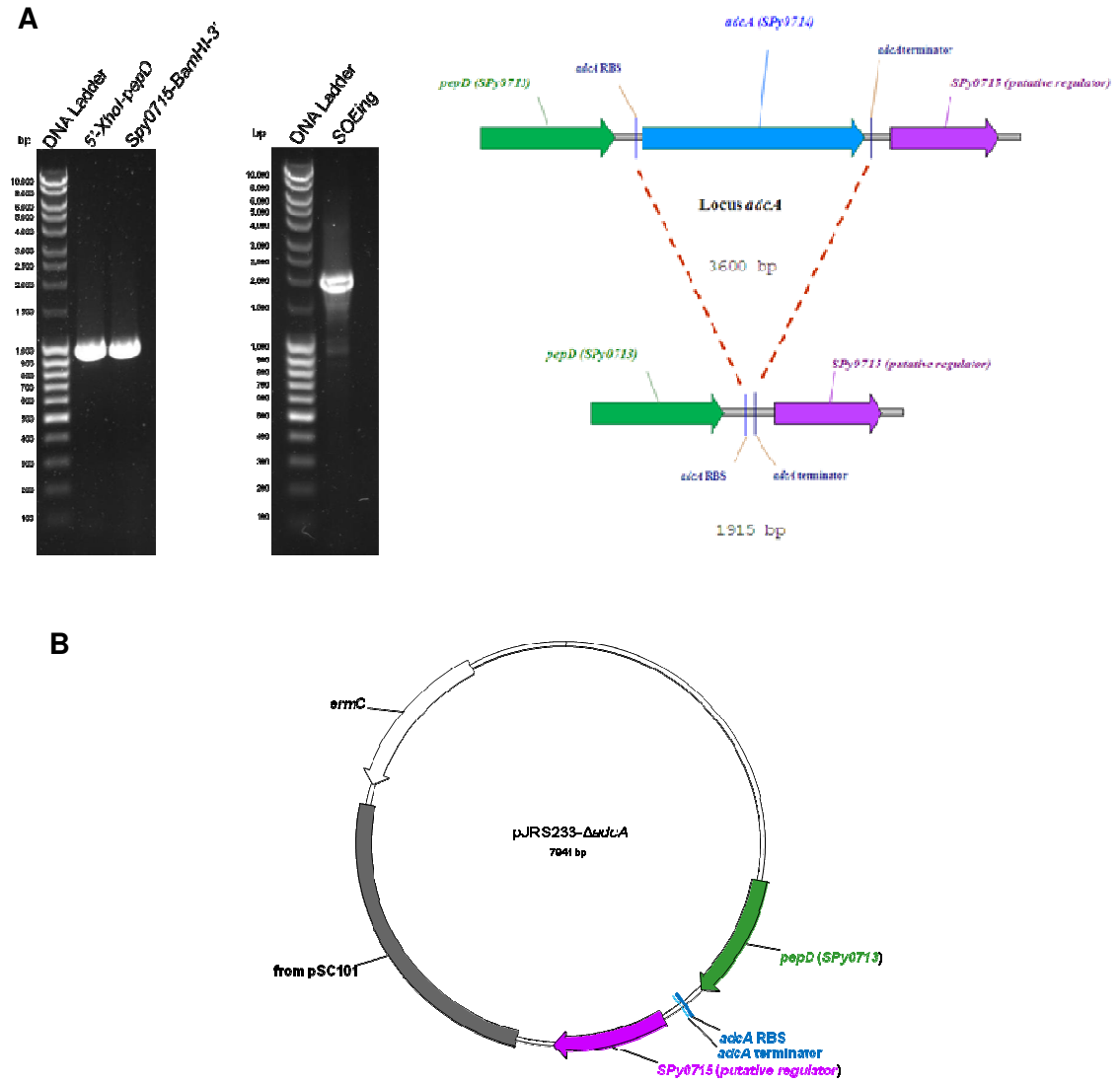



**Figure 7 Cloning of Δ*adcA* into pJRS233.**

**A: Amplification of the two flanking regions of *adcA* (left panel) and SOEing (right panel). B: Schematic representation of the SOEing for *adcA* knock-out. C: Map of pJRS233-Δ*adcA*.**

## Transformation of MGAS5005 with pJRS233-Δ*adcA*

*S. pyogenes* does not belong to the naturally competent streptococcal species able to take up foreign DNA, such as *S. pneumoniae*. In pneumococcus, competence is induced by the extracellular accumulation of the pheromone Competence Stimulating Peptide (CSP) (Claverys and Havarstein, 2002), whereas in *S. pyogenes* the biological pathway to uptake DNA remains unclear. Several procedures have been described in the literature to induce competence in GAS that can be grouped in two different approaches:

one that makes use of aminoacids (i.e. glycine or threonine) to destabilise the cell wall (Caparon and Scott, 1991; Sitkiewicz and Musser, 2006), and the other that uses several washes of the cells with a glycerol solution in order to eliminate all the electrolytes (Kimoto and Taketo, 2003). In this work both procedures were used to induce competence in strain MGAS5005 (see the Methods section) (Kimoto and Taketo, 2003; Sitkiewicz and Musser, 2006). Specifically, transformation of strain MGAS5005 with plasmid pJRS233-Δ*adcA* was carried out following the procedure described by Sitkiewicz (Sitkiewicz and Musser, 2006), while for complementation studies with vector pMU1328 transformants were obtained using the procedure from Kimoto (Kimoto and Taketo, 2003).

After preparation of electrocompetent cells, the transformation experiment was performed by using different amounts of DNA. After transformation, the bacteria were plated onto Tryptic Soy Agar (TSA) + 5% ram blood + 0.5 µg/ml erythromycin for antibiotic selection, and incubated at 30 °C, a temperature suitable for episomal replication of this plasmid in Gram-positive strains. The electroporation of pJRS233-Δ*adcA* into the electrocompetent cells gave a mean transformation efficiency of ≈ 4 colonies/µg DNA (Table 2).

| Sample | DNA | Transformants |
|---|---|---|
| 1 | no | 0 |
| 2 | 1.4 µg | 14 |
| 3 | 1.4 µg | 7 |
| 4 | 2.8 µg | 11 |
| 5 | 4.2 µg | 13 |
| 6 | 4.2 µg | 8 |
| *Average* | *2.3 µg* | *8.8 colonies/plate* |

**Table 2: Number of erythromycin-resistant transformants obtained after electroporation with plasmid pJRS233-Δ*adcA*.**

The erythromycin-resistant colonies were tested for the presence of the plasmid by PCR colony screening using the primers designed for SOEing. The presence of the Δ*adcA* construct should give a band of 1 kb in the samples containing pJRS233-Δ*adcA* as an episomal plasmid.

