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Characterization of Protective Antigens from *Streptococcus pyogenes* and their Contribution to Virulence

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1 SUMMARY

Streptococcus pyogenes, belonging to Group A Streptococci, is a gram-positive, extracellular bacterial pathogen and can cause a wide variety of human infections that range from asymptomatic colonization to life threatening invasive diseases. Although antibiotic treatment is very effective, when left untreated *S. pyogenes* infections can lead to post-streptococcal sequelae and severe diseases causing significant morbidity and mortality worldwide. Infectious diseases are the second leading cause of death worldwide, and the acquisition of antibiotic resistance by many pathogenic bacteria has incited interest in generating vaccines to cure or prevent disease. Therefore, Intercell` s Antigen Identification Program (AIP) has identified novel *S. pyogenes* protein antigens relying on human sera and bacterial surface display for a prophylactic vaccine based on recombinant proteins. Out of the 9 most promising protein candidates, selected by a multistep validation program and protection studies in mice, three vaccine candidates, Spy0416, Spy0895 and Spy1536, were chosen to evaluate their contribution to GAS disease. Spy0416, annotated as ScpC, was further characterized for its biochemical function. This demonstrated that the recombinant form of Spy0416 is capable of IL-8 degradation *in vitro* in a time and concentration dependent manner. Surprisingly, the isolated predicted proteinase domain did not exhibit IL-8 degrading activity, but was dependent on the presence of its ancillary C-terminal domains. The evaluation of possible contributions of the predicted C-terminal domains to IL-8 degradation showed that none of them in combination with the N-terminal proteinase domain degraded IL-8. This indicated the Spy0416 protein is in its entirety essential for the enzymatic activity *in vitro.*

For the two candidates Spy0895 and Spy1536 gene deletion mutants have been generated. While the ΔSpy0895 strain did not show a distinct phenotype, deletion of the *spy1536* gene revealed an important role for Spy1536 to mediate binding to human proteins such as fibrinogen, fibronectin, collagen I, plasminogen and laminin *in vitro*. Analysis of the expression of the two surface proteins, the M protein and Spy0269, indicated that the reduced binding of ΔSpy1536 streptococcal cells to human plasma-

and extracellular matrix-proteins is due to the abrogated localization of these and possibly other GAS proteins on the surface of ΔSpy1536. Interestingly, M protein was still detectable in the cytoplasm, but not in the bacterial cell wall fraction. This is in agreement with microarray analyses which revealed that transcription of the M protein and Spy0269 encoding genes was not affected. Furthermore, Microarray and Real Time RT-PCR also confirmed the observed phenotype of the *spy1536* gene deletion mutant. It revealed a ~10-100 fold reduced transcription of *fcrA*, encoding an M-related protein precursor, of *sclA* and *sclB*, encoding collagen-like surface proteins ScIA and ScIB, as well as reduced transcription of the *sfbX49* gene, encoding the fibronectin-binding protein SfbX49. Furthermore, *hasA* encoding hyaluronate synthase for the production of hyaluronidase capsule was found to be ~2 fold upregulated.

The work has therefore provided important evidence that Spy0416 and Spy1536 contribute to GAS pathogenesis which strongly supports their selection as vaccine candidates.

2 ZUSAMMENFASSUNG

Streptococcus pyogenes zählt zu Gruppe A Streptokokken und ist ein gram-positives extrazelluläres bakterielles Pathogen. Es ist fähig eine Vielzahl humaner Infektionen zu verursachen, die von asymptomatischer Kolonisierung bis hin zu lebensbedrohlichen invasiven Krankheiten reichen. Obwohl die Behandlung mit Antibiotika sehr effektiv ist, kann es im Falle einer unbehandelten *S. pyogenes* Infektion, zu schwerwiegenden Folgekrankheiten kommen, die zu signifikanter Morbidität und Mortalität führen können. Infektionskrankheiten sind die zweithäufigste Todesursache weltweit. Aufgrund zunehmender Antibiotikumresistenz vieler Pathogene, stellen Impfstoffe eine wichtige Alternative dar, um bestimmte Krankheiten zu heilen oder diesen sogar vorzubeugen. Darum hat Intercell's Antigen Identifizierungprogramm (AIP) neue Proteinantigene identifiziert, basierend auf humanen Seren und `bacterial surface display`, für die Entwicklung eines prophylaktischen, antibakteriellen Vakzins, beruhend auf rekombinanten Proteinen. Aus den 9 vielversprechendsten Kandidaten, die mittels umfassender Validierungsprozeduren und Protektionsstudien in Mäusen selektiert werden konnten, wurden drei Impfstoffkandidaten, Spy0416, Spy0895 und Spy1536, näher charakterisiert, um deren Rolle in der Krankheitenentwicklung durch Gruppe A Streptokokken zu evaluieren. Spy0416, annotiert als ScpC, wurde weiters in seiner biochemischen Funktion näher analysiert. Diese Analyse ergab, dass die rekombinante Form von Spy0416 Interleukin 8, abhängig von Inkubationszeit und Enzymkonzentration, *in vitro* abbauen kann. Überraschenderweise zeigte die Proteinasedomäne alleine keine IL-8 abbauende Aktivität, da diese von der Anwesenheit der C-terminalen Hilfsdomänen abhängig war. Die Evaluierung einer möglichen Beteiligung der C-terminalen Domänen am IL-8 Abbau zeigte, dass keine dieser Domänen in Kombination mit der N-terminalen Proteinasedomäne IL-8 abbaut. Das deutet darauf hin, dass Spy0416 in seiner Gesamtheit essenziell für die enzymatische Aktivität *in vitro* ist.

Mit weiteren zwei Kandidaten, Spy0895 und Spy1536, wurden Gendeletionsmutanten generiert. Während der Stamm ΔSpy0895 keinen deutlichen Phänotyp aufwies, ergab

die Deletion des *spy1536* Gens, dass Spy1536 eine wichtige Funktion in der Bindung an humane Proteine wie Fibrinogen, Fibronektin, Collagen I, Plasminogen und Laminin *in vitro* vermittelt. Die Analysen von Expression der beiden Oberflächenproteine, M Protein und Spy0269, deuteten darauf hin, dass die reduzierte Bindung von ΔSpy1536 Zellen an die humanen Plasma- und extrazellulären Matrix Proteine aufgrund einer außer Kraft gesetzten Lokalisierung dieser und möglicherweise anderer GAS Proteine auf der Oberfläche von ΔSpy1536 herrührt. Interessanterweise war das M Protein nach wie vor im Zytoplasma detektierbar, aber nicht in der bakteriellen Zellwand. Dies stimmt mit den Microarrayanalysen überein. In diesen war die Transkription von M Protein und Spy0269 kodierenden Genen nicht betroffen.

Weiters bestätigten Microarray und Real Time RT-PCR den beobachteten Phänotyp der *spy1536* Gendeletionsmutante. Es konnte eine 10-100 fach reduzierte Transkription von *fcrA*, das einen `M-related protein precursor` kodiert, von *sclA* und *sclB* gezeigt werden, welche die entsprechenden Collagen-ähnlichen Oberflächenproteine SclA und SclB kodieren, sowie eine reduzierte Transkription von *sfbX49*, welches das Fibronektin-bindende Protein SfbX49 kodiert. Zusätzlich wurde eine 2 fach erhöhte Transkription des *hasA* Gens gezeigt, das eine Hyaluronat Synthase zur Produktion der Hyaluronidase-Kapsel kodiert.

Diese Arbeit hat den Hinweis erbracht, dass Spy0416 und Spy1536 an der Krankheitsentwicklung durch Gruppe A Streptokokken beteiligt sind, was deren Selektion als Impfstoffkandidaten stark unterstützt.

3 INTRODUCTION

3.1 Streptococcal Pathogenesis and Disease

Streptococcus pyogenes is a gram-positive, extracellular, non-motile, β-hemolytic bacterial pathogen which belongs to Group A streptococci. It is capable of causing a wide variety of diseases globally with humans being their only known natural biological host. There is no environmental reservoir known for Group A streptococci and transmission of the bacteria occurs almost always from person to person [1]. The primary sites for colonization involve the nasal and oropharyngeal mucosal epithelium of the upper respiratory tract as well as the superficial layers of the epidermis where GAS can undergo successful reproductive growth and transmission to the next host. Two reasons why this bacterium presents such a challenge for the human body are the existence of more than 200 distinct serotypes and the enormous repertoire of different virulence factors. The ingenious way the bacterium counteracts host defenses largely contributes to the success of GAS in establishing infections in its human host.

3.1.1 Diseases Caused by *S. pyogenes* and Changes in Epidemiology

GAS can thrive in almost any body tissue of the upper respiratory tract and skin lesions serve as the primary sites of infection and principle reservoirs of transmission [2-4]. Bacteria are transmitted from human to human through respiratory droplets or through contact with infected wounds or sores on the skin [2-4].

Asymptomatic carriage can occur in humans, without triggering a vigorous host immune response to many GAS antigens [5]. This is the case for 5-15% of normal individuals who harbour the bacterium in their respiratory tract without developing any signs of disease. Clinically inapparent infections can also arise whereby the host lacks obvious clinical symptoms of illness although there is a significant immune response.

Once the bacterium has successfully colonized the upper respiratory tract or the epidermis, primary disease manifestations often include pharyngitis, impetigo and soft tissue infections. GAS is believed to be responsible for 15-30% of acute pharyngitis cases in children and also for 10% of cases in adults [6, 7]. Disease in people suffering from

skin infections or pharyngitis and tonsillitis is commonly referred to as "strep throat" and is most likely a source for spread of the infection. Asymptomatic carriers are less contagious. These non-severe infections are superficial self-limiting and usually cause only a mild illness. The treatment of an infected person with penicillin for 24 hours generally eliminates their ability to spread the bacteria and cures diseases within 10 days. In 1-3% of untreated infections rheumatic fever and glomerulonephritis may follow streptococcal disease. But these conditions and their pathology are not attributable to spreading of bacteria, but to aberrant immunological reactions to GAS antigens [8]. Intracellular invasion of epithelial cells on the other hand contributes to persistence of GAS in the human host because the bacteria get past the defenses of the person who is infected. The consequence may be invasive and toxin-mediated diseases [9]. Two of the most severe, but least common forms of invasive GAS disease are necrotizing fasciitis and streptococcal toxic shock syndrome (STSS). Necrotizing fasciitis is caused because bacteria gain access to deep tissue that is normally sterile leading to a widespread fascial necrosis with relative sparing of skin and underlying muscle [10]. STSS causes blood pressure to drop rapidly and organs (e.g., kidney, liver, lungs) to fail. About 20% of patients with necrotizing fasciitis, more than half with STSS and between 10-15% of patients with other forms of invasive group A streptococcal disease die.

Clearly the infectivity of a disease agent is complicated and relies on both, the virulence of the pathogen and the susceptibility of the host [1]. For example there are discrete populations of streptococci found to be commonly associated with either severe pharyngitis or impetigo [11]. Based on the nucleotide sequence differences in the region of the *emm* gene encoding the peptidoglycan-spanning domain, Bessen and colleagues found that streptococcal isolates from pharyngeal sites showed mainly the *emm* chromosomal pattern A (M1, M3, M6, M12, etc.), B (M5, M8, M14, M29) and C (M6, M18, M23, M38). In contrast, impetigo isolates were restricted to *emm* chromosomal pattern of groups D (M27, M28, M30, M32, etc.) and E (M2, M4, M9, M49, etc). Many pattern E isolates, such as M type 49 strains, are likely to colonize both tissue sites. Some serotypes have been clearly associated with epidemics of rheumatic fever such as M5 [12]. Also M3, 6, 14, 18, 19, 24 and a few others are well represented. An outbreak of acute rheumatic fever in Salt Lake City, Utah in the year 1987 for example was found to be mainly associated with M type 18 and M type 3 group A streptococcal strains [13].

Interestingly, prevalent pharyngeal types as M2, 4 and 12 were reported not to be associated with rheumatic fever [12]. Life-threatening infections like toxic shock syndrome were associated with GAS strains that frequently belonged to serotypes M1 and M3 most of which entered through the skin and soft tissue and only occasionally the pharynx.

Development of disease does not only depend on the streptococcal M type alone. It also depends on the host susceptibility. Differences in susceptibility may be attributable to genetic factors, frequency of underlying illness or living arrangements [14]. Some countries in Europe for example have reported higher incidence of GAS diseases particularly in ethnic groups. A study from north London found higher rates of pharyngeal carriage of GAS in orthodox Jewish children and adults attending primary care services than in other attendees, regardless of sore throat symptoms [15]. Furthermore, a study from southern Israel found a significantly higher risk of GAS disease in its Bedouin population compared to Jewish people among the population [16]. In case of acute rheumatic fever and rheumatic heart disease disproportionately high levels of appearance were found in the Pacific region and in some indigenous groups in developed countries. In Aboriginal communities in the Northern Terretory in Australia the prevalence of rheumatic heart disease was 11.8 per 1000 and the incidence of acute rheumatic fever in children was 254 per 100,000. In contrast the non-Aboriginal prevalence in the same region was 0.4 per 1000 and incidence was 1.3 per 100,000 [17-19]. Interestingly, in the Aboriginal groups in the Australian Northern Territory known to have high rates of rheumatic fever, serotypes of GAS traditionally associated with rheumatic fever, were not commonly found [19]. Therefore, it is possible that high prevalence rates of rheumatic heart disease in developing countries may be due to environmental factors including overcrowding, nutrition and access to medical services [19]. A study during the 1950s in the United States Air Force Base barracks provided a biological basis for a relationship between overcrowding and the incidence of acute rheumatic fever. It was found that rates of acquisition of streptococcal infections increased when beds were moved closer together [20]. It was also suggested that poor nutrition in early childhood plays a primary role in susceptibility to acute rheumatic fever. In regard to this, a case control study in Serbia described that a bodyweight 10% below average was found to increase the risk of acute rheumatic fever by 42% [21].

Access to medical services may be an explanation for the difference of rheumatic heart disease prevalence between developed and developing countries. In Costa Rica, the incidence of acute rheumatic fever fell in the 1970s concomitant with a national health strategy of penicillin prophylaxis [22]. Immunologic factors such as pre-existing M type-specific antibodies and other host characteristics may also be important in the clinical expression of illness.

Since there are many factors defined by the bacteria and the host described above that influence establishment of Group A streptococcal disease, changes in the epidemiology of GAS could depend on similar factors like the molecular basis of *Streptococcus pyogenes*, environment of the host and the host itself. The distinction between pharyngeal and impetigo strains briefly discussed before provides focus for addressing the molecular basis of the tissue tropism of *S. pyogenes* as one of the factors that might influence streptococcal epidemiology. If *emm* gene products define at least in part the tissue-specificity of infection the horizontal transfer of *emm* genes occurring between different group A streptococcal strains may be an important mechanism for the emergence of new clones. These new clones might have unique pathogenic qualities which may lead to a change in the principle reservoir for transmission [11]. Interestingly, it could be shown that changes in epidemiology of group A streptococcal disease in the USA was accompanied by a corresponding change in serotype distribution [23]. While proportions of invasive M types 1, 3 and 18 increased significantly between 1972 and 1988, less invasive strains of serotypes M4 and 12 decreased. Several reports in the literature have documented changes in the epidemiology of diseases caused by GAS, particularly in Northern Europe and North America [2, 24, 25]. For example significant changes occurred in the spectrum of invasive group A streptococcal infections in Arizona, between 1985 and 1990 [26]. Native Americans were at increased risk of acquiring these infections. Patients with the streptococcal toxic shock-like syndrome had epidemiologic features that distinguished them from patients with other invasive infections, including younger age and less underlying illness. In spite of that, the proportion of infections with hypotension, rash, desquamation, renal impairment and gastrointestinal involvement increased significantly.

In summary, group A streptococci cause various diseases (see Table 1) that range from non-severe to severe invasive diseases. Disease outcome is not only dependent on genetic factors but also strongly depends on the host and its environment.

Table 1: Diseases caused by *Streptococcus pyogenes*.