The PCR-positive colonies were then grown at 37°C, a non-permissive temperature for pJRS233-Δ*adcA,* and only the cells that integrate pJRS233-Δ*adcA* expressing the *ermC* gene could grow onto plates containing erythromycin.

The pJRS233-based mutagenesis relies on a double event of recombination, a first event of integration of the plasmid into the chromosome occurs by single crossover between one of the two identical flanking regions (on the plasmid and on the chromosome). This event leads to the integration of the plasmid that will result in a duplication of the two flanking regions in the chromosome and generates the intermediate strain MGAS5005::pJRS233-Δ*adcA* (Fig. 8).

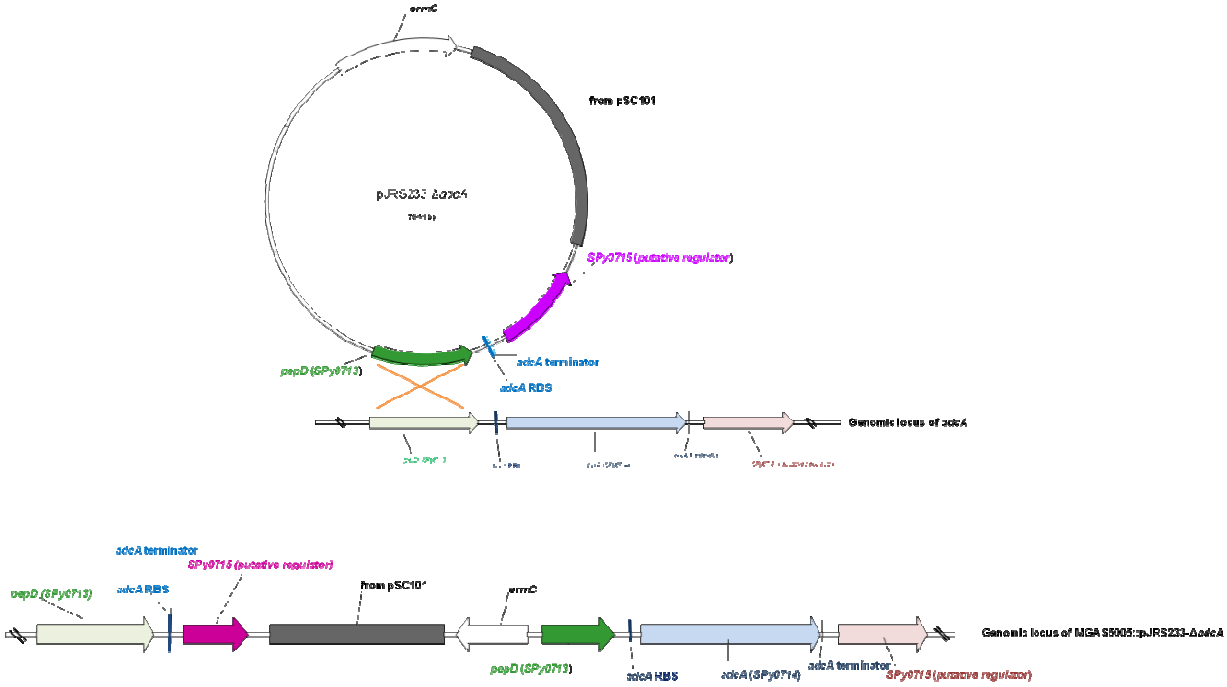



**Figure 8: Single crossing-over between pJRS233-Δ*adcA* and the genomic locus of *adcA*.**

**Upper panel: Integration through a single crossing-over. Lower panel: map of the genomic locus of intermediate strain MGAS5005::pJRS233-Δ*adcA*.**

The final null mutant Δ*adcA* can be obtained only after removal of the duplicated regions. This is achieved through a second event of recombination which allows the excision of the plasmid integrated into the chromosome together with the wild type *adcA* chromosomal gene in a theoretically expected 50 % of the cells.

The first screening was performed by PCR analysis on the chromosomal DNA of three transformants, named MGAS5005Δ*adcA*.2, MGAS5005Δ*adcA*.5 and MGAS5005Δ*adcA*.6, using primers external to the chromosomal regions flanking *adcA* but not present on the pJRS233-Δ*adcA* construct. The amplification products obtained are in agreement with the expected size for the two possible outcomes of the plasmid excision event: 2026 bp for the Δ*adcA* null mutant and 3574 bp for the re-established wild type locus (Fig. 9).

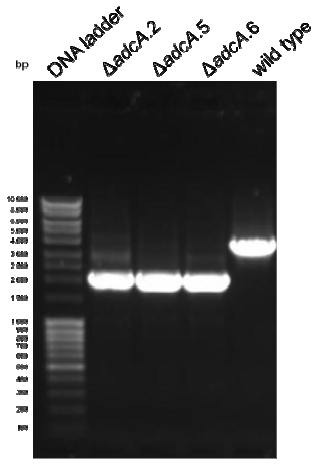



**Figure 9: Amplification products obtained from Δ*adcA*.2, Δ*adcA*.5, Δ*adcA*.6 and wild type genomic DNAs using primers specific for the genomic regions flanking *adcA*.**

Once pJRS233-Δ*adcA* had been excised from the genome, the three independent isogenic null mutants, MGAS5005Δ*adcA*.2, MGAS5005Δ*adcA*.5 and MGAS5005Δ*adcA*.6 were further characterised by sequencing the region surrounding the deletion and by Western Blot analysis on total cell extracts of the wild type and the Δ*adcA* strains. For extraction of total proteins, the wild type strain and the three independent null mutants were grown in THY to the same optical density (see Methods) and 5 µg of each total cell extract were used for Western Blot analysis. As a positive control 30 ng of recombinant protein were used. Although an aspecific signal close to the AdcA band is present in all the samples, only the extract from the wild type strain shows a specific band of the expected molecular mass (Fig. 10).

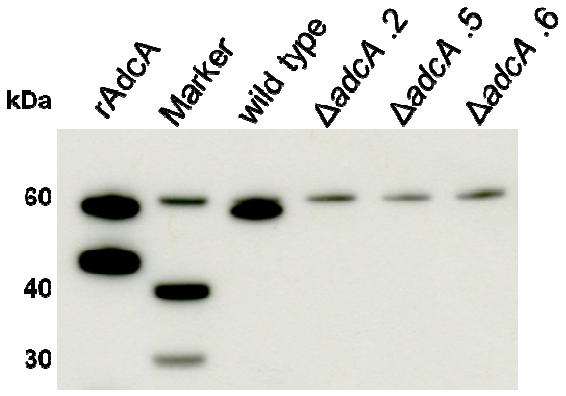



**Figure 10: Western Blot analysis using anti AdcA specific antibodies of total cell extracts of wild type, Δ*adcA*.2, Δ*adcA*.5 and Δ*adcA*.6 strains.**

## Complementation of Δ*adcA*

In order to obtain a complemented strain of the null mutant MGAS5005 Δ*adcA,* the wild type *adcA* gene was cloned into the pMU1328 vector (pMU1328-*adcA*), and for consistency the empty vector was electroporated also into the MGAS5005 wild type and Δ*adcA* mutant strains. The pMU1328 vector was designed for the identification of streptococcal DNA sequences that possess promoter activity (Achen *et al*., 1986). The vector was used succesfully also for the complementation of pneumococcus mutants (Gentile *et al*., 2011). Recently, this shuttle vector was employed for the overexpression and purification of pneumococcal proteins in *S. pneumoniae* (Lo *et al*., 2012). However, the use of this vector for complementation studies in *S. pyogenes* has never been described before in the literature.

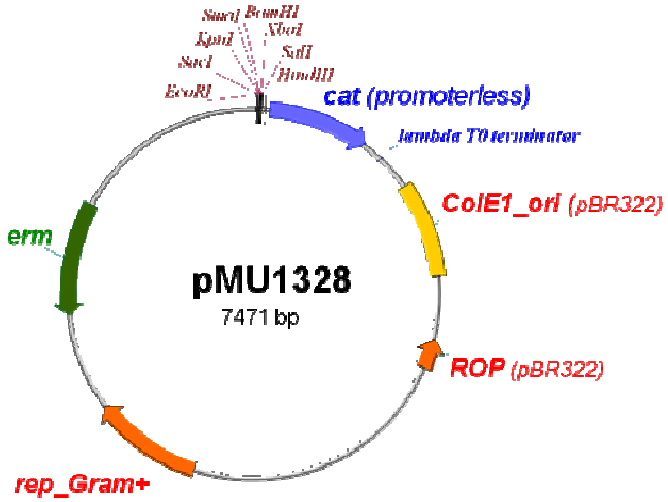



**Figure 11: Map of the pMU1328 vector.**

Deletion of the *adcA* gene in *S. pneumoniae* has been reported to lead to a dramatic decrease in competence (Dintilhac and Claverys, 1997). Thus, to overcome the loss of the capability to uptake exogenous DNA, transformation of the Δ*adcA* mutant with the pMU1328 complementation construct was obtained in the intermediate strain MGAS5005::pJRS233-Δ*adcA* that still contains the wild type *adcA* allele. Moreover, despite the fact that the two plasmids (pJRS233 and pMU1328) carry different *erm* genes (*ermC* for pJRS233 and *ermB* for pMU1328), they provide resistance to the same antibiotic (erythromycin). However, the excision of the plasmid pJRS233-Δ*adcA* from the chromosome (second crossover event giving rise to the deletion of *adcA)* with the

concomitant uptake of pMU1328 was obtained with a good frequency. This is possibly due to a greater instability of the integrated plasmid with respect to the episomal vector.

Analysis of the complemented Δ*adcA* strain was performed by PCR and by Western Blot.

The PCR analysis was performed by using primers that annealed on the chromosome externally to the flanking regions present on the pJRS233-Δ*adcA* construct. This analysis allowed to discriminate between the clones that have excised the plasmid sequences from the genome and those that still have the plasmid integrated.

Total cell extracts prepared from the wild type and the mutants (with or without the plasmid) do not apparently show differences in total proteins pattern (Fig. 12).

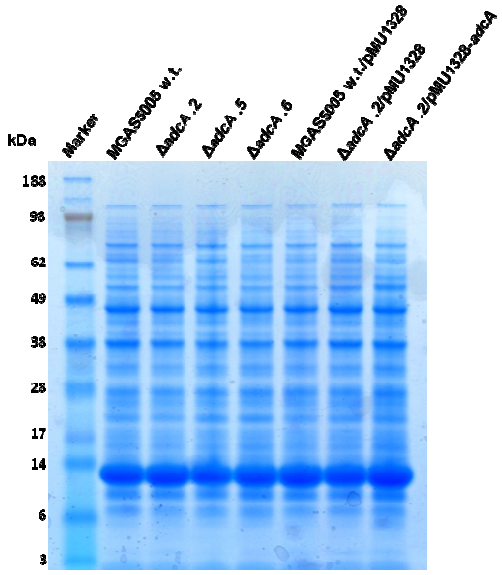



**Figure 12: Coomassie-stained SDS-PAGE of total cell extracts.**

The presence of the empty plasmid pMU1328 in the wild type does not seem to affect the expression of *adcA* when compared to the wild type without the plasmid. The complemented strain MGAS5005-Δ*adcA*/pMU1328-*adcA* expresses ≈ 50-fold more AdcA than the wild type, possibly due to the plasmid copy number. 5 µg of each total cell extracts were used for Western Blot analysis except for the complemented strain. In this case 0.1 µg were used because of the large amount of AdcA present in this strain as a result of the high plasmid copy number.

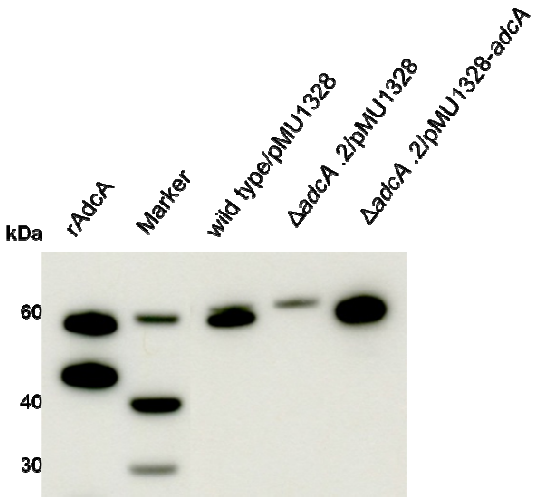



**Figure 13: Western Blot analysis of total cell extracts of wild type/pMU1328, Δ*adcA*.2 pMU1328 and Δ*adcA*.2 pMU1328-*adcA* using anti-AdcA specific antibodies.**

## Susceptibility to zinc starvation of Δ*adcA* isogenic null mutants

In order to test the susceptibility of MGAS5005*ΔadcA* null mutants to zinc starvation in a rich medium, the wild type strain and three independent isogenic null mutants were grown overnight in THY medium containing increasing concentrations of N,N,N'N'-Tetrakis (2-pyridylmethyl)-1,2-ethylenediamine (TPEN), a chelating agent (Fig. 14).