Category of Disease	Examples of Infections
Superficial	Pharyngitis, skin and soft tissue infections, impetigo, erysipelas, vaginitis, post-partum infections
Deep Infections	Bacteraemia, necrotizing fasciitis, deep tissue infections, cellulitis, myositis, puerperal sepsis, pericarditis, meningitis, pneumonia, septic arthritis
Toxin-mediated	Scarlet fever, toxic shock-like syndrome
Immunologically mediated	Rheumatic fever, post-streptococcal glomerulonephritis, Reactive arthritis

3.1.2 *S. pyogenes* - A Global Burden

Before the introduction of antimicrobials, serious infections caused by *S. pyogenes* were common [2]. This organism was responsible for as many as half of post-partum deaths before World War II and it was the major cause of death in burns patients. Due to the use of penicillin, Group A streptococcus was believed to be virtually eliminated as a pathogen, as over the last four decades, the industrialized world has witnessed a decline in the incidence of these diseases. In the 1970s, there was optimism that serious GAS disease had been almost completely eradicated in developed countries and it was noted that specific diseases, for example scarlet fever, were abolished in many European countries. In the mid to late 1980s, however, concerns about GAS disease were heightened since outbreaks of rheumatic fever occurred in different areas, not only in developing countries but also populations that had ready access to medical care within industrialized countries were affected [2, 27, 28]. For example, Salt Lake City, Utah registered an eightfold increase of rheumatic fever over the average annual incidences [13]. Regarding invasive diseases in the 1990s, Scotland marked year-on-year rises of GAS bacteraemia from the mid 1990s onwards [14]. Between 1993-1996 surveillance data

from Sweden showed a rise in invasive GAS infections, before they dropped back to a more stable annual rate. More pronounced changes are apparent in Iceland where rates of invasive GAS have swung from 1-2 per 100,000 to peaks above 6 per 100,000 which were the highest rates observed in any European country between 1992-1994 [14]. Regardless of the patterns of invasive GAS disease, there is a general suggestion of increasing invasive GAS across Europe over the past two decades.

According to WHO estimates in 2005 18.1 million people suffer from serious GAS disease and another 1.78 million cases occur each year [29]. These diseases are responsible for over 500,000 deaths each year. Added to this are over 111 million prevalent cases of streptococcal pyoderma and 616 million new cases of pharyngitis each year.

The Centers for Disease Control and Prevention (CDC, USA) estimates that invasive GAS disease accounts for ~ 10,000 cases per year in the USA. Among those are ~1350 deaths. Mortality that results from GAS invasive diseases varies depending on the disease manifestation and ranges from 20% for necrotizing fasciitis to ~45% for streptococcal toxic shock syndrome [7, 30]. The re-emergence of streptococcal toxic shock syndrome and necrotizing fasciitis has also been reported in several countries such as the United States, Canada, Northern Europe and New Zealand as well as Australia. The fatality rate of invasive disease ranges from 15 to 30%, but can exceed 50% in cases of STSS [31, 32]. In contrast, rheumatic fever and rheumatic heart diseases are rare in developed countries, while indigenous communities and developing countries are more affected due to overcrowding and poor access to health care [7, 19]. The global burden was estimated to 15.6 million existing cases of rheumatic heart disease (RHD), with 460,000 new cases being diagnosed each year and 349,000 RHD-related deaths each year [7].

The diversity of serotypes and the described changes in epidemiology of GAS indicates that GAS infections still remain a significant health problem in the twenty-first century. The regulated surveillance of GAS disease and of the associated distribution of *emm* types over time would be essential for a better protection against *S. pyogenes* by vaccination for example [32].

3.2 *S. pyogenes* Interactions with the Human Host

Human immune defense mechanisms are a complex and effective system to defend the body from unwanted intruders such as bacteria. Nevertheless from time to time bacteria manage to overcome defenses and to settle down in their new environment. This includes the adherence to, internalization into as well as persistence in host cells and tissue leading to long-term survival of the bacteria. But also the regulation of the processes involved in successful colonization and persistence and of the involved bacterial virulence factors are essential to guarantee its continued existence in the human host. In case of *S. pyogenes* the success in establishing disease can be attributed to its ability to colonize, rapidly multiply and spread in its host. This is facilitated by multiple adhesins that mediate attachment to host extracellular matrix components as well as by powerful invasins which contribute to the process of invasion (see Figure 1, page 18). The interaction with the human host gets even more complicated since many expressed virulence factors have multifunctional properties contributing to complex immune evasion mechanisms that outsmart the usually effective host immune system resulting in disease. The obstacles *S.pyogenes* has to face during the course of establishing infection in the host and the mechanisms it has to use in order to adapt to its new environment to ensure survival and consequently cause disease are briefly discussed below.

3.2.1 Adhesion of *S. pyogenes* to Human Extracellular Matrix

Streptococcal infections have as the common first step adherence of GAS to and colonization by GAS of epithelial cells of the pharynx or the skin. In order to successfully colonize the skin, GAS has to overcome several defense mechanisms/ barriers. For instance, fatty acids are secreted by the skin to inhibit bacterial growth. Additionally, non-specific host defense mechanisms prevent the bacteria from penetrating through the superficial epithelium of the upper respiratory tract [33]. To successfully colonize their host streptococci must replicate. The ability to replicate is affected by a variety of human factors such as the immunglobulins in the human saliva, specificity of those and normal flora of mucosal surfaces.

Despite the fact that *S. pyogenes* faces a variety of biological, mechanical and chemical forces, it is successful in circumventing these obstacles by the production of many

surface components which mediate interaction with host cell receptors. As soon as streptococci overcome unspecific interaction, a temporally limited, weak attachment of the bacteria mediated by lipoteichoic acid (LTA), firm adherence occurs in a second step. Firm adherence relies on the presence and expression of specific adhesins on the bacterial surface [34] which primarily target host extracellular matrix (ECM) proteins. These proteins [35, 36] include among others collagens, which represent the major structural components of the eukaryotic ECM forming different types of interstitial or basement membrane networks [37] as well as laminin, exposed to pathogenic bacteria most frequently seen in damaged or inflamed tissue. and fibronectin, which is not only a substrate for adhesion but serves also as the prototype of adhesion proteins that bind specifically to microorganisms [38, 39]. Prebound fibronectin on the streptococcal surface for instance supports binding to collagen [40]. Also human plasma glycoproteins such as fibrinogen and plasminogen are targeted by *S. pyogenes* to maintain themselves in the host environment.

Among the streptococcal adhesins is the M protein which is the most prominent protein on the streptococcal surface and also the major virulence factor of GAS. It is the basis for more than 100 distinct serotypes defined. The M protein of group A streptococci is an α-helical coiled coil bacterial surface protein often seen as a mosaic of various functional domains: a conserved C-terminal, a variable amino-terminal domain and sequence repeats making up the central helical rod. The sequence repeats bind a variety of host factors [41]. The M protein mediates mainly binding to collagen and fibronectin but it also interacts with the plasma proteins fibrinogen and plasminogen [42, 43].

Since adherence plays a major role for the colonization of *S. pyogenes*, several other surface proteins are expressed which guarantee successful interaction with host extracellular matrix components. Collagen binding proteins that have been described to mediate adherence of streptococcal cells to collagen rich tissues are Cpa, directly binding to collagen type I [44, 45]. This protein is also an example for conceivable tissue site preferences since it displays high affinity binding to human collagen type I [44], a form that is abundant in the dermis of human skin. Also SclA and SclB, both containing collagen-like domains, bind collagen [46-49]. One of the best studied molecules involved in adherence is SfbI protein or its allelic variant protein F1 both binding fibronectin [50-53]. The involvement of protein F1/SfbI in adhesion was shown by the introduction of

the Protein F encoding gene *prtF* into an enterococcal strain which proved solely protein F on itself and not the bacterial background is sufficient for adherence [54]. In contrast, insertional mutagenesis of the *prtF* gene resulted in loss of the ability to bind fibronectin and reversely could be complemented by the introduction of the respective gene on a plasmid [50]. Besides protein F1/SfbI also serum opacity factor mediates fibronectin-binding [55]. Furthermore, it is known to mediate the opacity reaction in human serum [56, 57]. Lbp has been described to bind laminin [58], which is the major noncollagenous adhesive glycoprotein in basement membranes [59, 60]. PAM [61, 62] as well as α-enolase [63], have been reported to bind plasminogen, a key component of the fibrinolytic system. The different adhesins expressed in a given environment on the surface of *S. pyogenes* additionally define the preferential adhesion to a certain type of cells and therefore are responsible for tissue tropism [34]. In summary, colonization of and adherence to epithelial cells represents the first stage of GAS infection mediated by the expression of many various surface molecules interacting with their specific ligand on host cells. The fate of *S.pyogenes*, whether it will be cleared, remains surface bound or will induce its internalization depends on many host factors, such as receptors and immune responses, the strength of physical interaction that occurs between host and GAS as well as the signal GAS sends to either stimulate or antagonize internalization.

3.2.2 Invasion by *S. pyogenes* of Host Cells

Binding of host ECM proteins during streptococcal adherence may lead to intracellular invasion by GAS of host cells. This is a dynamic process that implies the activation of host cell signal transduction pathways by GAS and active participation of the host cell cytoskeletal structure. To initiate host cell signalling, GAS interacts with various host cellular receptors such as integrins, CD46 or CD44.

The interaction with integrins on host cells is crucial for skeletal rearrangements to induce uptake of adherent *S. pyogenes* cells by an actin-mediated "zipper phagocytosis". It can be induced by laminin bound to the surface of *S. pyogenes* [64], but also by plasma-fibronectin bound either to protein F1 or to M protein [65].

The M protein can also bind host CD46, a membrane bound complement regulatory protein, related to factor H which serves as a cellular receptor on keratinocytes [66]. This contact may also initiate proinflammatory responses during infection [67]. Further

signalling events to the host cell are established by binding of streptococcal hyaluronic acid capsule to the host CD44 receptor. This interaction contributes to translocation first, by disrupting intercellular junctions facilitating the uptake of streptococci into deeper tissue and secondly it prevents streptococci from being trapped within epithelial cells [68].

The process of streptococcal invasion is very complex and also involves the secretion of various invasins and toxins which may result in the destruction of host cells and allows *S. pyogenes* to spread among tissues by dissolving host fibrin and intercellular ground substances [8]. The secreted invasins and toxins act in a variety of ways, leading to lysis of eukaryotic cells, including red blood cells and phagocytes and to damage of other host macromolecules like enzymes and informational molecules. Examples for such virulence determinants are Streptolysin S (SLS) and Streptolysin O (SLO). Both are potent cytolytic toxins produced by almost all clinical isolates of group A streptococci [69]. SLO is a 540-amino-acid secreted protein toxin that binds to cholesterol in eukaryotic cell membranes, where it oligomerizes to produce large transmembrane pores leading to cell lysis [70]. Recently, it was demonstrated that SLO acts as a vehicle for translocation of a second GAS protein, NAD-glycohydrolase (NADase), into the cytoplasm of host epithelial cells [71]. SLS is primarily responsible for beta-hemolysis observed surrounding colonies grown on the surface of blood agar media [72, 73]. It can exist in intracellular, cell surface-bound and extracellular forms [74] and can damage other cell membranes, including those of lymphocytes [75], neutrophils and platelets [74] as well as subcellular organelles such as lysosomes [76]. Its toxicity to erythrocytes, leukocytes and smooth muscle has qualified it as an accessory virulence factor [76]. Streptococcal hyaluronidase plays an important role to aid host tissue invasion and is capable of degrading hyaluronic acid, a major component of the extracellular matrix of body tissues [77]. GAS are capable of producing 2 types of hyaluronidase, a bacteriophage associated enzyme and an extracellular one that is secreted from the cell [78]. Two further toxins contribute to the large repertoire of virulence factors used by *S. pyogenes*, Streptodornase and Streptokinase, which possess deoxyribonuclease activity and participate in fibrin lysis, respectively [79].

In summary, the process of invasion by GAS is mediated by a number of streptococcal surface proteins which interact with host cellular receptors to induce their uptake into

host cells but invasion of the host also involves the expression of various invasins and toxins which allow *S. pyogenes* to spread into deeper tissues resulting in damage and destruction of host cells.

3.2.3 Streptococcal Immune Evasion Mechanisms

The interaction of streptococcal cells with host cell surfaces and receptors induces mechanisms of innate and adaptive immunity. Innate immunity includes for example proinflammatory responses, complement activation and phagocytosis. Gram-positive bacteria contain peptidoglycan in their cell wall that activates the alternative complement pathway. One result of complement activation is opsonization and enhanced phagocytosis of the bacteria. Complement by-products such as C5a stimulate inflammatory responses by recruiting and activating leukocytes. Phagocytes use various surface receptors to recognize appropriately opsonized bacteria and upon activation of the phagocytes cytokines are secreted to induce leukocyte infiltration. On the other side virulence of bacteria has been linked to a number of mechanisms that mediate resistance to innate immunity including antiphagocytic mechanisms and inhibition of complement or inactivation of complement products.

Adaptive immunity on the other hand is mainly mediated by antibodies and represents the principle protective immune response to GAS. It functions to block infection, eliminate the microbes and neutralize their toxins. The antibody responses against bacteria are directed against cell wall antigens, secreted and cell-associated toxins, which may be polysaccharides or proteins. Effector mechanisms used by antibodies to combat streptococcal infections include neutralization, opsonization and phagocytosis as well as activation of the classical complement pathway. Neutralization is mediated by high affinity IgG and IgA isotypes, opsonization by IgG1 and IgG3 and complement activation by IgM and IgG1 and IgG3 isotypes. Activation of $CD4^+$ helper T cells leads to the production of cytokines to stimulate antibody production, to induce local inflammation (tumor necrosis factor, lymphotoxin) and to enhance the phagocytic and microbicidal activities of macrophages (IFN-γ) and neutrophils which participate in defending *Streptococcus pyogenes*.

S. pyogenes has evolved various virulence mechanisms that counteract human host defenses which contribute to GAS pathogenesis and guarantee their successful survival within its host. These immune evasion mechanisms are briefly described below.

3.2.3.1 Inhibition of the Alternative Complement System by *S. pyogenes*

The complement system represents an important component of the host defense system as the first line of defense against invading microorganisms [80]. It consists of a set of plasma proteins which interact in a complex cascade of events. The result is the generation of bioactive molecules which mediate opsonic, chemotactic and lytic functions against invading microorganisms. The capsule of *S. pyogenes* inhibits complement activation and the M protein supports streptococcal infections by binding fibrinogen. Interaction of plasma fibrinogen with M protein masks C3b-binding sites on the streptococcal surface [81]. Binding of factor H, a serum control protein of the alternative complement pathway, leads to the inhibition of the alternative C3b convertase [82]. The deposition of large numbers of molecules of complement fragment C3b on the surface of microorganisms which thereby become accessible to uptake by phagocytic cells is essential for opsonising the bacteria [41, 83, 84]. Binding of fibrinogen or factor H prevents complete opsonisation of the Streptococcus. However immune opsonisation is not inhibited by either fibrinogen or factor H.

3.2.3.2 GAS Inhibits Polymorphonuclear Neutrophil Recruitment

Circulating polymorphonuclear neutrophils (PMNs) are recruited to sites of infection by chemokines, cytokines, matrix metalloproteases and products produced by the invading microorganisms. Host derived chemotactic factors are critical in the regulation of inflammatory responses. Disrupting PMN recruitment is one strategy used by GAS to evade innate immunity By the expression of streptococcal C5a peptidase, ScpA, GAS attacks complement derived chemotaxins C5a [85]. C5a peptidase is highly specific for the alternative complement component C5a and is strategically bound to the bacterial surface near the source of C5a which is generated by interaction of streptococci with the alternative complement pathway. Although several virulence factors are known to attack complement components, they are non-specific and act on a variety of proteins [86, 87]. ScpA is unique in that it acts on C5a specifically which delays infiltration of phagocytes, retarding clearance of bacteria from mucosal surfaces [88].