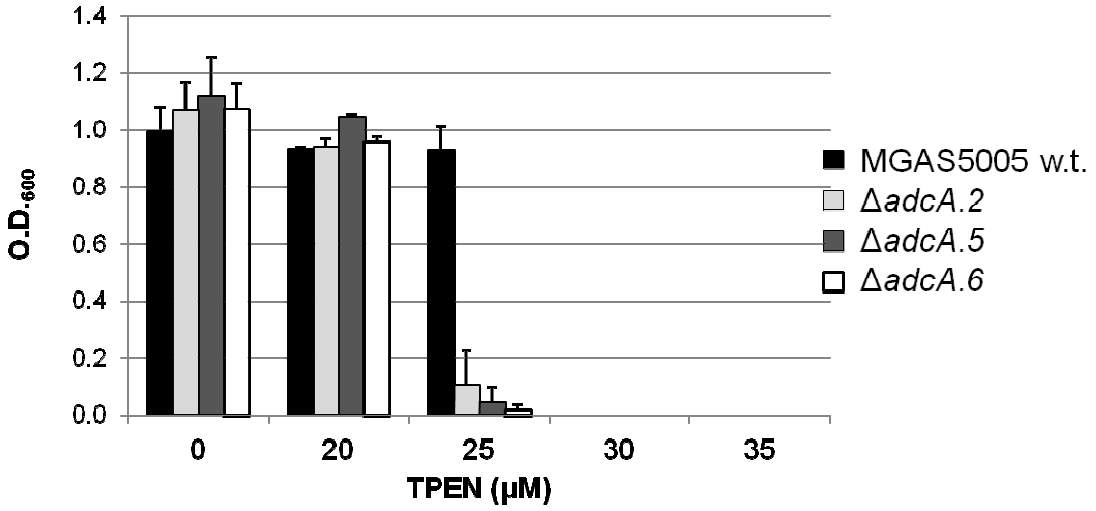



**Figure 14: Susceptibility to $Zn^{2+}$ starvation of wild type and three isogenic null mutants.**

The cultures of the Δ*adca* null mutants clearly show a higher sensitivity to zinc starvation compared to the wild type strain in the presence of 25 µM TPEN, which chelates the free $Zn^{2+}$ ions in the culture medium.

To verify if complementation is sufficient to restore growth, the plasmid-complemented strain of MGAS5005Δ*adcA.2*, was also grown in THY in the presence of increasing concentrations of TPEN (Fig. 15). For consistency, the wild type and *ΔadcA*.2 strains containing the empty vector pMU1328 were used as controls in this experiment.

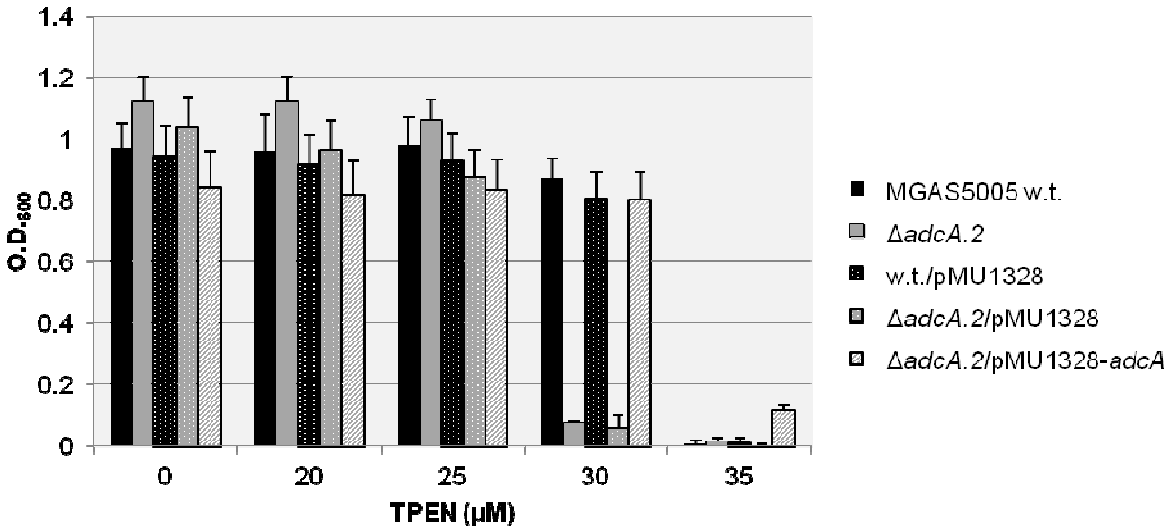



**Figure 15: Susceptibility to $Zn^{2+}$ starvation of wild type, mutants and complemented strains.**

The different susceptibility to zinc starvation of the wild type and Δ*adc.2* strains shown in Fig.10 and Fig.11 is due to small differences in THY preparation and TPEN stability.
The Δ*adcA.2* mutant, as well as the mock Δ*adcA.2*/pMU1328, do not grow in the presence of 30 µM TPEN, while the wild type (with or without the empty plasmid pMU1328) does not grow when 35 µM TPEN is added to the medium. However, the complemented strain shows some growth still at 35 µM TPEN, probably due to the high expression of AdcA from the pMU1328-*adcA* plasmid.

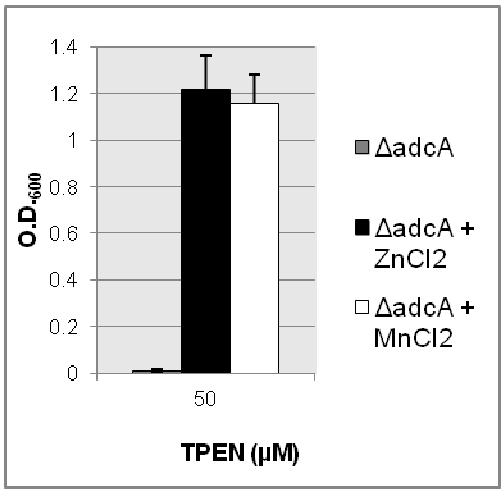



**Figure 16: Growth rescued by adding $Zn^{2+}$ or $Mn^{2+}$**

When the Δ*adcA* mutant is grown in the same conditions (following the approach described by Weston *et al.*) growth is inhibited in the presence of 50 µM TPEN, but is rescued by adding an equimolar concentration of $ZnCl_2$ or $MnCl_2$ (Fig. 16).

## Influence of zinc starvation on GAS $Zn^{2+}$ transporters

As already mentioned above, import of $Zn^{2+}$ ions into *S. pyogenes* seems to be carried out by the two Metal Binding Substrate (MBS) proteins AdcA and Lmb, although the latter could be involved also in $Mn^{2+}$ uptake (Weston *et al.*, 2009).
The gene coding for the metal transporter Lmb is located in an operon upstream of the *htpA* gene (Fig. 17). The Histidine Triad Protein A (HtpA) is a 92 kDa surface-exposed protein and was shown to bind $Zn^{2+}$ *in vitro* (Kunitomo *et al.*, 2008). The binding is due probably to the five histidine triad motifs (HXXHXH) contained in the protein sequence. The co-transcription of *lmb* and *htpA* and their common zinc-binding properties suggest a possible role in zinc homeostasis for both.