Besides targeting inflammatory responses by the elimination of C5a at the bacterial surface [88], IL-8, the neutrophil activating peptide 1, was recently shown in association with necrotizing fasciitis to be targeted for degradation [89]. IL-8 is generally produced as a consequence of neutrophil activation which further amplifies the inflammatory response [90] by mediating endothelial translocation and subsequent recruitment of neutrophils to the site of infection. The cell envelope serine proteinase Spy0416 (ScpC) was identified to specifically cleave IL-8 between ^{59}Q and ^{60}R resulting in conversion from an 8 kDa to a lower molecular-weight species of 6 kDa [91]. This lead to a paucity of neutrophils at the site of infection, because the inactivated form of IL-8 lacks the necessary biological activity to promote neutrophil transmigration.

3.2.3.3 Antiphagocytic Behavior of *S. pyogenes*

Phagocytes seem to be very efficient in eliminating streptococci since the bacteria do not produce catalase or significant amounts of superoxide dismutase to inactivate the oxygen metabolites (e.g. hydrogen peroxide, superoxide) produced by the oxygen-dependent mechanism of phagocytes. Therefore, pathogens can be quickly killed after engulfment by phagocytes. The streptococcal defense must be one to stay out of phagocytes [8]. It is well established that group A streptococcus causes diseases attributed partially to its ability to resist phagocytosis by human neutrophils due to surface exposed M protein and hyaluronic acid capsule. Inhibition of phagocytosis is principally due to the ability of M protein to bind fibrinogen [81] or complement control protein factor H which inhibits non-immune opsonisation [83]. Furthermore, streptococcal cell surface structures including hyaluronic acid capsule contribute to resistance to phagocytosis [92]. Hyaluronic acid capsule does not seem to block C3 deposition on the surface of GAS. Rather, it acts as a physical barrier to prohibit direct interaction of PMNs with opsonins on the bacterial surface [93].

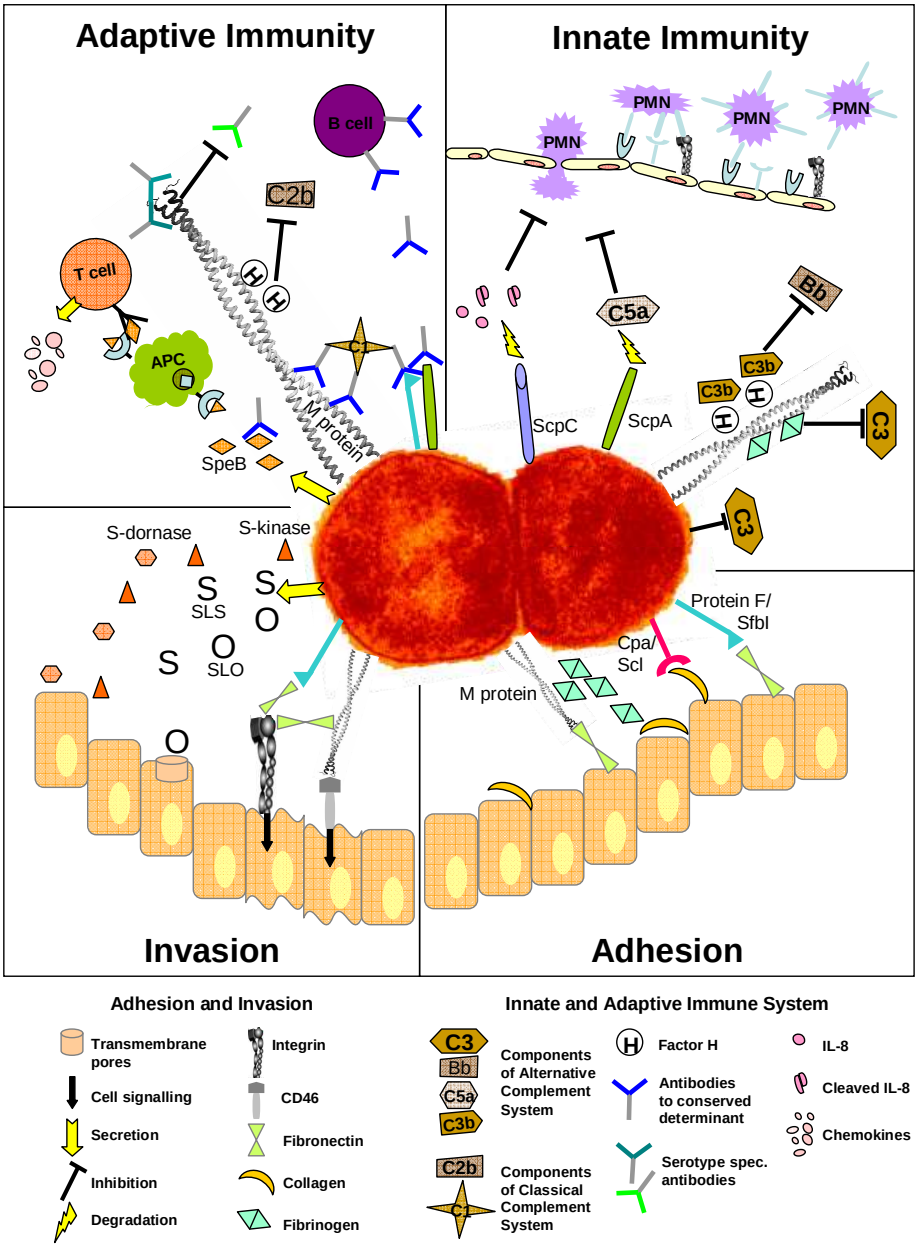

Figure 1: Interactions of *S. pyogenes* with the human host in terms of adhesion, invasion, innate and adaptive immunity

Adhesion processes involve interaction of *S. pyogenes* M protein and Protein F/SfbI with fibronectin as well as Cpa or Scl with collagen. Interaction with plasma glycoproteins (fibrinogen and plasminogen) is mediated via the M protein. Invasion processes involve M protein interaction with CD46 cellular receptor and/or interaction of M protein and SfbI via plasma fibronectin with integrins to establish signalling events into the cell and induce actin-mediated "zipper phagocytosis". Invasion is supported by secretion of toxins by *S. pyogenes* including streptodornase (S-dornase), streptokinase (S-kinase) involved in fibrin lysis as well as potent cytolytic toxins including streptolysin S (SLS) and streptolysin O (SLO).

To protect itself from innate immunity, proinflammatory actions for the recruitment of phagocytes to the infection site are reduced by ScpA and ScpC degrading C5a and IL-8, respectively. The alternative complement system is inhibited by M protein that binds factor H, a complement control protein and fibrinogen. Adaptive immunity is the principle protective immune response. Factor H binding to streptococcal M protein inhibits the classical complement pathway. Antibodies against conserved determinants found in the C-terminal region of the M protein, Protein F/SfbI, ScpA and also toxins are effective in inducing opsonisation and neutralization, respectively. Humoral immune defenses can be avoided by genetic sequence variation of the M protein N-terminal domain. Strong cytokine secretion is induced by binding of SpeB (pyrogenic exotoxin) to MHC class II molecules outside the peptide binding cleft of APCs (antigen presenting cells) which leads to activation of $CD4^+$ T cells.

3.2.3.4 Evasion of Humoral Immune Responses

The major mechanism used by bacteria to evade humoral immunity is genetic variation of surface antigens. An example is the M protein. Type-specific M protein antibodies directed to the exposed extreme amino-terminal ends of M protein epitopes result in activation of the classical complement pathway through deposition of C3b and subsequent phagocytosis [8, 79]. Extreme genetic variation at exactly the amino-terminal ends of the M protein protects strains of different M types from being immediately eliminated by antibodies. Even in the situation of increased defense by the host *S. pyogenes* again demonstrates adaptation. Furthermore, late complications of humoral immune response to bacterial infection can be the generation of disease-producing antibodies attributed to the conserved C-terminal region of the M protein. Some of the antibodies that target the C-terminus of the streptococcal M protein cross react with myocardial sarcolemmal proteins and myosin and are deposited in the heart and subsequently cause carditis [94].

T cells are targeted by streptococcal pyrogenic exotoxins (SpeA, SpeB, SpeC, SpeD) which can trigger and increase cytokine release of T cells. These exotoxins do not require processing by antigen presenting cells due to their ability to stimulate T cells by binding class II MHC molecules outside the peptide binding cleft. The independence of polymorphic differences in MHC leads to a massive detrimental cytokine release [8, 95-97] promoting injury to surrounding tissues. These exotoxins are powerful toxins as they can potentially trigger diseases such as toxic shock and autoimmunity and therefore play an important role in *S. pyogenes* survival within its human host. Cytokines such as tumor necrosis factor, IL-1, IL-6, and IFN-γ have been implicated as central contributors to tissue destruction and death due to shock triggered by streptococcal infection [79]. Primarily chemokines stimulate the activation and migration of neutrophils into tissue from peripheral blood.

This summary only briefly described some of the streptococcal immune evasion mechanisms used by GAS to successfully establish disease and are not the only ones used in the repertoire of Group A streptococcal pathogenesis. There are other mechanisms described in literature [79, 98-100] and there might be discovered many more in the future.

3.3 Treatment and Prevention

Penicillin, a beta-lactam antibiotic, is to date the drug of choice for treating most GAS infections and resistance to penicillin was not reported until today. The primary goal of antibiotic therapy for patients with streptococcal pharyngitis is eradication of the organism [101]. Nevertheless the evaluation of the efficacy of penicillin for treatment of GAS pharyngitis has revealed lower than expected eradication rates from the upper respiratory tract [101]. The primary treatment for GAS infections in patients with β-lactam hypersensitivity or chronic, recurrent pharyngitis due to prior treatment failure are thus macrolides and lincosamides. In recent years, resistance to macrolides such as erythromycin, the second drug of choice, was reported to emerge [102]. Interestingly, the increased use of macrolide antibiotics is correlated with an increased rate of resistance to erythromycin among group A streptococcal isolates [103, 104]. In Finland, the rates of erythromycin resistance among group A streptococci prompted an intense effort to decrease the use of macrolide antibiotics, which in turn led to a dramatic reduction in the prevalence of erythromycin-resistant strains [105]. Although there is no resistance to penicillin registered so far, reports about increasing antibiotic resistance in different bacteria such as *Streptococcus pneumoniae* [106] or *Staphylococcus aureus* [107] and also recently observed increased properties of insusceptibility to penicillin for example in group B streptococci [108] are concerning [109]. Penicillin resistant *S. pyogenes* strains would increase the disease burden dramatically since penicillin is uniquely effective in the treatment of GAS infections and still this treatment is not optimal because treatment failures do occur [1]. One problem with antibiotic treatment might be the intracellular localization of GAS which probably protects the bacteria from β-lactam action and might account for recurrent pharyngotonsillitis [110]. *In vitro* studies show that the ability of GAS to persist in the throat following antibiotic therapy is in accordance with their capacity to adhere to and be internalized by epithelial cells [1]. For instance, it could be shown that the proportion of *prtF1*-positive strains was significantly higher among patients with eradication failure than among those with successful eradication of bacteria, thus promoting a carrier state [111]. Neeman's data indicated that *prtF1*-positive strains isolated from carriers enter cultured epithelial cells much more efficiently than strains lacking this gene. Inadequately or untreated streptococcal pharyngitis can lead to rheumatic fever and rheumatic heart disease which is the case

for about 3% of individuals. Even though in the United States for example outbreaks of rheumatic fever are relatively rare [13], as many as 1% of school-age children in developing countries are estimated to have rheumatic heart disease [112].

Another problem represents the excessive global usage of antibiotics. Whenever symptoms of a sore throat and high fever occur, symptoms are quickly eradicated with antibiotics, without careful diagnosis, which increase concerns that penicillin-resistant strains may appear since there might occur selective pressure for resistance. Therefore, there is a strong motivation and need to develop a safe and effective vaccine against group A streptococcal pharyngitis [1].

3.4 Vaccine Approaches to Protect Against GAS Pharyngitis

Prevention of severe diseases relies on the diagnosis and efficient treatment with penicillin. Although so far *S. pyogenes* remains susceptible to penicillin, resistance to different antibiotics has been reported with an increasing frequency [102, 113-115]. Most significantly, GAS pharyngitis contributes to approximately 20% to antibiotic prescriptions for acute respiratory illnesses in the Unites States [116]. The eradication or at least significant reduction of carriage of *S. pyogenes* in the human pharynx would have profound effect on the dissemination of this organism in the population and on the initiation of streptococcal disease. Even in cases of the highly invasive strains of streptococci, there is not much evidence that they originate from other than pharyngeal sources [1]. Therefore, vaccination clearly constitutes an attractive alternative strategy to control GAS infections not only to significantly reduce the burden of invasive and non-invasive disease, but also to reduce antibiotic use and thus development of resistance in group A streptococci.

Vaccines take advantage of the adaptive immune system which is based on a specific repertoire of B cell-derived antibodies and T cell presented receptors that bind to antigen of an intruding microbe [117]. The pathogen-specific antigens formulated in vaccines are capable of inducing protective B and T cell responses. Traditional vaccines contain inactivated/attenuated microbes in which whole microbes seem to deliver the set of antigens needed to induce a repertoire of protective antibodies and T cells such as in case of vaccines directed against *Bordetella pertussis* and *Vibrio cholerae*. These traditional

vaccines indeed disclosed that a proper set of antibodies optimally mounted against a single antigenic structure like surface carbohydrate or a particular protein can be sufficient to provide protective immunity. In case of *S. pyogenes* it is getting more complicated due to the complexity of the pathogen as a single antigen is not sufficient to provide protection against the whole range of serotypes.

3.4.1 M protein Based Vaccine Approaches

A prime vaccine candidate has been the M protein since it could be shown that M protein-specific human and animal antibodies have the capacity to opsonize streptococci in preparation for phagocytic clearance [1, 46]. Therefore, studies were designed to develop a GAS vaccine focusing on the variable amino-terminal and conserved carboxy-terminal regions of the M protein [118]. Although the variable N-terminal region of the M protein induces opsonic and protective antibodies which overcome the antiphagocytic property, protection is only serotype specific. On the other hand, vaccines based on conserved domains located in the carboxy-terminal region have been reported to evoke protection against strains of heterologous M serotypes, but also may induce harmful cross-reacting antibodies [119, 120]. This cross reactivity is based on significant sequence homology of M protein with mammalian fibrillar proteins including type I keratin, myosin and human α-tropomyosin [121]. B cell epitopes have been identified within the M protein that induce antibodies that show cross reactivity with human heart tissue, joint but also kidney and brain [120-124].

Thus multivalent M protein vaccines were designed which contain epitopes from the amino-terminal type specific regions of different M proteins in tetravalent, octavalent and 26-valent vaccines [125-127]. In the development of type-specific epitope-based vaccines, still one has to consider the prevalence of specific serotypes within a population [126] and the variability of M protein structures within a serotype [128-130]. It is unrealistic to predict that highly complex multivalent M protein based vaccines will prevent all group A streptococcal infections [126].

Vaccine approaches using the conserved C-terminal region of the M protein were also taken into consideration [131, 132]. For instance overlapping synthetic peptides of the conserved region of the M6 protein were covalently linked to the mucosal adjuvant cholera toxin B subunit [131]. Another approach was investigated by Olive and

colleagues with a peptide vaccine that contains a conserved M protein B cell epitope and lacks a T cell autoepitope which elicited protective immunity at the mucosal level in mice [133]. Further attempts using M protein conserved region as a streptococcal vaccine were undertaken with vaccinia virus as vector system [1, 134].

Combination of multiple M protein amino terminal peptides and a conserved region peptide demonstrated complete protection in mice parenterally upon challenge with different GAS strains [135, 136].

Although approaches to design multivalent M protein vaccines containing epitopes from the amino-terminal type specific regions of different M proteins [127], the conserved C-repeat region of this antigen [131, 133] or combinations of them [135-137] have proven to be reasonably effective in inducing protective immunity against *S. pyogenes* infections in experimental animal models, individual consideration led to the conclusion that they either do not fulfil all the requirements of broad coverage and safety or they have failed conferring full protection against a lethal challenge [138].