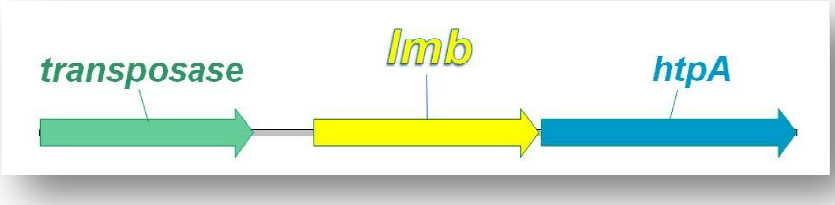



**Figure 17: Genetic organisation of the *lmb* operon in GAS**

Bacterial cells have to adjust the expression of the two proteins in response to the extracellular availability of free zinc ions. Thus, in order to test if the level of expression of *adcA* in wild type *S. pyogenes* is dependent on the concentration of zinc, the accumulation of AdcA was analysed in the total extracts of cells grown in media supplemented with increasing amounts of $ZnCl_2$. The culture media were pre-treated with 35 µM TPEN, a concentration sufficient to inhibit growth by chelating all the free $Zn^{2+}$ ions essential for cell viability.

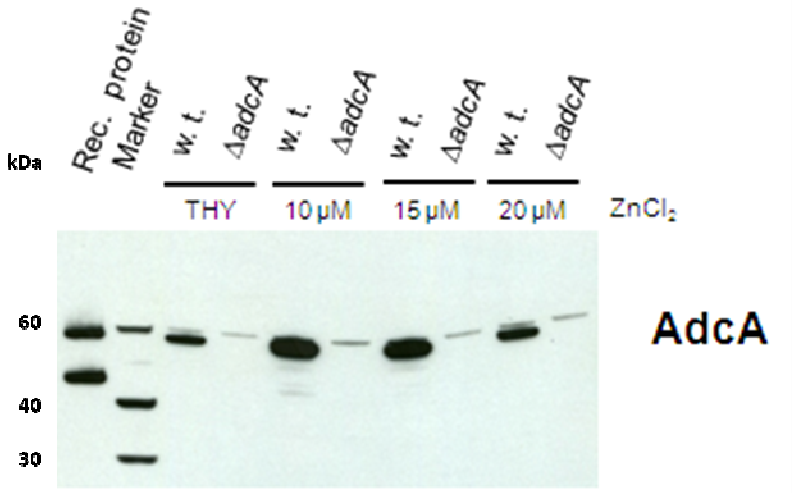



**Figure 18: Western Blot using anti-AdcA specific antibodies of total cell extracts from wild type and Δ*adcA* mutant grown in different conditions**

The expression of AdcA, Lmb and HtpA in different culture conditions was tested by Western Blot analysis of 5 µg of the total cell extracts of the wild type and Δ*adcA*.2 mutant, using 30 ng of the recombinant protein as a positive control.

The zinc-depleted culture medium (THY + 35 µM TPEN + 10 or 15 µM $ZnCl_2$) induces over-expression of the zinc transporters in order to scavenge as many zinc ions from the extracellular milieu as possible (Fig. 18).

At a concentration of 20 µM $ZnCl_2$, the wild type strain displays very low expression, very similar to that obtained in cells grown in the rich medium THY, indicating that this concentration of zinc does not require high expression of Lmb and HtpA since AdcA alone can supply the required amount of zinc (Fig. 18).

The mutant strain Δ*adcA* in the presence of 20 µM $ZnCl_2$ expresses a larger amount of Lmb and HtpA than wild type cells, possibly because it lacks AdcA (Figs. 19 and 20). Clearly, the mutant cells have to counterbalance the absence of AdcA by means of increasing the expression of Lmb.

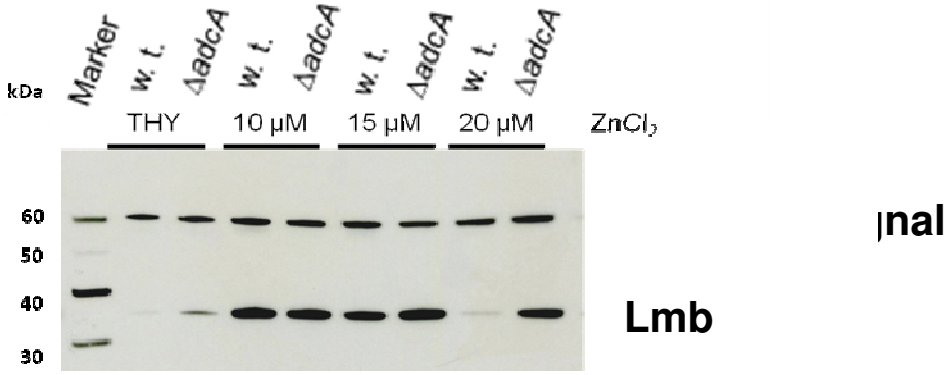



**Figure 19: Western Blot analysis of wild type and Δ*adcA* mutant total cell extracts grown in different conditions using anti- Lmb specific antibodies.**

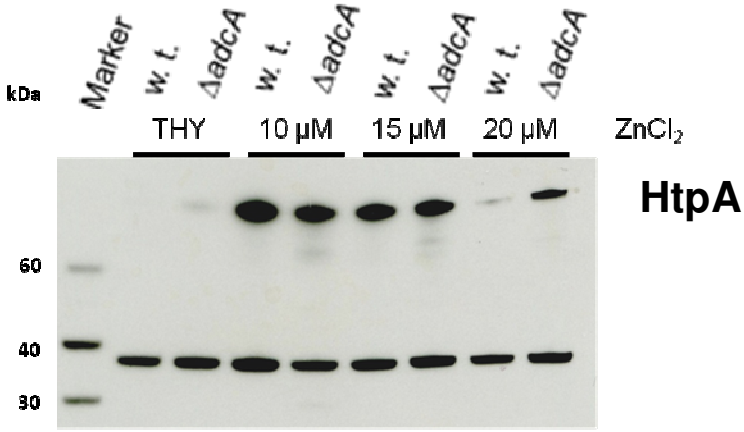



**Figure 20: Western Blot of wild type and Δ*adcA* mutant total cell extracts grown in different conditions using anti-HtpA specific antibodies.**

# Discussion

The ability to modulate the uptake of $Zn^{2+}$ from the environment is a crucial step for the survival of the pathogen in the different anatomical sites. The bacteria have to adapt their import/export systems to each particular milieu. In fact, $Zn^{2+}$ concentration ranges from 5 µM in the nasopharynx to 300 µM in the lung (Jacobsen *et al.*, 2011). Although zinc is a micro-element fundamental to the activity of many enzymes and proteins, its intracellular concentration has to be accurately regulated by the microorganism because it displays toxicity at concentrations lower than other cations such as iron or copper.