3.4.2 Protective *S. pyogenes* Antigens Other than M protein

To circumvent problems that might occur with vaccines solely based on M protein, attempts have been made to identify novel protective GAS antigens. Several protective antigens were found to induce protective immune responses in animals and were therefore considered as vaccine candidates. For example the group A streptococcal C5a peptidase, which cleaves the human chemotaxin C5a and prevents its binding to polymorphonuclear neutrophils, was targeted as a potential vaccine candidate, since it was shown to lack serotypic diversity [118, 139]. The intranasal administration of a defective form of streptococcal C5a peptidase molecule to mice showed protection when challenged with heterologous serotypes [140]. Furthermore it stimulated significant levels of specific salivary secretory IgA which increased clearance of streptococci from intranasally infected mice [140]. C5a peptidase antibodies in acute and convalescent sera from children with streptococcal pharyngitis were also detected [141]. Studies have also shown that more than 70% of adults have measurable IgA directed at C5a peptidase (ScpA) in their saliva [142].

A different strategy was targeted by Musser and his group working on the group A streptococcal cysteine protease SpeB [143]. Since it was found that a mutant lacking SpeB

expression lost nearly all its ability to cause death in mice they could show that both passive and active immunization with rabbit IgG to cysteine protease and cysteine protease itself, respectively, foreshortened time to death but did not prevent it. Also pyrogenic exotoxin A and C (SpeA and SpeC) are considered as potential vaccine candidates. Selected multiple-site mutants of SpeA as toxoids [144] and two toxoid mutants of pyrogenic exotoxin C [145] were shown to be protective when used as immunogens in rabbits challenged with a lethal dose of wildtype SpeA or wildtype SpeC, respectively. Other streptococcal proteins shown to produce protective immunity to heterologous streptococcal serotypes are for instance serum opacity factor [146] and the fibronectin binding domain of SfbI [147]. SfbI components were even described to have immune modulatory properties resulting in improved cellular and humoral immune responses at both systemic and mucosal levels [147, 148]. In addition, streptococcal cell surface heme-binding protein, Shp, has been shown to induce production of anti-Shp antibodies in mice with bactericidal activity [149]. Furthermore SLO was found to elicit antibody responses with markedly different subclass profiles than seen for the M protein which supports its utility as a vaccine candidate and possible implications for IgG subclass regulation [150]. Antibodies to SLO were predominantly IgG1 with only minor contributions from other subclasses while M protein specific antibodies were distributed between IgG1 and IgG3 subclasses. In recent years more and more potential candidates have been identified for a vaccine against group A streptococcal infections. Genome sequencing coupled with advances in technology has permitted development of rapid strategies to identify potential new vaccine candidates. Postgenomic analyses for example led to the identification of *spy0843* which encodes a cell surface protein and can confer protection against intraperitoneal challenge in a mouse model of GAS infection [151]. Attempts to find new GAS candidate vaccine antigens among putative lipoproteins were made by Lei and colleagues since lipoproteins are a major class of cell surface exposed proteins and play critical roles in nutrient uptake, antibiotic resistance, adhesion as well as protein secretion [152]. Five extracellular lipoproteins were identified: Spy0385, a lipoprotein component of an ABC transporter possibly involved in iron acquisition, Spy1245, part of a putative phosphate transporter, Spy1274, a component of a putative amino acid ABC transporter, Spy1390, a homologue of the protease maturation protein PrtM of *L. lactis*

and Spy1558, a thioredoxin homologue. All five proteins stimulated the production of antibodies that significantly inhibited *in vitro* growth of GAS in blood as assessed by commonly used bactericidal activity assay. Recently another vaccine candidate was published, which has also already been identified by our ANTIGENome Technology [153]. The IL-8 degrading cell envelope serine proteinase Spy0416 (ScpC) was shown to mediate protection against lethal *S. pyogenes* infection in mice [154].

The increasing knowledge about pathogens shows that streptococcal infection is a highly complex process and that a vaccine targeting a single antigen might not be sufficient to control all pathogenic aspects of the bacteria and protect the host against their colonization. A type-specific vaccine which is capable of protecting against a streptococcal infection therefore necessitates a multivalent antigen corresponding to stable immunodeterminants on serotypes that together account for the majority of nasopharyngeal isolates prevalent within the population at a given time and the absence of cross reactivity with its host tissue [1].

3.5 Antigenome Technology Applied to GAS

Several approaches were recently applied to identify novel vaccine candidates from GAS based on proteomic methodologies, also taking advantage of the availability of several genomic GAS sequences [154-156]. These studies have provided evidence for the surface localization of numerous group A streptococcal proteins, some of them without predictable signatures for surface localization. In spite of these efforts, so far only one of the identified surface proteins, Spy0416, was shown to mediate protection against *S. pyogenes* infection [154].

By the use of the ANTIGENome technology (see Figure 2) protective vaccine candidates have already successfully been identified from *S. aureus* [157], *S. pneumoniae* [158] and several additional bacterial pathogens (unpublished data). This technology was also employed to *S. pyogenes* for the comprehensive identification of novel conserved and protective antigens suitable for vaccine development to prevent GAS infections. The underlying technology combines genome-derived peptide libraries which comprise nearly the complete protein repertoire of a pathogen with comprehensive collections of antibodies which derived from individuals exposed to *S. pyogenes* [117]. For the library

construction the streptococcal strain SF370 was chosen. After randomly shearing the pathogen's genome into 30-300 bp DNA fragments and introduction of the fragments into a frame-selection vector system [157, 159] for the elimination of those random sequences that possess a stop codon within the expressed reading frame, the library was directly and in-frame transferred into a display vector. This allowed efficient presentation of fragment-encoded peptides on the surface of *Escherichia coli* in context of outer membrane proteins LamB, FhuA and BtuB [117, 153] which are characterized by flexible loops. In this context it could be assumed that the foreign peptides inserted within such a loop may potentially adopt a structural configuration that comes close to its natural structure. The three genomic libraries were constructed in the LamB, BtuB and FhuA platforms, representing 0.5, 0.15 and 0.35 million clones with average sizes of 40, 100 and 350 bp, respectively, covering the *S. pyogenes* SF370 genome more than 50 times [153, 159].

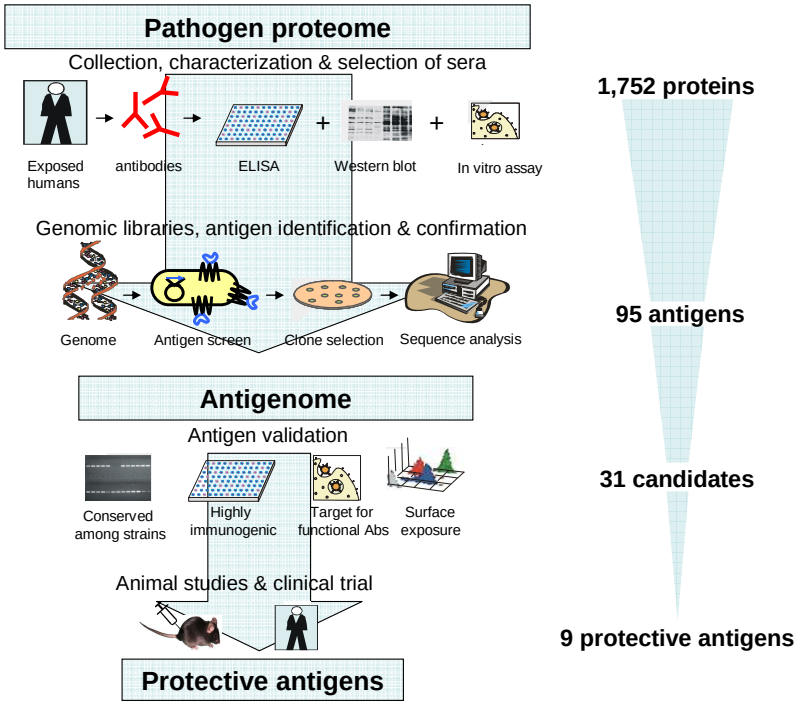

Figure 2: Scheme of the antigen identification and validation approach [117]
Selection procedure of protective streptococcal antigens out of a pool of proteins encoded by the pathogen.

Human sera which provide imprints of pathogen encounters were used to screen the previously generated libraries for antigens expressed by the pathogen during infection *in vivo*. Sera have been collected from patients with acute *S. pyogenes* infections, such as pharyngitis, wound infection and bacteraemia (based on medical microbiological tests).

As another source for human sera served uninfected healthy adults, since group A streptococcal infections are common and antibodies are present as a consequence of natural immunization from previous encounters with streptococci. The serum samples from patients and healthy individuals respectively were characterized for anti-*S. pyogenes* antibodies by immune assays to select the most appropriate screening reagents. For the assessment of anti-bacterial antibody-levels of high affinity (IgA and IgG) ELISA was performed using total bacterial extracts or whole cells as well as secreted components isolated from culture supernatant. Sera with the best reactivity were further used for the antigen identification. This selection process was performed via magnetic bead cell sorting (MACS screening) where *E. coli* cells displaying the genomic peptide library from SF370 strain were incubated with purified and biotinylated antibodies from previous selections. Those cells that displayed the peptides recognized by the specific antibodies were selected by binding to streptavidin coupled paramagnetic beads allowing the separation by magnetic force. The antigenicity of the selected bacterial clones was then confirmed by western blot analyses. The screens identified 95 antigen candidates annotated in the *S. pyogenes* SF370 genome and 55 peptides that could not be assigned to an annotated open reading frame.

In order to select the most promising candidate antigens for further evaluation in animal models of group A streptococcal disease, several independent selection criteria based on a variety of *in vitro* assays were used:

» The presence of the corresponding genes was tested by PCR and sequence analysis in a set of 50 clinical GAS isolates encompassing 15 different serotypes.

» Peptide ELISA served to evaluate the reactivity of antigenic peptides corresponding to the identified antigenic epitopes with multiple individual sera to identify the most immunogenic epitopes.

» Surface localization of the antigenic proteins on the pathogen was evaluated by bioinformatics and FACS analysis.

» All antigens were analyzed by bioinformatics, lack of homology with human proteins and for their intellectual property status.

The described validation steps generated a ranking list of approximately 31 candidates that were recombinantly expressed in *E. coli* to test their potency to protect against *S. pyogenes* in animal models. Three different animal models were established and survival of the mice was monitored as readout for protection. These animal models included systemic immunization of CD-1 mice 3 times in an interval of 2 weeks with respective recombinant antigens applied with CFA/IFA or Aluminum hydroxide and challenge one week after the last booster immunization either with *S. pyogenes* AP-1 intravenously or alternatively with *S. pyogenes* A20-MA intranasally. The third animal model consisted of intranasal immunization of BALB/c mice with the recombinant antigen co-administered with IC31®-low and challenged with A20-MA intranasally. Based on the data from all three animal models nine group A streptococcal proteins have been selected, Spy0269, Spy0292, Spy0416, Spy0488, Spy0872, Spy0895, Spy1536, Spy1666 and Spy1727 as the most promising candidates for further characterization and vaccination studies. In this work the focus is particularly set on Spy0895, Spy1536 and Spy0416.

In summary, the antigen identification approach thus reduced the complex GAS proteome (1752 proteins) to an 'antigenome' consisting of a smaller subset of approximately 100 antigenic proteins. Among the most frequently identified antigens were most of the previously published antigens and protective proteins, such as M1 protein (Spy2018) [46], C5A peptidase (Spy2010) [160], Streptolysin O (Spy0167) [150] and Exotoxin B (Spy2036) [161], Spy0843 [151] as well as in the meantime published ScpC (Spy0416) [154]. This latter observation clearly confirmed that the ANTIGENome technology selects valuable vaccine candidates and thereby giving credibility to the approach [153].

4 STUDY OBJECTIVES

The aim of the present work was the characterization of protective antigens identified by Intercell's ANTIGENome Technology and selected as candidates for a prophylactic vaccine for the prevention of infections caused by group A streptococcus. Out of nine promising vaccine candidates, Spy0416, Spy0895 and Spy1536 have been chosen for further characterization.

The further biochemical characterization of Spy0416 was based on the work of my diploma thesis [162, 163], where I could demonstrate for the first time *in vitro* degradation of IL-8 by recombinant Spy0416. Particular focus was set on the contribution of the distinct C-terminal domains of Spy0416 to IL-8 degradation. Additionally, other CXC and CC chemokines were examined as substrate for degradation by Spy0416.

Secondly, by generation of gene deletion mutants I aimed to assess the contribution of the encoded Spy0416, Spy0895 and Spy1536 proteins to GAS pathogenesis. Due to the large size and complex protein structure of Spy0416 it was speculated that it has additional functions besides its IL-8 degradation activity. In case of Spy0895 which is annotated as putative histidine protein kinase, I was hoping to reveal a potential involvement into a two component system. For Spy1536 on the other hand, annotated as conserved hypothetical protein, not even a putative function was assigned so far. Therefore, the availability of deletion mutants was expected to facilitate a thorough characteristic including the assessment of growth defects under different environmental conditions and the contribution of the genes to virulence in mice. Further it was planned to investigate the interaction of the mutants with host proteins as well as the effect of gene deletion on expression of surface proteins and transcription.

5 MATERIALS AND METHODS

5.1 Materials

5.1.1 Laboratory Equipment

Blockheater QBT2	Grant Instruments Ltd, England
Centrifuges	HERAEUS Megafuge 2.0, Kendro Lab. Products, England
	HERAEUS Multifuge 3S-R, Kendro Lab. Products, England
	HERAEUS Biofuge Fresco, Kendro Lab. Products, England
	EPPENDORF Centrifuge 5415 R, Eppendorf, Germany
E. coli Pulser	Bio-Rad, USA
FluorChem SP Imaging System	Biozym, Austria
GFL Rocking Shaker 3013	Gesellschaft für Labortechnik, Germany
Hybridization shaker	Amersham,
Incubator	HERA Cell 150, Kendro Lab. Products, England
	HERAEUS Kelvitron-T
Laminar flow	HERA Safe Laminar Flow KS12, Kendro Lab. Products, England
Microplate Reader	Tecan Sunrise, Austria
Microscope	Nikon Eclipse E200, Austria
Optimax X-Ray Film	Protec Medizintechnik GmbH & Co KG, Germany
pH-meter	Mettler Toledo Seven-easy, Germany
Powersupply Power Pac	Bio-Rad, USA
Power washer PW 96	Tecan, Austria
Scales	Sartorius TE1502S, Germany
	Sartorius CP224S, Germany

Sonoplus HD2070, UW2070	Bandelin, Germany
Spectrophotometer - Ultrospec 3300 pro	Amersham Biosciences, England
Synergy2 ELISA reader	BioTek, Germany
Thermocycler	Biometra T3000, Germany
	Biometra T3, Germany
	Biometra T-Gradient, Germany
Thermomixer compact	Eppendorf, Germany
Trans-Blot® SD semi-dry	Bio-Rad, USA
UV Transilluminator	Life Technologies, Germany
Vacuum pump	Neuberger, KNF Comfort

5.1.2 Additional Equipment

Amersham™ CyScribe™ cDNA Post labelling Kit	GE-Healthcare
BCA™ Protein Assay Kit	Thermo Scientific
Blood Agar plates	BioMerieux
Bromphenolblue	Neolab Migge Laborbedarf Vetriebs GmbH
Chemiglow™	Alpha Innotech Corp.
Centrifugal device Nanosep 10K Omega	PALL Life Sciences
Chang cells (clone 1-5c-4, Wong Kilbourne Derivative (D) of Chang conjunctiva)	ATCC
Detroit 562 cells	ATCC
DIG Easy Hyb	Roche
DIG labelled RNA Molecular Weight Marker	Roche
DIG Wash and Block Buffer Set	Roche
DNA Molecular Weight Marker II	Roche
DNaseI, amplification grade	Invitrogen
ECL detection kits	Amersham Biosciences
E. coli Pulser® Cuvettes	Bio-Rad
Electroporation cuvettes, 2mm	Peqlab
EMEM	BioWhittaker

Expand High Fidelity PCR System	Roche
F-12K Medium (Kaighn's Modification of Ham's F-12 Medium)	ATCC
Human collagen I and IV	Sigma
Human fibrinogen	Sigma
Human fibronectin	Sigma
Human haptoglobin	Sigma
Human laminin	Sigma
Human plasminogen	Sigma
iBlot® Gel Transfer Device	Invitrogen
iBlot® Gel Transfer Stacks (nitrocellulose, regular)	Invitrogen
Illustra™ CyScribe™ GFX™	GE-Healthcare
1 kb Plus DNA Ladder	Invitrogen
Lane Marker reducing sample buffer	Pierce
Light Cycler 480 SYBR Green I Master	Roche
Luminescent Detection Kit and ready to use CSPD	Roche
Ni-NTA agarose beads	BioRad
Nitrocellulose membranes (0.45 Micron)	Hybond™
Page Ruler™ Plus Prestained Protein Ladder	Fermentas
PCR DIG Probe Synthesis kit	Roche
Poly Prep™ slides	Sigma
ProLong gold antifade reagent containing DAPI	Invitrogen
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR purification Kit	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
Restriction enzymes	New England Biolabs® Inc.
RiboPure™–Bacteria Kit	Ambion
SDS-Page PAGEr® Duramide Precast Gels	Cambrex
Sigma Wide Range marker	Fermentas
Simply Blue Safe Stain	Invitrogen
Slide-A-Lyzer dialysis cassette	Pierce
SOC medium	Invitrogen

SuperScript III First-Strand Synthesis System	Invitrogen
TC Microwell 96F plates	Nunc
TOPO-TA Cloning Kit	Invitrogen
Twin.Tec real-time PCR Plates 96	Eppendorf
Wizard® Genomic DNA Purification kit	Promega

5.1.3 Chemicals and Reagents

ABTS	Sigma-Aldrich
Acetic acid	Fluka
Aceton	Sigma-Aldrich
AEBSF	Serva
Agarose, electrophoresis grade	Invitrogen
Albumin Bovine Serum (BSA) Fraction V	Biomol
Ammonium Chloride	Sigma-Aldrich
Ammonium Hydroxide	Sigma-Aldrich
Amphotericin B	Invitrogen
Aprotinin	Sigma-Aldrich
APS (Ammoniumperdisulfat)	Sigma-Aldrich
Bestatin	Sigma-Aldrich
Bromphenol Blue	Sigma-Aldrich
BugBuster® Protein Extraction Reagent	Novagen
Calcium carbonate	Sigma-Aldrich
Complete Freund´s Adjuvant	Difco
DEPC	Invitrogen
DMEM (1x) high Glucose	PAA
E-64	Serva
EDTA	Sigma-Aldrich
Ethanol	Merck
Ethidium Bromide	Fluka
Erythromycin	Sigma-Aldrich
Fetal Bovine Serum	Sigma-Aldrich
FITC Isomer I	Sigma-Aldrich

Formaldehyd Lösung, 37%	Neolab Migge Laborbedarf Vetriebs GmbH
Formamide	Sigma-Aldrich
Gentamicin (10 mg/ml), liquid	Invitrogen
D-(+) Glucose Ultra, 99.5%	Sigma-Aldrich
Glutaraldehyde	Merck
Glycerol, 87 %	Fluka
Glycerol, 99%	Sigma-Aldrich
Glycine	Fluka
HBSS	Gibco
Hydrochloric Acid, 37%, A.C.S. reagent	Fluka
HEPES	Fluka
Imidazole	Sigma-Aldrich
Incomplete Freund´s Adjuvant	Difco
IPTG	Sigma-Aldrich
Kanamycin Sulfate	Gibco
Leupeptin	Serva
L-Glutamine	Invitrogen
Lysozyme	Sigma-Aldrich
Magnesium chloride	Sigma-Aldrich
MEM nonessential amino acids	Invitrogen
2-Mercaptoethanol (50 mM)	Invitrogen
Methanol	Fluka
MOPS, minimum 99.5 titration	Sigma-Aldrich
Mutanolysin from Streptomyces globisporus	Sigma-Aldrich
Ni-NTA-Agarose®	Qiagen
PEG- Solution	Sigma-Aldrich
Penicillin-Streptomycin-Glutamine (100 x, liquid)	Invitrogen
Pepstatin A	Serva
PMSF	Sigma-Aldrich
Ponceau Solution (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid)	Sigma-Aldrich

2-Propanol	Fluka
Simply Blue Safe Stain Solution	Invitrogen
Skim Milk Powder	Fluka
Sodium Acetate	Merck
Sodium Carbonate	Sigma-Aldrich
Sodium Chloride	Sigma-Aldrich
Sodium Dodecyl Sulfate (SDS)	Gibco™
Sodium Hydrogen Phosphat	Merck
Sodium Hydroxide	Sigma-Aldrich
Sodium Pyruvate	Invitrogen
TEMED (N,N,N,N-Tetra-methyl-ethylendiamine)	Bio-Rad
TRIZMA®base, minimum 99.9%	Sigma-Aldrich
Trypsin, 0.05% (1X) with 0.53 mM EDTA 4Na	Invitrogen
Tween® 20	Sigma-Aldrich
Triton X100	Fluka
UltraPure™ Phenol:Water (3.75:1 v/v)	Invitrogen
X-Gal (5-Bromo-4-chloro-3-indolyl β-D-galactoside)	Sigma-Aldrich

5.1.4 Buffers

Binding Buffer	0.5 M NaCl, 20 mM NaH_2PO_4, 20 mM Imidazole/PBS
Coating buffer:	0.1 M sodium carbonat, pH 9.3
Deionized formamide	500 ml formamide stirred with 50 g AG 501-X8 ion exchange resin, filtrated and stored at RT
Denaturation solution	0.5 M NaOH, 1.5 M NaCl
10 x DNA loading buffer	250 mg bromphenol blue dissolved in 40 ml water containing 20 g sucrose in a total volume of 50 ml
Electroporation buffer	0.3 M glucose in PBS
Electrophoresis/TAE buffer:	196 mM glycine, 0.1% SDS in 50 mM Tris-HCl pH 8.3 for one liter
High Stringency buffer	0.5x SSC, 0.1% SDS or 0.1% SSC, 0.1% SDS
Low Stringency buffer	2x SSC, 0.1% SDS

10x MOPS buffer	200 mM MOPS, 50 mM sodium acetate, 20 mM EDTA, pH 7
Neutralization solution	0.5 M Tris-HCl pH 7.5, 1.5 M NaCl
PBS-T	PBS/ 0.1% Tween-20
PIC I (100x)	Leupeptin (10 mg/ml), Pepstatin A (1 mg/ml in EtOH), Aprotinin (5 mg/ml in HEPES, pH 8.0)
PIC II (100x)	4 mM Bestatin, 1 mM E-64, 10 mM AEBSF
SDS-running buffer	1xPBS/ 0.1% SDS
20x SSC	3 M NaCl, 0.3 M sodium citrate $2H_2O$
Western blot transfer buffer	39 mM glycine, 48 mM TRIZMA®base, 0.037% SDS, 20% methanol

5.1.5 Antibodies

Dilutions used in Western blot and Immunofluorescence experiments are indicated in brackets.

Penta-His antibodies (1:5,000)	Qiagen
Polyclonal goat anti-human CXCL8/IL-8 (1:1000)	R&D Systems
Polyclonal goat anti-human CXCL11/I-TAC (1:1000)	R&D Systems
Polyclonal goat anti-human IP-10 (1:1000)	R&D Systems
Polyclonal goat anti-human MCP-1 (1:1000)	Santa Cruz Biotechnology
Polyclonal goat anti-*S. pyogenes* SpeB (1:1000)	Santa Cruz Biotechnology
Polyclonal mouse anti-M1, -M23, -Spy0269, -Spy1666 serum (1:25 or 1:1000)	in house
Secondary goat anti-mouse IgG/HRP (1:5,000)	Amersham Biosciences
Secondary rabbit anti-goat IgG/HRP (1:5,000)	DakoCytomations
Texas Red dye conj. AffiniPure goat anti-mouse (1:100)	JacksonImmunoResearch Laboratories

5.1.6 Cytokines

CXCL8/IL-8	R&D Systems
MCP-1	PeproTech Inc.
IP-10	PeproTech Inc.

I-TAC	PeproTech Inc.

5.2 General Methods of Molecular Biology

5.2.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out for analytical purposes to separate DNA according to its size and for isolation of specific DNA fragments. Corresponding to the size of the DNA, 0.8-1% agarose gels were prepared with TAE buffer. DNA was visualized with 0.5 µg/ml ethidiumbromide added to the liquid agarose. A DNA gel loading buffer was added to the DNA sample and the 1 kb Plus DNA Ladder was used as standard size marker. For cloning procedures DNA fragments were excised from the gel and purified using the QIAquick Gel Extraction Kit.

5.2.2 SDS Polyacrylamid Gel Electrophoresis

For the separation of proteins according to their size SDS-PAGE was carried out using 4-20% gradient PAGEr® Duramide Precast Gels. Protein samples were mixed with 5 µl Lane Marker reducing sample buffer and heated for 5 minutes to 95°C or for 20 minutes to 75°C. Sigma Wide Range marker or Page Ruler™ Plus Prestained Protein Ladder served as a molecular weight markers. The proteins were separated at 150 V for 1 hour or at 200 V for 45 minutes. The gradient gels were further used either for Western blot analysis (see 5.2.3) or were stained with Simply Blue Safe Stain Solution.

5.2.3 Western Blot

Proteins were separated by one dimensional SDS-PAGE as described under 5.2.2 and were transferred onto a Hybond™ nitrocellulose membrane via a semi-dry transfer system (Trans-Blot® SD) using western blot transfer buffer or via dry blotting utilizing iBlot® Gel Transfer Device and iBlot® Gel Transfer stacks according manufacturer's instructions. After the proteins have been transferred on the nitrocellulose membrane they were visualized with Ponceau S. Membranes were blocked with 5% milk in PBS or PBS-T at room temperature for one hour or at 4°C overnight. Primary antibodies were diluted in 5% milk in PBS or PBS-T for 1 hour at room temperature (see 5.1.5). The membranes were washed three times for 15 minutes with PBS-T and were incubated

with horseradish-peroxidase (HRP) conjugated secondary antibodies in PBS or 5% milk in PBS-T for one hour at room temperature. Prior to detection, the membranes were washed with PBS-T and PBS. For detection either the ECL detection kit was used where the membranes were exposed to a Hyperfilm™ ECL in an X-ray film cassette for 3-15 minutes or Chemiglow™ served as substrate for the measurement of chemiluminescence with the FluorChem SP Imaging System with a CCD digital camera.

5.2.4 Southern Blot

For the generation of DIG-dUTP labelled probes labelled M49591 *S. pyogenes* genomic DNA served as template. The probes were amplified with Expand High Fidelity Kit under standard conditions with primers (see Table 3, Appendix I) 6074 and 6075 resulting in a 313 bp, with 6076 and 6077 resulting in a 117 bp fragment. Additionally pGhost5 served as template for amplification of a specific probe with primers 6333 and 6334 resulting in a 1032 bp PCR product. After purification using QIAquick Gel Extraction Kit the PCR products served as templates for the DIG labelling PCR reaction using PCR DIG Probe Synthesis Kit. DIG labelling required the following PCR conditions: 1.5 mM $MgCl_2$ and a ratio of 1 to 2 of 1.5 mM dNTPs and PCR DIG probe synthesis mix.

Genomic DNA was isolated using Wizard® Genomic DNA Purification kit (as described under 1.8) and was digested for 8 hours at 37°C with Eco*RI* or Eco*RV* restriction enzymes to generate an average size of 5000 bp fragments. The digested genomic DNA (0.5 µg/ sample) was separated on a 0.8% agarose gel at 80 V for 5 hours. The DIG-labelled DNA Marker II served as DNA ladder. All incubations were carried out at room temperature unless otherwise stated. For the transfer of the DNA on the nylon membrane the agarose gel was submerged twice for 15 minutes in Denaturation solution. The gel was rinsed with sterile water and immediately submerged twice for 15 minutes in Neutralization solution. Finally the gel was equilibrated in 20x SSC for at least 10 minutes. Capillary transfer of DNA on a nylon membrane was carried out overnight using 20x SSC. For DNA crosslinking the wet membrane was exposed for 3 minutes to UV light with DNA site up. Further steps were adapted from Roche using DIG Easy Hyb and DIG Wash and Block Buffer Set. Pre-hybridization and hybridization steps were carried out at 42°C using DIG. DIG labelled probes were diluted 1:10 in

ddH$_2$O for hybridization. Post-hybridization washes with Low and High Stringency Buffer were carried out twice under constant agitation for 5 minutes at room temperature and for 15 minutes at 65°C, respectively. Visualization was carried out using DIG Luminescent Detection Kit according to the manufacturer's instructions.

5.2.5 Northern Blot

For Northern blot analysis all steps were carried out under RNase free conditions using DEPC treated water. The probes that have been generated for Southern blot were used and additional probes were generated using primers listed in Table 3 (see Appendix I). For probe amplification M49591 *S. pyogenes* genomic DNA (see 5.8) served as template DNA. Amplification was carried out using primers 7268 and 7269 resulting in a 419 bp fragment, 7270 and 7271 resulting in a 152 bp PCR product, and 7272 combined with 7273 generating a 258 bp PCR fragment. PCR conditions of the DIG labelling PCR reactions were the same as described for the Southern blot probes (see 5.2.4).
RNA was isolated from mid-exponential growth phase (OD_{600nm} ~0.5) using RiboPure™–Bacteria Kit (see 5.9) and was separated on a 1-2% agarose gel in 1x MOPS buffer containing 2% formaldehyde. DIG labelled RNA Molecular Weight Marker was used as Marker. Transfer of RNA on the positively charged nylon membrane was carried out the same way as for Southern blot (see 5.2.4). Further steps were adapted from Roche using DIG Easy Hyb and DIG Wash and Block Buffer Set, as described under 1.2.4. Exceptions were the increase of pre-hybridization and hybridization temperatures to 50°C and post-hybridization wash with High Stringency Buffer was carried out at 50°C. Visualization of specific probes again proceeded the same way as described under 5.2.4 using DIG Luminescent Detection Kit.

5.3 Bacterial Strains and Plasmids

The following *S. pyogenes* strains were used for *in vitro* and *in vivo* studies

» AP-1: M1 serotype, procured from Dr. Lars Björck, Department of Cell and Molecular Biology, Section for Molecular Pathogenesis, Lund University, Lund, Sweden

» SF370: M1 serotype

» A20-MA: M 23 serotype

» M49591: M49 serotype, received from Bernd Kreikemeyer, University of Rostock Medical Faculty Inst. of Med. Microbiology, Virology and Hygiene Dept. of Med. Microbiology and Hospital, Rostock, Germany

The following *E. coli* strains were utilized for cloning and expression of recombinant proteins:

» Competent ElectroMAX™DH5α-E™ (genotype *F-ϕ80ΔlacZΔM15(lacZYA-argF) U169 recA1 hsdR17(rk-, mk+)gal- phoA supE44λ-thi-1gyrA96 relA1*): Invitrogen

They were used for transformation via electroporation and plasmid maintainance. The ElectroMAX™DH5α-E™ host is particularly suitable for cloning because of a high plasmid yield and quality due to the *end*A1 mutation besides high transformation efficiency. Additionally, the stability of inserts is ensured due to the *rec*A1 mutation.

» One Shot® TOP10 chemically competent *E. coli* cells (genotype F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G): Invitrogen

TOP10 chemically competent cells do not require IPTG for induction of the lac operon. For blue white screening only 40 µl of 40 mg/ ml X-Gal in dimethylformamide is required.

» BL21 Star™ (genotype F^- *ompT hsd*S_B (r_B^-, m_B^-) *gal dcm rne*l31, DE3): Stratagene

They were used for the expression of recombinant proteins. Besides the λDE3 lysogen, which allows high-level expression of T7-regulated genes, BL21 Star™ contains the *rne131* mutation which enhances the expression capability of the strain. The *rne* gene encodes the RNase E enzyme which is usually involved in rRNA maturation and mRNA degradation. The *rne131* mutation encodes a truncated RNase E not able to degrade mRNA. Consequently the RNase E-defective BL21 Star™ strain exhibits upon induction increased mRNA stability.