The absence of a zinc transporter affects the capacity to uptake zinc from the culture medium and the mutant susceptibility to zinc starvation. When three independent null mutants of *adcA* are grown in the presence of increasing concentrations of TPEN, at 25 µM TPEN growth is completely inhibited while growth of the wild type strain is impaired only by 30 µM TPEN (Fig. 14). In another experiment, upon complementation of the Δ*adcA* mutant, the concentration of TPEN that blocks its growth increases from 30 µM for the mutant to 35 µM for wild type and complemented strains (Fig. 15).

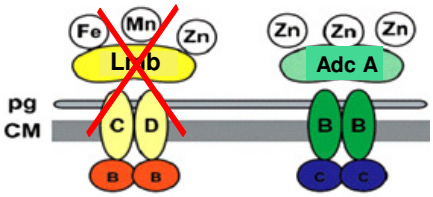
The import of zinc from the external milieu to the intracellular compartment seems to be the general activity of the two transporters, AdcA and Lmb. Weston *et al.*, studied the role of Lmb (or Lbp as they named it) in zinc homeostasis in *S. pyogenes* strain HSC5 and demonstrated that the deletion of Lmb gives rise to a phenotype that is growth deficient in a zinc-depleted medium (Weston *et al.*, 2009).

To confirm their hypothesis, Weston *et al*. grew the Δ*lmb* mutant in the presence of an equimolar concentration of TPEN and $Mn^{2+}$ or $Zn^{2+}$ (50 µM). In particular, the concentration of TPEN used in their experiment inhibited growth in the absence of added $Mn^{2+}$ or $Zn^{2+}$. The Δ*lmb* mutant resumed growth only in the presence of $Zn^{2+}$ but not in the presence of $Mn^{2+}$. This phenotype was explained as being a demonstration that Lmb has a role only in zinc homeostasis (Weston *et al.*, 2009).

In our experimental conditions, when *adcA* is deleted from the genome, the presence of equimolar concentrations of $Zn^{2+}$ or $Mn^{2+}$ can rescue the growth of the null mutant (Fig.12). These two phenotypes could be explained in terms of the metal specificity of the two transporters. If *lmb* is absent, the import of $Zn^{2+}$ is rescued by AdcA, which however cannot compensate for the absence of Lmb in $Mn^{2+}$ import, as highlighted by the failure to rescue growth in the Δ*lmb* mutant (Weston *et al.*, 2009). Alternatively, if *adcA* is absent, the expression of *lmb* is essential to rescue growth upon the addition of

$Zn^{2+}$ or $Mn^{2+}$ in the presence of 50 µM TPEN (Fig.12). This result clearly indicates that GAS has only one $Mn^{2+}$ transporter, Lmb, while AdcA and Lmb show a redundant role in zinc uptake,as is illustrated in the scheme shown below:

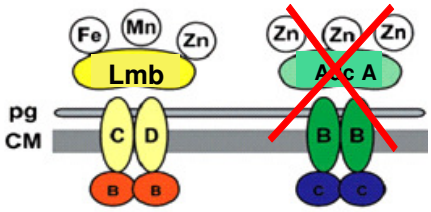
**Expression of AdcA**




**+Zn $^{2+}$: growth rescued by AdcA**

**+Mn $^{2+}$: growth NOT rescued by AdcA**

**Expression of Lmb**




**+$Zn^{2+}$ : growth rescued by Lmb**

**+$Mn^{2+}$ : growth rescued by Lmb**

When the zinc uptake systems are under stress due to the zinc-depleted growth conditions, both AdcA and the operon coding for Lmb and HtpA are expressed at levels higher than in complete medium. In fact, in the presence of 35 µM of TPEN and 20 µM zinc, the wild type strain displays a pattern of expression of AdcA, Lmb and HtpA comparable to that shown in THY medium (Fig. 18, Fig. 19 and Fig. 20). The mutant strain, since it lacks *adcA,* shows a clear overexpression of Lmb and HtpA for the same conditions (Fig. 19 and Fig. 20).

The operon coding for Lmb and HtpA, as well as the *adcA* gene, are under the control of AdcR, which is a zinc-binding negative regulator, and the presence of limiting amounts of zinc in the culture medium stimulates the increase in expression of *lmb* and *htpA* to counterbalance the absence of *adcA*.

In conclusion, in wild type cells AdcA is always expressed at a high level, whereas Lmb and HtpA are highly expressed only in zinc-depleted medium or in Δ*adcA* mutants. This finding supports the notion that AdcA is functionally homologous to ZnuA, the major high affinity zinc transporter in many bacteria, and like ZnuA is responsible for the efficient recruitment of zinc in most conditions.

# Addendum

| Oligonucleotide name | Sequence |
|---|---|
| P1for*Xho*I_*adcA* | GCGGCCTCGAGGAAGATTACCTTTGCTCAGCTGA |
| P2rev_*adcA* | GAAGATTTGCTTAGTGAGTTAAGAGATTCCTCCTTTGTTATTAACTG |
| P3for_*adcA* | CAGTTAATAACAAAGGAGGAATCTCTTAACTCACTAAGCAAATCTTC |
| P4rev*Bam*HI_*adcA* | GCGGCGGATCCTTCCTTGGTAGTGATAGCTGCAC |
| P5for_*adcA* | GCCATTTAATACCATGGTGCC |
| P6rev_*adcA* | CTGCAATCCTTAGGCGTTCTAA |
| 5'*adcA*_Bam_for | GCTAAGGATCCGCAACTGCTTAGCC |
| 3'*adcA*_Sal_rev | GCGGCGTCGACAAAGAAAAAGCAAACCTCCTTAAAAG |
| pMU1328-for | GCTATATGCGTTGATGCAATTT |
| pMU1328-rev | TCGGTATAAAACACTAATATCA |

## PCR programs

| | | |
|---|---|---|
| 94 ℃ | 5 min | |
| 94 ℃ | 30 sec | |
| 54 ℃ | 50 sec | x 5 cycles |
| 72 ℃ | 120 sec | |
| 94 ℃ | 30 sec | |
| 65 ℃ | 50 sec | x 25 cycles |
| 72 ℃ | 120 sec | |
| 72 ℃ | 7 min | |

**Program A**

| | | |
|---|---|---|
| 94 ℃ | 5 min | |
| 94 ℃ | 30 sec | |
| 58 ℃ | 50 sec | x 30 cycles |
| 72 ℃ | 120 sec | |
| 72 ℃ | 7 min | |