» BL21-CodonPlus® (DE3)-RIPL (*genotype* B F– *ompT hsdS*(rB– mB–) *dcm*+ Tetr *galλ*(DE3) *endA* Hte): Stratagene

They produce an increased supply of rare *E. coli* tRNAs that correspond to codons used more frequently by other organisms which helps to overcome expression problems due to codon bias.

The following plasmids were used for cloning procedures and gene deletion (see Appendix II):

» pET28b(+) and pET28bn-His: Novagen and Intercell construct

They were used as vectors for expression of recombinant proteins. The pET28(+) expression vector carries a C-terminal His-Tag/thrombin/T7-Tag configuration and an optional N-terminal His-Tag sequence. Transcription is carried out by T7 RNA polymerase. When heterologous genes are expressed from T7-based expression vectors, the yields of recombinant proteins increase. Vector pET28bn-His is a modification of pET28b(+) and carries an N-terminal His-Tag. This vector expresses a His-tag at the N-terminus of the recombinant protein.

» pGhost5:

This temperature sensitive shuttle vector was used for the generation of gene deletion mutants in M49591 *S. pyogenes* strain. It harbours an erythromycin resistance cassette for selection in *E. coli* and *S. pyogenes* as well as an origin of replication (pBR322) for maintenance and propagation in *E. coli* at 37°C. It was kindly provided by Prof. Dr. Dieter J. Reinscheid, Bonn-Rhein-Sieg University of Applied Sciences, 53359 Rheinbach, Germany.

5.4 Cloning of Genes and Expression of Proteins

5.4.1 Preparation of Insert DNA by PCR

The gene fragment of interest was amplified from *S. pyogenes* SF370 or M49591 genomic DNA (see 5.8) using the Expand High Fidelity PCR System. Gene specific primers for all amplified DNA fragments were designed with Vector NTI and are presented in Appendix I. PCR products obtained were digested with the respective enzymes at 37°C for 1-3 hours, unless otherwise stated. After digestion the DNA was purified using the QIAquick PCR purification or Gel Extraction Kit from Qiagen according to manufacturer's instructions.

5.4.2 Ligation of DNA Fragments into Plasmids

The digested, purified plasmids (pET28b(+), pET28bn-His and pGhost5) and generated PCR fragment were ligated containing maximum 50 ng pET28b(+) or pET28bn-His and

50-100 ng insert DNA fragment, 2 µl ligase buffer, 1 unit T4 DNA Ligase and 1 µl distilled H_2O. In case of pGhost5 cloning procedures a minimum of 200 ng vector and 250 ng insert were used. The ligation was carried out for 16 hours at 16°C. Prior to transformation into *E. coli* (see 5.4.4) the Ligation reaction was precipitated by adding distilled water to a total volume of 100 µl. To the 100 µl 0.1 volumes 3 M sodium acetate (pH 5.3), 0.015 volumes glycogen and 2 volumes of chilled ethanol (100%) were added. The mixture was vortexed and incubated for 10 minutes at 80°C. This was followed by 5 minutes centrifugation at 15,700 g. The received pellet was washed with 200 µl 70% ethanol and again centrifuged for 2 minutes at 15,700 g. The pellet was dried at 37°C to remove ethanol and finally it was resuspended in 1-10 µl water.

5.4.3 TOPO Cloning

To facilitate cloning into pET28b(+), pET28bn-His or pGhost5 PCR selected amplified DNA fragments have been subcloned into the TOPO® vector from Invitrogen's TOPO-TA Cloning Kit. The procedures were carried out according to the manufacturer's instructions. The TOPO vector with its insert was transformed into One Shot® TOP10 chemically competent *E. coli* cells via heat shock (see 5.4.4).

5.4.4 Transformation of Electrocompetent and Chemically Competent *E. coli* Cells

Transformation was done in pre-chilled electroporation cuvettes with a 0.1 cm electrode gap. Electrocompetent *E. coli* cells (DH5-α-E™, BL21 Star™ and BL21-CodonPlus® (DE3)-RIPL competent cells, see 5.3) were thawed on ice, and aliquotes of 19 µl were gently mixed with 1 µl ligation mixture (5-50 ng DNA) by tapping. After transfer to chilled cuvettes the cells were electroporated with an *E. coli* Pulser setting the voltage to 2.0 kV. Cells were immediately resuspended in 1 ml SOC medium and incubated shaking for 60 minutes at 37°C.

Chemical transformation was carried out with One Shot® TOP10 chemically competent *E. coli* cells via heat shock. Therefore one aliquot of *E. coli* cells was gently mixed by tapping with 5-50 ng DNA and incubated on ice for 30 minutes. The heat shock was carried out for 30 seconds at 42°C and the cells were immediately put on ice, resuspended with 250 µl SOC medium and incubated at 37°C shaking for 1 hour. Transformants were selected by plating on LB plates containing 50 µg/ml Kanamycin for

pET28b(+), pET28bn-His and TOPO® vector (also X-gal for selection) or in case of pGhost5 500 µg Erythromycin. Presence of recombinants was ascertained by colony PCR. Plasmid isolation from recombinant colonies was done with the QIAprep Spin Miniprep Kit and inserts were checked by restriction digestion and sequencing. For restriction enzyme mapping, an aliquot of 0.05 - 0.2 µg was digested with the appropriate enzymes for 2 hours at 37°C. The size of the restriction fragments was confirmed on a 0.8-1% agarose gel (see 5.2.1).

5.4.5 Analysis of Protein Expression

After plasmids were transferred into the expression host *E. coli* BL21 Star™ (see 5.4.4), expression was tested in small scale with IPTG induction. Bacteria grown overnight in LB medium supplemented with 50 µg/ml Kanamycin at 37°C were diluted 1:4 and cultured until an OD_{600nm} 0.6 was reached. Cells were induced with 1 mM IPTG for 2 hours at 37°C. Cells were pelleted and lysed by incubation with 100 µl Bug Buster™ lysis buffer for 1 hour at room temperature. After centrifugation for 5 minutes at 15,700 g, the culture supernatant (soluble fraction) was taken off and analysed by SDS-PAGE (see 5.2.2). The expressed proteins were visualized with Simply Blue Safe Stain and Western blot analysis using Penta-His™ antibodies (see 5.2.3).

5.4.6 Expression of Recombinant *S. pyogenes* Proteins

For expression of recombinant proteins, *E. coli* cells (BL21 Star™ One Shot® or BL21-CodonPlus® (DE3)-RIPL competent cells) which harbour the pET28b(+) or pET28bn-His vector with the respective gene as insert were cultivated as a starter culture in 25 ml LB medium supplemented with Kanamycin (50 µg/ml) at 37°C shaking overnight. The overnight culture was diluted 1:40 in one litre LB-Kanamycin (50 µg/ ml) at a starting OD_{600nm} ~0.01 and the *E. coli* cells were further incubated until they reached an OD_{600nm} of 0.8. Recombinant protein expression was induced by adding IPTG (final concentration of 0.5 mM) and an additional growth period of 3 hours was necessary to produce sufficient protein for purification. Following induction, the cells were harvested by centrifugation for 40 minutes at 1,800 g at 4°C. The cell pellets were collected, resuspended in PBS and pellets from two liters of culture were combined. After an additional centrifugation step to decant the supernatant efficiently, the cells were frozen at -80°C overnight. In the

pET28b(+) and pET28bn-His vectors all recombinant proteins are expressed with a tag of six histidines at the C-terminus and the N-terminus respectively. Purification was carried out by affinity chromatography using Ni-NTA-Agarose® bead.

5.4.7 Purification of Recombinant *S. pyogenes* Proteins

For purification of the expressed recombinant proteins *E.coli* cell pellets generated previously (see 5.4.6) were thawed and resuspended in 25 ml BugBuster® Protein Extraction Reagent. This liberates intracellular proteins by disrupting the *E. coli* cell wall. Protease inhibitor cocktail I and II (PIC I and PIC II, see 5.1.4) as well as PMSF (1 mM final concentration) were added to avoid protein degradation by proteases. The cell lysates were incubated rotating 45 minutes at room temperature. Meanwhile a 1.5 ml aliquot of Ni-NTA-Agarose® beads was washed twice with 1 ml distilled water. After the washing step the beads were washed two times with binding buffer (0.5 M NaCl, 20 mM NaH_2PO_4, 20 mM Imidazol in PBS) and centrifuged for 2 minutes at 4°C at 1,300 g. To separate soluble and insoluble fractions of the *E. coli* lysates the suspension was centrifuged and the supernatant was kept as soluble fraction. Furthermore, it was mixed with Ni-NTA-Agarose® beads in binding buffer and after addition of 0.1% Tx100 it was incubated rotating for at least 2-3 hours at room temperature. The mixture of cell lysate and Ni-NTA-Agarose® beads was applied on a column and its flow through was collected. The tube which had contained the lysate-bead mixture was rinsed with 2 column volumes of 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1% TX100 and 0.5 mM DTT and the rinse was poured onto the column, to loose as little as possible of the recombinant protein.

The column was subsequently washed with the following buffers:

» 2 column volumes of 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1% TX100 and 0.5 mM DTT

» 10 column volumes using 50 mM Tris/HCl (pH 8.0), 150 mM NaCl and 0.5 mM DTT

» 5 column volumes using 20 mM Imidazol in 50 mM Tris/HCl pH 8.0, 150mM NaCl and 0.5 mM DTT

» 5 column volumes using 40 mM Imidazol in 50 mM Tris/HCl pH 8.0, 150mM NaCl and 0.5 mM DTT

Proteins bound to the Ni-NTA-Agarose® were harvested in 4 elution steps using the following buffers:

» 3 column volumes of 500 mM imidazole, 20 mM NaH_2PO_4, 0.5 mM NaCl in 50 mM Tris/HCl pH 8.0

» 3 column volumes of 500 mM imidazole, 20 mM NaH_2PO_4, 0.5 mM NaCl in PBS pH 7.4

» 3 column volumes of 500 mM imidazole, 20 mM NaH_2PO_4, 0.5 mM NaCl in 50 mM HEPES pH 7.0

» 3 column volumes 500 mM imidazole, 20 mM NaH_2PO_4, 0.5 mM NaCl in 50 mM HEPES pH 6.2

In order to remove everything that was bound to the Ni-NTA agarose beads a final wash using 3 column volumes of 500 mM imidazole, 8 M urea in Tris/HCl pH 8.0 with 0.5 mM DTT was carried out.

The efficiency of protein purification was analysed by SDS-PAGE (see 5.2.2) using between 1 and 5 µl of soluble fraction, wash or flow through and eluate fractions and visualized with Simply Blue Safe Stain. The fractions containing overexpressed proteins were pooled and dialyzed against PBS. The amount of recombinant protein was evaluated by SDS-PAGE using BSA as quantitative standard.

5.5 Cultivation of *S. pyogenes* Strains

S. pyogenes strains were grown overnight in THY medium in a humidified atmosphere at 37°C and 5% CO_2 without shaking. The cultures grown overnight were diluted 1:20 in the respective growth medium. For the determination of the growth behaviour of the different strains a growth curve was generated. Therefore the optical density of the *S. pyogenes* strains was measured at 600 nm every hour except during logarithmic growth phase (around OD_{600nm} ~0.5) when the optical density was measured every 30 minutes. To evaluate the colony forming units at an optical density of 0.5 serial dilutions of the bacteria were plated in triplicates on blood agar plates.

5.6 Generation of Competent *S. pyogenes* Cells

For the generation of electrocompetent M49591 *S. pyogenes* cells the bacteria were grown overnight in 3 ml THY supplemented with 20 mM glycine. After a 1:10 dilution in 30 ml

fresh THY medium containing 20 mM glycine the bacteria were grown until they reached an optical density of ~0.2 and were incubated on ice for 10 minutes. The bacteria were pelleted by centrifugation at 1,300 g for 10 minutes and washed twice in ice cold electroporation buffer. The washed pellet was taken up in 500 µl cold electroporation buffer and left on ice until the competent *S. pyogenes* cells were used for electroporation.

5.7 Preparation of *S. pyogenes* Subcellular Fractions for SDS-PAGE and Western Blot Analysis

S. pyogenes strains were grown as described under 1.5 and the bacterial pellets of a 50 ml mid-exponential (OD_{600nm} ~0.5) or late stationary (overnight culture) culture were harvested by centrifugation at 1,800 g for 15 minutes. The pellets were washed once with PBS and stored overnight at -80°C. After being frozen the bacterial cell pellets were taken up in 10 mM Tris HCl, pH 7.6 and lysed by sonication (6x 30 seconds, pulse 5, 100%). Lysates were centrifuged and the supernatants containing cytoplasmic proteins were concentrated using Centrifugal device Nanosep 10K Omega. The pellet from the lysate was washed twice with 10 mM Tris/HCl pH 7.6, resuspended in 50 mM Tris, 5 mM EDTA, 10 mM NaCl, pH 8.1, containing 250 µg Lysozym and 50 U Mutanolysin, and was incubated at 37°C for 4 hours. After centrifugation, cell wall proteins contained in the supernatant were concentrated the same way as the cytoplasmic fractions. The protein concentration of lysates, cytoplasmic and cell wall fractions was measured using the BCA™ Protein Assay Kit.

5.7.1 TCA Precipitation of Culture Supernatants

For concentration of proteins that were secreted into the culture supernatant, TCA-precipitation was selected as procedure. First 100% TCA was added to each supernatant, to give a final concentration of 12% and the mixture was incubated on ice for one hour. After incubation on ice the samples were centrifuged at 1,800 g for 40 minutes at 4°C. The harvested pellets were washed twice with freeze cold acetone (-20°C) and the supernatant after centrifugation at 1,800 g for 15 minutes at 4°C was removed carefully. The samples were dried at 37°C until all the acetone was completely evaporated. The

precipitated samples were dissolved in PBS and kept for further use at -20°C. The protein concentration was measured using the BCA™ Protein Assay Kit.

5.8 Preparation of *S. pyogenes* Genomic DNA

Genomic DNA of *S. pyogenes* strains was purified using Wizard® Genomic DNA Purification kit following the manufacturer's instructions except that the double amount of lysozyme was used and lytic enzyme was not used at all. The isolated genomic DNA was further used for PCR or Southern blot analysis (see 5.2.4).

5.9 Isolation of *S. pyogenes* RNA

RNA was isolated from *S. pyogenes* strains for Northern blot analysis (see 5.2.5), Microarray analysis (see 5.21) and Real Time RT-PCR (see 5.22) using RiboPure™-Bacteria Kit. Therefore, overnight cultures of *S. pyogenes* strains were diluted 1:50 in fresh THY medium and grown until they reached mid-exponential phase at OD_{600nm} ~0.5. All steps were carried out as described in the kit's protocol for RNA used in Northern blot experiments including DNase treatment.

As for transcriptome analyses a higher concentration of high quality RNA was desirable the protocol was slightly changed: The pellet of a 50 ml culture was taken up in 1.4 ml RNAwiz and portioned on 4 provided tubes containing zirconia beads. The 10 minutes step where bacteria are lysed by the zirconia beads during vortexing was increased to 20 minutes. The RNA was eluted in 2x 25 ml elution buffer and DNase treatment was carried out using 1 U DNaseI, amplification grade, from Invitrogen per microgram RNA. This reaction was inactivated by addition of 2.5 M EDTA and heating to 65°C for 10 minutes. Absence of DNA was verified by PCR. The RNA of one 50 ml pellet was pooled and precipitated. For precipitation UltraPure™ Phenol:Water (3.75:1 v/v) was added 1 to 1 and centrifuged for 5 minutes at 15,700 g. LiCl (5 M) was added to the supernatant 1 to 1 followed by incubation overnight at 4°C. The RNA was harvested by centrifugation (15,700 g for 15 minutes) and washed with ice cold 70% ethanol. The dried pellet was taken up in 50 µl DEPC-treated H_2O. RNA was checked for degradation by agarose gel electrophoresis (see 5.2.1). Additionally, RNA quality was examined using SuperScript® III First-Strand Synthesis System which was also used for the reverse

transcription of RNA into cDNA. RNA was reverse transcribed as suggested by the manufacturer and degraded by RNaseH. Controls were treated the same way but missing the reverse transcriptase leading to the fact that during the RNase treatment potential DNA contaminants are left in the samples that are amplified during PCR and visualized on the agarose gel.

5.10 Generation of Polyclonal Antibodies

Polyclonal mouse antibodies have been generated and mainly used for Western blot analysis (see 5.2.3) as well as immunofluorescence analysis (see 5.20).
For the production of antigen specific antibodies ten 6-8 weeks old CD-1 mice were immunized with 50 μg recombinant protein emulsified with 1% Alum as adjuvant in their flank after taking pre-immune sera from their tail vein. After 14 and 28 days the mice received a booster dose through the same route with the same dose of Alum. Seven days after the second boost immune sera were taken, pooled and tested for total IgG by ELISA and for antigen specific antibody levels by Western blotting. Presence of protein specific antibodies was tested either using 1-5 μg of recombinant protein or lysates comprising 20-30 μg protein via Western blot analysis (see 5.2.3). All studies were done according to the local animal ethics guidelines (Tierschutzgesetz BGBl. Nr. 501/1989).

5.11 Intraperitoneal Murine Challenge Model

For the comparison of virulence between various *S. pyogenes* strains, bacteria were grown overnight in THY medium and diluted 1:50 in fresh THY medium at 37°C and 5% CO_2. The bacterial cultures were pelleted after they have reached an optical density of ~0.5 by centrifugation at 1,800 g for 10 minutes. The pellet was taken up in ice cold PBS to reach a final concentration of $5x10^8$ cfu/ 100 μl. CD-1 mice were challenged intraperitoneally with $5x10^8$ cfu/ 100μl and the survival of the CD-1 mice was monitored over a period of 2 weeks. Serial dilutions of the bacterial suspension used for challenge were plated from 1:10 to $1:10^{-6}$ in triplicates on blood agar plates.

5.12 CXC Chemokine Degradation Assay

The assay for chemokine degradation was always carried out at 37°C. Interleukin 8 was used at a final concentration of 6.6 μg/ ml in PBS containing 0.1% BSA. This corresponds to an amount of 150 ng chemokine per assay sample (22.8 μl). BSA, present in the IL-8 solution at 100 μg/ ml (228 ng per sample), served as a standard showing the use of equal amounts of IL-8. MCP-1, IP-10 and I-TAC were utilized at a final concentration of 8.8 μg/ ml corresponding to an amount of 200 ng chemokine per assay sample (22.8 μl). The Spy proteins Spy0416A, Spy0416B, Spy0416B$_{Fn2Fn3}$, Spy0416B$_{Fn2'Fn3'}$, Spy0416B$_{Y}$, Spy0416B$_{Fn2'Fn3'+Y}$ and Spy0416B$_{\Delta Fn1}$ were used at a concentration of 44 μg/ ml each or in combination at a concentration of 22 μg/ml, unless otherwise stated. Chemokine degradation was visualized via semi-dry Western blot analysis (see 5.2.3.) using the respective primary and secondary antibodies listed under 1.1.5.

5.13 Protein Interaction Studies by Surface Plasmon Resonance (SPR)

Interaction studies were performed in collaboration with Andreas Kungl (Department of Pharmaceutical Sciences, University of Graz and ProtAffin Biotechnologie AG, 8020 Graz, Austria) as described in Fritzer et al., 2009 [162]. In brief, BIAcore X–100 biosensor system was used to perform binding experiments. FC2 (flow cell 2) of a BIAcore C1 sensor chip–surface was initially activated with a mixture of 0.4 M EDC and 0.1 M NHS. IL–8 was immobilized via amine coupling at a concentration of 25 μg/ ml in 10 mM acetate puffer pH 5.5. At a flow rate of 10 μl/ minute and a temperature of 25°C the injection time was 7 minutes, resulting in an immobilization level of 342 RU after blocking for 7 minutes with ethanolamine. FC1 served as control surface that underwent the same chemistry except immobilization of IL–8. Analyses were performed by injecting different concentrations of Spy0416A, Spy0416B, Spy0416A1 and Spy0416AΔ (ranging from 0 – 1 μM in PBST buffer) over the IL-8 surface for 3 minutes at a flow rate of 30 μl per minute. Considering the high stability of the formed complexes, the dissociation time was set to 7 minutes, followed by a regeneration step with 0.5% SDS at the end of each cycle. The sensorgrams were analyzed using the BIAcore X-100 evaluation software. Therefore reference subtracted association and dissociation phases of all binding curves were simultaneously fitted to different binding models.

5.14 Isothermal Fluorescence Titration (IFT)

Isothermal Fluorescence Titration was performed in collaboration with Andreas Kungl (Department of Pharmaceutical Sciences, University of Graz and ProtAffin Biotechnologie AG, 8020 Graz, Austria) as described in Fritzer et al., 2009 [162]. In brief, 39 nM of Spy0416A or Spy0416B were titrated with IL-8 in the presence of 1 μM bis-ANS. Excitation was set at 395 nm and fluorescence emission spectra were collected between 450 nm and 550 nm. Fluorescence quenching of bis-ANS emission was detected due to competition of IL-8 for Spy0416 binding. The excitation-/ emission slit widths were set to 15 nm and 20 nm, respectively; the temperature was adjusted to 22°C and a 430 nm cut off filter was inserted into the emission path to avoid stray light. After background correction of the spectra, areas were integrated and mean values of the fluorescence intensity, *F*, resulting from two independent experiments plotted against the volume corrected concentration of the IL-8 ligand. The resulting binding isotherms were analysed by nonlinear regression as described previously [164, 165]. The fitted parameters were F_{max} and K_D.

5.15 Preparation of Human Neutrophils and Intracellular [Ca^{2+}]-Measurement

Preparation of human neutrophils and intracellular [Ca^{2+}]-measurement was performed in collaboration with Andreas Kungl (Department of Pharmaceutical Sciences, University of Graz and ProtAffin Biotechnologie AG, 8020 Graz, Austria) as described in Fritzer et al., 2009 [162]. Human whole blood was obtained from healthy volunteers by venipuncture into tubes containing K_3EDTA. Neutrophils were isolated by Ficoll-Paque centrifugation (400x g, 30 minutes, 4°C) followed by dextran sedimentation of the red blood cell (RBC) pellet containing PMNs for 1 h at RT. The PMN-rich supernatant was aspired, washed twice with HBSS(-) at 600x g for 10 minutes followed by hypotonic lysis of the RBCs and 2 further washing steps. Finally the cells were diluted with HBSS(+) at 10^7 cells/ml, taking into account that only 60% of the counted cells were neutrophils. Fluorescence measurements were performed on a Perkin Elmer LS50B fluorometer. The diluted neutrophils were supplemented with 0.5% BSA and loaded with 3 μM FURA-2AM for 30 minutes at 37°C in the dark. Subsequently, the cells were washed twice with HBSS(+) (500x g, 4 minutes) and stored in HBSS(+)/ 0.5% BSA. Immediately before

measurement, 1 ml aliquots were centrifugated for 3 minutes at 500x g and resuspended in 1 ml HBSS(+). The cells were placed in a continuously stirred cuvette maintained at 37°C. After an equilibration period of 10 minutes, a stable fluorescence signal was reached. The fluorescence was monitored at an excitation of 345 nm and an emission of 495 nm, as determined in an initial measurement. The excitation/emission slits were set at 10/4. Cells were stimulated with 100 nM protein 60 sec after pre-equilibration, and maximum and minimum fluorescence were determined using the following solutions: Minimum buffer (100x): 0.18 M EGTA, 1.4 M Tris, 10% Triton X-100 and Maximum buffer (100x): 1M HCl, 0.5M $CaCl_2$.

5.16 Generation of Gene Deletion Mutants in Strain M49591 using pGhost5

Construction of gene deletion mutants using the temperature sensitive shuttle vector pGhost5 was adapted from Biswas *et al.* [166]. In brief, 150 µl of competent *S. pyogenes* cells (see 5.6) and 1 µg of pGhost5 vector were used for each transformation. Electroporation was carried out at 2 kV. The transformed cells were kept on ice for 5 minutes and incubated for 5 hours in prewarmed THY medium at 28°C, the temperature at which the shuttle vector pGhost5 is maintained extra-chromosomally. *S. pyogenes* Cells were plated on THY agar plates containing 5 µg/ ml Erythromycin and incubated at 28°C with 5% CO_2. For the integration of the construct into the chromosome of *S. pyogenes*, cells were grown in THY containing 5 µg/ml Erythromycin overnight at 28°C and 5% CO_2 and diluted 1:100 in THY without Erythromycin until they reached an optical density (600 nm) between 0.3 and 0.5. A temperature shift from 28°C to 37°C for 150 minutes induced integration of the vector into the GAS chromosome and serial dilutions of cells were plated on THY containing 5 µg/ml Erythromycin and incubated overnight at 37°C and 5% CO_2. Successful integration of the vector into the chromosome was confirmed via PCR. After verification of pGhost5 integration into the chromosome, another temperature shift back to 28°C overnight induced excision of pGhost5. Cells were plated on agar plates with and without Erythromycin for the selection of erythromycin sensitive cells with the desired recombination event and hence gene deletion. Positive cells were identified by PCR. Gene deletion was further verified by sequencing and Southern blot analysis (see 5.2.4).

5.17 Electron Microscopy of *S. pyogenes* Cells

S. pyogenes strains were grown overnight in THY medium at 37°C and 5% CO_2. The cultures were diluted in fresh THY medium and grown till they reached an optical density of 0.5. The cell pellets were harvested by centrifugation at 1000 g for 15 minutes and washed twice with PBS. The cell pellets were resuspended in PBS and fixed using of 5% paraformaldehyde, 5% glutaraldehyde and 5 mM $CaCl_2$ in PBS for 1 hour at room temperature. *S. pyogenes* cells were finally visualized with an analytical high-resolution scanning electrone microscope of the type FEI Quanta 200F with a 50,000 and a 100,000 fold magnification at the Technical University of Vienna.

5.18 Adhesion Assay for Human Detroit 562 and Chang cells

Detroit 562 cells were grown in EMEM supplemented with 10% fetal bovine calf serum (heat inactivated), 50 µM 2-Mercaptoethanol, 1% MEM nonessential amino acids, 1% gentamicin (10 mg/ ml, liquid) and 1% sodium pyruvate giving the complete growth medium. For Chang cells F-12K Medium (Kaighn's Modification of Ham's F-12 Medium) supplemented with 10% heat inactivated fetal bovine calf serum, 100 units penicillin G, 0.25 µg/ml amphotericin B and 100 µg/ml streptomycin was used. Passage of Detroit 562 and Chang cells was carried out every 3-4 days. Therefore the cells were washed twice with HBSS containing 10 mM glucose and were trypsinized for 7 minutes at 37°C and 5% CO_2 using Trypsin, 0.05% (1X) with 0.53 mM EDTA 4Na. The trypsinized cell suspension was taken up in fresh complete medium and was washed once. Cells were harvested by centrifugation at 1000 g for 7 minutes and diluted 1 to 5 in fresh cell culture medium. Before the adherence assay, cells were passaged into antibiotic-free medium.

For the adhesion assay cells were seeded into wells of 24-well tissue culture plates at a density of 2.5x 10^5 cells per well in 1 ml antibiotic-free medium containing 10% FCS and incubated overnight or for 2 days at 37°C and 5% CO_2 to reach about 95% confluence. Meanwhile, *S. pyogenes* strains were cultured overnight at 37°C and 5% CO_2 without shaking. On the next day the bacterial cells were harvested and washed once with PBS. Bacteria were taken up either in EMEM or F-12K medium supplemented with 10% FCS without antibiotics, to reach 3.4x 10^8 cfu/ ml. For infection 100 µl of the previously

prepared bacterial cell suspension was added to the washed Detroit 562 or Chang cells and incubated for 2 hours at 37°C and 5% CO_2.

After incubation, medium was decanted followed by intensive washing with PBS to remove unbound bacteria. The Detroit 562 and Chang cells together with the attached bacterial cells were trypsinized with 100 µl trypsin for 5 minutes and taken up in 400 µl H_2O or directly taken up in 500 µl H_2O and incubated for 10 minutes at room temperature. Immediately, 100 µl of serial dilutions from 1:10 to $1:10^{-6}$ were plated in triplicates on blood agar plates and incubated overnight at 37°C and 5% CO_2. To consider also bacterial growth during the process of attachment a control consisting of *S. pyogenes* strains incubated in wells which did not contain Detroit 562 or Chang cells was used. Afterwards the suspension was plated also on blood agar plates, using 100 µl of 6-fold serially diluted bacteria in PBS.

5.19 Protein Binding Assays

Human plasma- and extracellular matrix-proteins (fibronectin, fibrinogen, laminin, collagen type I and type IV, plasminogen and haptoglobin) were coated on 96 well plates (10 µg in 100 µl) using 100 mM $NaHCO_3/Na_2CO_3$, 50 mM NaCl (pH 9.2) as coating buffer and incubated overnight at 4°C. The plates were washed once with PBS and blocked with 10% BSA in PBS for 2 hours at 37°C. For labelling with Fluoreszein Isothiocyanate (FITC) Isomer I overnight cultures of *S. pyogenes* strains were washed once with PBS and incubated with 1 ml FITC Isomer I (1 mg/ ml Na_2CO_3) for 20 minutes at room temperature in the dark. After incubation, the culture was washed with PBS until the supernatant appeared transparent again and $6x\ 10^7$ cfu/100 µl were incubated with the previously coated human proteins on the 96 well plates for 1 hour at 37°C. A control of $6x\ 10^7$ cfu/100 µl FITC-labelled bacteria was incubated for the same time separated to take bacterial growth during the incubation time into account. Finally, the plates were washed 4 times with 0.1% Tween20 in PBS and measured with the BioTek Synergy2 ELISA reader using the following settings: Excitation at 485/20 nm, Emission 560/60 nm, Gain: 72, 80, 75, 77.

5.20 Immunofluorescence Analysis using Confocal Microscopy

Immunostaining was adapted from Harry *et al*. [167]. In brief, bacterial cells from an overnight culture were harvested by centrifugation at 1000 g for 10 minutes and washed once with PBS. After the cells were fixed for 15 minutes at room temperature and 45 minutes on ice using 5 ml 3% (v/v) paraformaldehyde, 30 mM sodium phosphate (pH 7.5), they were washed three times using 100 mM glycine in PBS and once with PBS only. For the detection of streptococcal proteins on the bacterial surface, cells were resuspended in 50 mM glucose, 20 mM Tris-HCl pH 7.5, 10 mM EDTA and ~5x 10^7 cfu/100 µl were transferred immediately onto poly L-lysine coated Poly Prep™ slides and allowed to settle for 15 minutes. The slides were washed twice with PBS and dipped in 0.1% Triton X100 in PBS for 5 minutes. After another wash with PBS the slides were blocked overnight at 4°C with 2% BSA in PBS. For the one hour-incubation with the primary antibody at room temperature, M23, M1, Spy0269 and Spy1666 specific hyper-immune mouse sera (see 5.1.5) generated in CD-1 mice (see 5.10) were diluted 1:25 in 100 µl 1% BSA in PBS. Slides were washed three times for 5 minutes with 0.1% Tween 20 in PBS, dried and incubated with Texas Red dye conjugated AffiniPure goat anti mouse antibody, diluted 1:100 with 1% BSA in PBS for 1 hour in the dark at room temperature. Slides were washed three times with 0.1% Tween 20 in PBS for 5 minutes followed by another 3 washes with PBS for 5 minutes. Control cells were stained with DAPI by mounting the slides with ProLong gold antifade reagent containing DAPI. Polymerization was allowed for 24 hours at room temperature in the dark. Immunostaining was visualized with a Zeiss Axiovert 200M microscope equipped with a Zeiss LSM510META confocal laser-scanning unit, a Plan-Fluor 100x1.45 oil or a Plan-Apochromat 63x1.40 oil DIC MC27 (aperture, 0.19 mm) objective (Zeiss, Germany).