**Program B**

| | | |
|---|---|---|
| 94 ℃ | 5 min | |
| 94 ℃ | 30 sec | |
| 52 ℃ | 50 sec | x 30 cycles |
| 68 ℃ | 120 sec | |
| 68 ℃ | 7 min | |

**Program C**

| | | |
|---|---|---|
| 94 ℃ | 5 min | |
| 94 ℃ | 30 sec | |
| 59 ℃ | 50 sec | x 5 cycles |
| 72 ℃ | 90 sec | |
| 94 ℃ | 30 sec | |
| 66 ℃ | 50 sec | x 25 cycles |
| 72 ℃ | 90 sec | |
| 72 ℃ | 7 min | |

**Program D**

| | | |
|---|---|---|
| 94 ℃ | 5 min | |
| 94 ℃ | 30 sec | |
| 52 ℃ | 50 sec | x 30 cycles |
| 68 ℃ | 90 sec | |
| 68 ℃ | 7 min | |

**Program E**

# References


Achen, M. G., Davidson, B. E., and Hillier, A. J. (1986) Construction of plasmid vectors for the detection of streptococcal promoters. *Gene* **45,** 45-49.

Ammendola, S., Pasquali, P., Pistoia, C., Petrucci, P., Petrarca, P., Rotilio, G., and Battistoni, A. (2007) High-affinity Zn2+ uptake system ZnuABC is required for bacterial zinc homeostasis in intracellular environments and contributes to the virulence of Salmonella enterica. *Infect. Immun.* **75,** 5867-5876.

Bayle, L., Chimalapati, S., Schoehn, G., Brown, J., Vernet, T., and Durmort, C. (2011) Zinc uptake by Streptococcus pneumoniae depends on both AdcA and AdcAII and is essential for normal bacterial morphology and virulence. *Mol. Microbiol.*

Bisno, A. L., Brito, M. O., and Collins, C. M. (2003) Molecular basis of group A streptococcal virulence. *Lancet Infect. Dis.* **3,** 191-200.

Brenot, A., Weston, B. F., and Caparon, M. G. (2007) A PerR-regulated metal transporter (PmtA) is an interface between oxidative stress and metal homeostasis in Streptococcus pyogenes. *Mol. Microbiol.* **63,** 1185-1196.

Caparon, M. G. and Scott, J. R. (1991) Genetic manipulation of pathogenic streptococci. *Methods Enzymol.* **204,** 556-586.

Carapetis, J. R., Steer, A. C., Mulholland, E. K., and Weber, M. (2005a) The global burden of group A streptococcal diseases. *Lancet Infect. Dis.* **5,** 685-694.

Carapetis, J. R., Steer, A. C., Mulholland, E. K., and Weber, M. (2005b) The global burden of group A streptococcal diseases. *Lancet Infect. Dis.* **5,** 685-694.

Claverys, J. P. (2001) A new family of high-affinity ABC manganese and zinc permeases. *Res. Microbiol.* **152,** 231-243.

Claverys, J. P. and Havarstein, L. S. (2002) Extracellular-peptide control of competence for genetic transformation in Streptococcus pneumoniae. *Front Biosci.* **7,** d1798-d1814.

Cole, J. N., Barnett, T. C., Nizet, V., and Walker, M. J. (2011) Molecular insight into invasive group A streptococcal disease. *Nat. Rev. Microbiol.* **9,** 724-736.

Commons, R., Rogers, S., Gooding, T., Danchin, M., Carapetis, J., Robins-Browne, R., and Curtis, N. (2008) Superantigen genes in group A streptococcal isolates and their relationship with emm types. *J. Med. Microbiol.* **57,** 1238-1246.

Davidson, A. L. and Chen, J. (2004) ATP-binding cassette transporters in bacteria. *Annu. Rev. Biochem.* **73,** 241-268.

Davidson, A. L., Dassa, E., Orelle, C., and Chen, J. (2008a) Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol. Mol. Biol. Rev.* **72,** 317-64, table.

Davidson, A. L., Dassa, E., Orelle, C., and Chen, J. (2008b) Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol. Mol. Biol. Rev.* **72,** 317-64, table.

Davis, L. M., Kakuda, T., and DiRita, V. J. (2009) A Campylobacter jejuni znuA orthologue is essential for growth in low-zinc environments and chick colonization. *J. Bacteriol.* **191,** 1631-1640.

Dintilhac, A. and Claverys, J. P. (1997) The adc locus, which affects competence for genetic transformation in Streptococcus pneumoniae, encodes an ABC transporter with a putative lipoprotein homologous to a family of streptococcal adhesins. *Res. Microbiol.* **148,** 119-131.

Ferretti, J. J., McShan, W. M., Ajdic, D., Savic, D. J., Savic, G., Lyon, K., Primeaux, C., Sezate, S., Suvorov, A. N., Kenton, S., Lai, H. S., Lin, S. P., Qian, Y., Jia, H. G., Najar, F. Z., Ren, Q., Zhu, H., Song, L., White, J., Yuan, X., Clifton, S. W., Roe, B. A., and McLaughlin, R. (2001) Complete genome sequence of an M1 strain of Streptococcus pyogenes. *Proc. Natl. Acad. Sci. U. S. A* **98,** 4658-4663.

Galeotti, C. L., Bove, E., Pezzicoli, A., Nogarotto, R., Norais, N., Pileri, S., Lelli, B., Falugi, F., Balloni, S., Tedde, V., Chiarot, E., Bombaci, M., Soriani, M., Bracci, L., Grandi, G., and Grifantini, R. (2011) Surface Interactome in Streptococcus pyogenes. *Mol. Cell Proteomics. in press*

Gentile, M. A., Melchiorre, S., Emolo, C., Moschioni, M., Gianfaldoni, C., Pancotto, L., Ferlenghi, I., Scarselli, M., Pansegrau, W., Veggi, D., Merola, M., Cantini, F., Ruggiero, P., Banci, L., and Masignani, V. (2011) Structural and functional characterization of the Streptococcus pneumoniae RrgB pilus backbone D1 domain. *J. Biol. Chem.* **286,** 14588-14597.

Grosse, C., Anton, A., Hoffmann, T., Franke, S., Schleuder, G., and Nies, D. H. (2004) Identification of a regulatory pathway that controls the heavy-metal resistance system Czc via promoter czcNp in Ralstonia metallidurans. *Arch. Microbiol.* **182,** 109-118.

Guerra, A. J., Dann, C. E., and Giedroc, D. P. (2011) Crystal Structure of the Zinc-Dependent MarR Family Transcriptional Regulator AdcR in the Zn(II)-Bound State. *J. Am. Chem. Soc.* **133,** 19614-19617.

Hantke, K. (2005) Bacterial zinc uptake and regulators. *Curr. Opin. Microbiol.* **8,** 196-202.

Jacobsen, F. E., Kazmierczak, K. M., Lisher, J. P., Winkler, M. E., and Giedroc, D. P. (2011) Interplay between manganese and zinc homeostasis in the human pathogen Streptococcus pneumoniae. *Metallomics.* **3,** 38-41.

Kimoto, H. and Taketo, A. (2003) Efficient electrotransformation system and gene targeting in pyogenic streptococci. *Biosci. Biotechnol. Biochem.* **67,** 2203-2209.

Klein, J. S. and Lewinson, O. (2011) Bacterial ATP-driven transporters of transition metals: physiological roles, mechanisms of action, and roles in bacterial virulence. *Metallomics. in press*

Kloosterman, T. G., van der Kooi-Pol MM, Bijlsma, J. J., and Kuipers, O. P. (2007) The novel transcriptional regulator SczA mediates protection against Zn2+ stress by activation of the Zn2+-resistance gene czcD in Streptococcus pneumoniae. *Mol. Microbiol.* **65,** 1049-1063.

Kunitomo, E., Terao, Y., Okamoto, S., Rikimaru, T., Hamada, S., and Kawabata, S. (2008) Molecular and biological characterization of histidine triad protein in group A streptococci. *Microbes. Infect.* **10,** 414-423.

Linke, C., Caradoc-Davies, T. T., Young, P. G., Proft, T., and Baker, E. N. (2009) The laminin-binding protein Lbp from Streptococcus pyogenes is a zinc receptor. *J. Bacteriol.* **191,** 5814-5823.

Lo, S. M., Hilleringmann, M., Barocchi, M. A., and Moschioni, M. (2012) A novel strategy to over-express and purify homologous proteins from Streptococcus pneumoniae. *J. Biotechnol.* **157,** 279-286.

Loo, C. Y., Mitrakul, K., Voss, I. B., Hughes, C. V., and Ganeshkumar, N. (2003) Involvement of the adc operon and manganese homeostasis in Streptococcus gordonii biofilm formation. *J. Bacteriol.* **185,** 2887-2900.

Ma, Z., Jacobsen, F. E., and Giedroc, D. P. (2009) Coordination chemistry of bacterial metal transport and sensing. *Chem. Rev.* **109,** 4644-4681.

Mitchell, T. J. (2003) The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nat. Rev. Microbiol.* **1,** 219-230.

Musser, J. M. and Shelburne, S. A., III (2009a) A decade of molecular pathogenomic analysis of group A Streptococcus. *J. Clin. Invest* **119,** 2455-2463.

Musser, J. M. and Shelburne, S. A., III (2009b) A decade of molecular pathogenomic analysis of group A Streptococcus. *J. Clin. Invest* **119,** 2455-2463.

Nanamiya, H., Akanuma, G., Natori, Y., Murayama, R., Kosono, S., Kudo, T., Kobayashi, K., Ogasawara, N., Park, S. M., Ochi, K., and Kawamura, F. (2004) Zinc is a key factor in controlling alternation of two types of L31 protein in the Bacillus subtilis ribosome. *Mol. Microbiol.* **52,** 273-283.

O'Farrell, P. H., Kutter, E., and Nakanishi, M. (1980) A restriction map of the bacteriophage T4 genome. *Mol. Gen. Genet.* **179,** 421-435.

Olsen, R. J., Shelburne, S. A., and Musser, J. M. (2009) Molecular mechanisms underlying group A streptococcal pathogenesis. *Cell Microbiol.* **11,** 1-12.

Outten, C. E. and O'Halloran, T. V. (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* **292,** 2488-2492.

Panina, E. M., Mironov, A. A., and Gelfand, M. S. (2003) Comparative genomics of bacterial zinc regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. *Proc. Natl. Acad. Sci. U. S. A* **100,** 9912-9917.

Pattery, T., Hernalsteens, J. P., and De, G. H. (1999) Identification and molecular characterization of a novel Salmonella enteritidis pathogenicity islet encoding an ABC transporter. *Mol. Microbiol.* **33,** 791-805.

Patzer, S. I. and Hantke, K. (1998) The ZnuABC high-affinity zinc uptake system and its regulator Zur in Escherichia coli. *Mol. Microbiol.* **28,** 1199-1210.

Perez-Casal, J., Price, J. A., Maguin, E., and Scott, J. R. (1993) An M protein with a single C repeat prevents phagocytosis of Streptococcus pyogenes: use of a

temperature-sensitive shuttle vector to deliver homologous sequences to the chromosome of S. pyogenes. *Mol. Microbiol.* **8,** 809-819.

Petersson, K., Forsberg, G., and Walse, B. (2004) Interplay between superantigens and immunoreceptors. *Scand. J. Immunol.* **59,** 345-355.

Rees, D. C., Johnson, E., and Lewinson, O. (2009) ABC transporters: the power to change. *Nat. Rev. Mol. Cell Biol.* **10,** 218-227.

Schwan, W. R., Warrener, P., Keunz, E., Stover, C. K., and Folger, K. R. (2005) Mutations in the cueA gene encoding a copper homeostasis P-type ATPase reduce the pathogenicity of Pseudomonas aeruginosa in mice. *Int. J. Med. Microbiol.* **295,** 237-242.

Shafeeq, S., Kloosterman, T. G., and Kuipers, O. P. (2011) Transcriptional response of Streptococcus pneumoniae to Zn2+) limitation and the repressor/activator function of AdcR. *Metallomics.* **3,** 609-618.

Sitkiewicz, I. and Musser, J. M. (2006) Expression microarray and mouse virulence analysis of four conserved two-component gene regulatory systems in group a streptococcus. *Infect. Immun.* **74,** 1339-1351.

Sumby, P., Porcella, S. F., Madrigal, A. G., Barbian, K. D., Virtaneva, K., Ricklefs, S. M., Sturdevant, D. E., Graham, M. R., Vuopio-Varkila, J., Hoe, N. P., and Musser, J. M. (2005) Evolutionary origin and emergence of a highly successful clone of serotype M1 group a Streptococcus involved multiple horizontal gene transfer events. *J. Infect. Dis.* **192,** 771-782.

van der Heide, T. and Poolman, B. (2002) ABC transporters: one, two or four extracytoplasmic substrate-binding sites? *EMBO Rep.* **3,** 938-943.

Ward, S. K., Abomoelak, B., Hoye, E. A., Steinberg, H., and Talaat, A. M. (2010) CtpV: a putative copper exporter required for full virulence of Mycobacterium tuberculosis. *Mol. Microbiol.* **77,** 1096-1110.

Weston, B. F., Brenot, A., and Caparon, M. G. (2009) The metal homeostasis protein, Lsp, of Streptococcus pyogenes is necessary for acquisition of zinc and virulence. *Infect. Immun.* **77,** 2840-2848.