5.21 Microarray Analysis

In order to evaluate differential gene expression between different strains, dual colour microarray hybridization analysis was chosen. Microarray chips were obtained from Bernd Kreikemeyer (Department of Medical Microbiology, Virology and Hygiene, University Hospital Rostock, Germany). The whole *S. pyogenes* genome DNA arrays contained 50-mer 5' amino-oligonucleotides and were designed and produced by MWG

Biotech. As sequence information for the target selection served the published M1 *S. pyogenes* sequence comprising 1964 ORFs, a partial genome sequence of the serotype M18 strain 818 ORFs of 1889 ORFs and the partial serotype M49 [168].

5.21.1 Generation and Purification of Cy3- and Cy5-Labelled cDNA

RNA from three independent isolations as described under 1.9 was prepared for hybridization: First, 25 µg of RNA were precipitated overnight at -20°C using 0.1 volume of 4 M LiCl (pH 7.0) and 2.5 volumes of 100% ethanol. This was followed by centrifugation at 15,700 g for 10-15 minutes at 4°C and resuspension of the dried pellet in 9 µl RNase-free water. The generation of CyDye labelled cDNA was carried out in two steps, the first one involves the incorporation of amino allyl-dUTP (AAdUTP) during cDNA synthesis using an optimized nucleotide mix and the second step involves chemically labeling of the amino allyl modified cDNA. Therefore the Amersham™ CyScribe Post labelling Kit was utilized according to manufacture's instructions. Synthesis of cDNA and indirect labelling via aminoallyl integration was performed as follows: Random primers with the previously precipitated RNA were heat denatured for 5 minutes at 70°C to eliminate secondary structures and incubated for 10 minutes at room temperature for annealing a mixture of anchored oligo(dT) and random nonamer primers. Immediately a mix of 5x CyScribe buffer, 0.1 M DTT, dNTP mix, aminoallyl-dUTPs and CyScribe Reverse transcriptase as described by the manufacturer were added and heated for 90 minutes at 42°C. For the purification of aminoallyl-labelled cDNA to remove RNA from the single stranded cDNA, mRNA is degraded into short oligomers by the treatment with 2 µl of 2.5 M NaOH and incubated at 37°C for 15 minutes. Neutralization was induced by the addition of 10 µl 2 M HEPES. The newly synthesized cDNA was precipitated by the addition of 0.1 volume 4 M LiCl (pH 7.0), 2.5 volumes of 100% ethanol and 1 µl acrylamid (Ambion) and incubation at -20°C for 30 minutes. The pellet harvested by centrifugation at 15,700 g for 15 minutes was resuspended in 120 µl Na_2CO_3 (pH 9.0). cDNA concentration was measured and 20-30 µg were expected for optimal incorporation of Cy3- and Cy5-labelled nucleotides into the cDNA. GE Healthcare's Illustra™ CyScribe™ GFX purification kit was utilized for the post-labelling coupling reaction which was carried out as described by the manufacturer except that 4 M hydroxylamine/HCl was increased from 15 µl to 30 µl. To

minimize hybridization background and improve the sensitivity of detection of low abundance targets unincorporated CyDye molecules were removed with the Illustra™ CyScribe™ GFX purification kit.

5.21.2 Preparation of Microarray Chips and Data Analysis

For the preparation of the chips, unspecific probe binding was prevented by blocking the slides with 4x SSC, 1% BSA and 0.5 % SDS at 42°C for 45 minutes. The chips were washed five times with sterile water and dried by centrifugation at 1000 g for 3 minutes. For the dual colour microarray hybridization the Cy3- and Cy5- labelled probes (Cy3-labelled M49591 wildtype and Cy5-labelled gene deletion mutant per chip and vice versa) were denatured for 5 minutes at 99°C and immediately cooled in an ice bath. The probes were pipetted carefully on the microarray chips and covered by a plastic foil. Hybridization was performed at 42°C overnight in the dark. Chips were washed in the dark with 2x SSC, 0.1% SDS for 5 minutes at 42°C, with 1x SSC for 5 minutes at 42°C and finally with 0.1x SSC for 5 minutes at room temperature, rinsed with water and dried by centrifugation. Each experiment was performed in triplicates including separate sample preparation as well as labelling and hybridization. Hybridization was detected with a DNA Microarray Scanner from Agilent Technology instrument (Affymetrix). Data analysis was carried out using GeneSpring GX Software also from Agilent. Data for individual genes (values) was expressed as the fold change of normalized wildtype/ experimental signal (gene deletion mutant).

5.22 Real Time RT-PCR

Real Time RT-PCR was performed to verify the previously generated microarray data. The mRNA expression was analyzed from two independent RNA isolations as described under 1.9 and reverse transcribed into cDNA with random hexamer primers using the SuperScript® III First-Strand Synthesis System following the manufacturer's instructions. Gene-specific oligonucleotide primers (see Table 6, Appendix I) were designed using Vector NTI (Invitrogen) and were tested in PCR using M49591 genomic DNA and standard conditions to determine amplification specificity. PCR analyses were performed using Real Time PCR LightCycler® 480 SYBR Green I Master for the incorporation of SYBR Green I dye into double-stranded DNA during each phase of

DNA Synthesis. 5 µl of 1:25 diluted cDNA were used for one PCR reaction. Real Time RT-PCR was carried out in *twin.tec* real-time PCR using the Mastercycler from Eppendorf with the following conditions: 95°C for 5 minutes to activate the FastStart Taq DNA polymerase, 40 cycles running 95°C for 10 seconds, primer annealing at 55°C for 15 seconds, elongation at 72°C and SYBR Green I fluorescence was measured at 72°C for 10 seconds after every cycle. In the end 95°C for 20 seconds was followed by a melting curve analysis where the temperature was increased stepwise from 60-95°C for 30 minutes and finally 95°C for 15 seconds. The temperature at which the amplified DNA is separated into single strands which leads to the loss of the fluorescent SYBR Green is characteristic for each PCR product. The housekeeping gene *mutS*-mRNA (encoding DNA mismatch repair protein) expression was used as internal control as it produced more stable results than using *gki* (glucose kinase) or *recP* (transketolase) as internal controls. Data analysis was supported by the Eppendorf RealPlex software and quantitative differences for each sample were determined using the 2-(ΔCTsample-ΔCTcalibrator) method [169].

6 RESULTS

6.1 Selection of Nine Protective Antigens from *Streptococcus pyogenes* Identified via Intercell's ANTIGENome Technology

In order to identify novel group A streptococcal antigens for vaccine development the ANITGENome technology was employed using human sera collected from uninfected healthy adults [153, 159]. Bacterial surface display screens led to the identification of 95 antigen candidates as annotated by the *S. pyogenes* SF370 genome and 55 peptides that could not be assigned to an annotated open reading frame [153]. The most promising candidate antigens for further evaluation in animal models of group A streptococcal disease were selected by *in vitro* assays such as peptide ELISA with human sera and gene conservation analysis. Three different animal models were performed to reduce the number of identified candidates to the most promising ones. Mice were either immunized with the adjuvant CFA/IFA followed by intravenous challenge with *S. pyogenes* AP-1 or with Aluminum hydroxide following intranasal challenge with *S. pyogenes* strain A20-MA and the third immunization was carried out via the intranasal route with the adjuvant IC31™ before challenging with A20-MA. The data from all three animal models provided ample evidence for the selection of nine group A streptococcal proteins, Spy0269, Spy0292, Spy0416, Spy0488, Spy0872, Spy0895, Spy1536, Spy1666 and Spy1727 as the most promising candidates for further vaccine development.

The nine selected vaccine candidates had not been characterized previously in more detail at the time I started this work. While this work was performed, Spy0416 was shown to possess IL-8 degrading activity [89, 91, 162]. The other eight proteins have either been assigned no or only a putative function so far (see Figure 3). Spy0488, Spy1536, Spy1666 and Spy1727 were described as hypothetical or conserved hypothetical proteins and show very limited similarity to proteins with known functions. However, Spy0269 was described as a putative surface exclusion protein and possesses a short 130 amino acid region showing similarity with the EzrA protein that interacts with the cell-division protein FtsZ [170]. Spy0292 was annotated as Penicillin

binding protein, but no beta-lactamase activity was yet reported for this protein. The Spy0872 protein shows similarity to secreted 5'-nucleotidases, enzymes located at the cell surface, such as UshA of *E. coli* [171], with an important function in nucleotide salvage or growth on nucleosides as carbon source. Spy0895 displays weak similarity with several proteins and was thus annotated as putative histidine kinase and transcription regulator LytR, none of which functions was so far experimentally confirmed. Due to this very limited knowledge about the proteins the aim was to gain more information about their potential function and contribution to streptococcal virulence and pathogenesis.

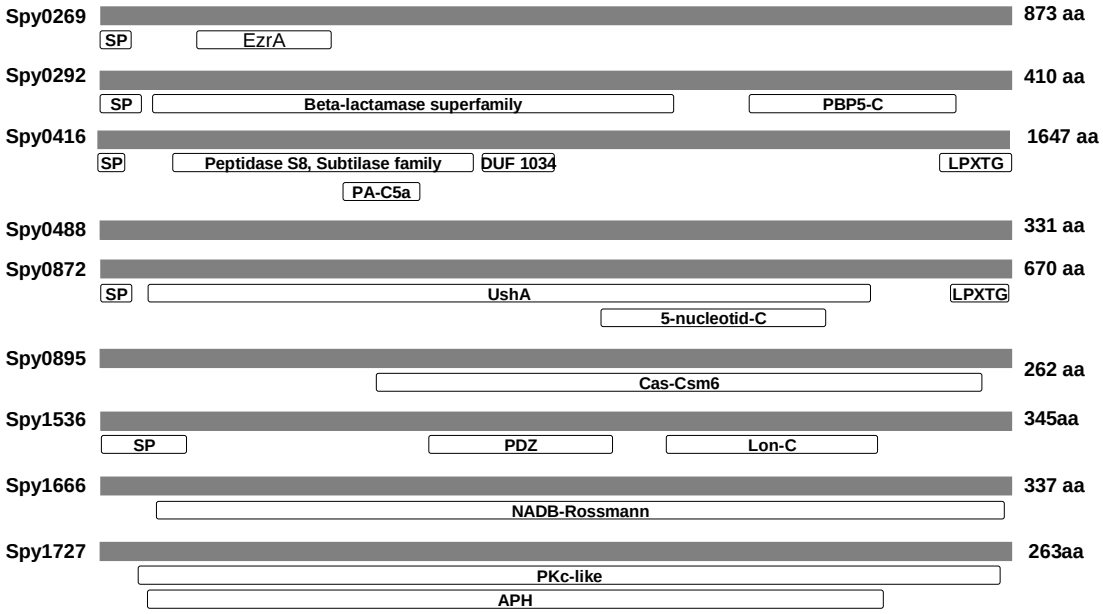

Figure 3: Structural features of the nine protective candidates
The schematic drawings are based on the protein sequences encoded by the SF370 genome. SP, Signal peptide; EzrA, septation ring formation regulator EzrA; PBP5-C, Penicillin-binding protein 5, C-terminal domain; PA-C5a, Protease-associated domain of C5a-like proteins; DUF1034, Domain of Unknown Function; LPXTG, cell wall anchor motif; UshA, 5'-nucleotidase/2',3'-cyclic phosphodiesterase and related esterases; 5-nucleotid-C, C-terminal domain of 5'-nucleotidases; Cas-Csm6, CRISPR (Clustered regularly interspaced short palindromic repeats) -associated protein; PDZ, PDZ ('post synaptic density disc-large zo-1') domain, also called DHR (discs-large homologous region); Lon-C, Lon protease (S16) C-terminal proteolytic domain; NADB-Rossmann, Rossmann-fold of NAD(P)(+)-binding proteins; PKc-like, catalytic domain of protein kinase C superfamily; APH, Aminoglycoside-2''-phosphotransferase enzyme family.

6.2 Focus on Three of the Nine Candidates Spy0416, Spy0895 and Spy1536

The three candidates Spy0416, Spy0895 and Spy1536 have been chosen for further characterization for several reasons.

Spy0416 was selected for more detailed characterization prior to its description as the IL-8 degrading cell envelope serine proteinase ScpC [89, 91, 162], because this antigen was

among those candidates with the widest and highest range of immune reactivity with human sera. Further it showed high levels of protection in several mouse models against streptococcal infection. In addition the sequence of the *spy0416* gene displayed at least 98% amino acid sequence identity in genomes of 50 GAS isolates encompassing 15 different serotypes and bioinformatic analysis revealed a C-terminal LPXTG motif predictive of surface localization of Spy0416. Biochemical characterization had already started during my diploma thesis where I could demonstrate that the recombinantly expressed form of Spy0416 cleaved IL-8 *in vitro* in a site-specific manner which led to inactivation of the chemokine [162, 163]. Interestingly, IL-8 degradation was only observed in presence of both, the catalytic N-terminal domain and the accessory C-terminal domain, for which a function could not be demonstrated so far. Therefore, I continued my work on the characterization of Spy0416 by evaluating possible contributions of the predicted individual domains within Spy0416B to IL-8 degradation.
A collaboration with Bernd Kreikemeyer (Rostock, Germany) was established on the generation of *spy0416*, *spy0872* and *spy0292* gene deletion mutants. For my thesis I further focused on the generation of deletion mutants for two additional candidates, *spy1536* and *spy0895* in order to assess their possible contribution to streptococcal virulence and pathogenesis. The first set of candidates *spy0416*, *spy0872* and *spy0292* for gene deletion was selected based on their high protection observed in the mouse models and their location on the GAS genome as independently transcribed genes. In contrast, *spy0895* and *spy1536* are located within an operon flanked by one and two genes, respectively (see Figure 14B and 14C, page 74). Spy0895, annotated as putative histidine protein kinase, Spy1536, a conserved hypothetical protein which is similar to the ATP-dependent proteinase La, were selected due to their consistent protection in the animal models and high conservation in the GAS genome - Spy0895 with more than 98.9% amino acid sequence identity and Spy1536 with more than 99.1% amino acid sequence identity in 13 GAS strains of 10 different M serotypes [153].

6.3 Characterization of Spy0416

The identification of the Spy0416 protein as a prominent antigen [153] using the ANTIGENome technology [172] together with the at that time recent reports showing

that the Spy0416 protein of invasive *S. pyogenes* strains possesses human chemokine degrading activity [89, 91], has prompted us to further investigate the function of this putative serine proteinase. Several attempts to express and purify the full length recombinant protein failed. Apart from the fact that correct amplification of the gene sequence was not possible, cloning into an *E. coli* expression vector was not successful [162]. Therefore I generated two subfragments, Spy0416A & B overlapping with 132 amino acids (see Figure 4) for the analysis of IL-8 degradation. The design of these fragments was supported by bioinformatic analyses to avoid hindrance of efficient expression. It also revealed that the Spy0416 protein consists of multiple domains of which the C-terminal region entails several domains with unknown functions (see Figure 4). We could previously demonstrate that Spy0416 cleaves IL-8 in a site specific manner that leads to inactivation of the chemokine *in vitro* [162]. Furthermore, site directed mutagenesis revealed that the amino acid residues, Asp^{151}, His^{279} and Ser^{617}, predicted to form the active site in the N-terminal domain of Spy0416 (see Figure 4) in analogy to Subtilisin-like proteinases [173], are essential for enzymatic activity. The abolishment of IL-8 degradation activity by the elimination of the PA and Fn1 domain in Spy0416A also indicated a contribution of these two domains. As we could show that the C-terminal domain of Spy0416 is also required for proteolytic activity we wanted to further investigate the contribution of the individual domains to IL-8 degradation.

6.3.1 Recombinant Spy0416 Possesses IL-8 Cleavage Activity

IL-8 degradation could be reconstituted *in vitro* with the two generated subfragments Spy0416A and Spy0416B, as already published [162, 163]. We could further show that IL-8 degradation was dependent on enzyme concentration and degradation time visualized by immunoblotting since only active IL-8 was detected with anti CXCL-8 antibodies.

In order to confirm this data and provide a more detailed kinetic on the process, a degradation assay was performed based on the measurement of chemiluminescence.

Therefore, Spy0416A and Spy0416B were incubated in increasing concentrations for each protein with IL-8 for various time intervals at 37°C and IL-8 was quantified on a nitrocellulose membrane using FluoreChem SP Imaging System and a CCD digital camera.

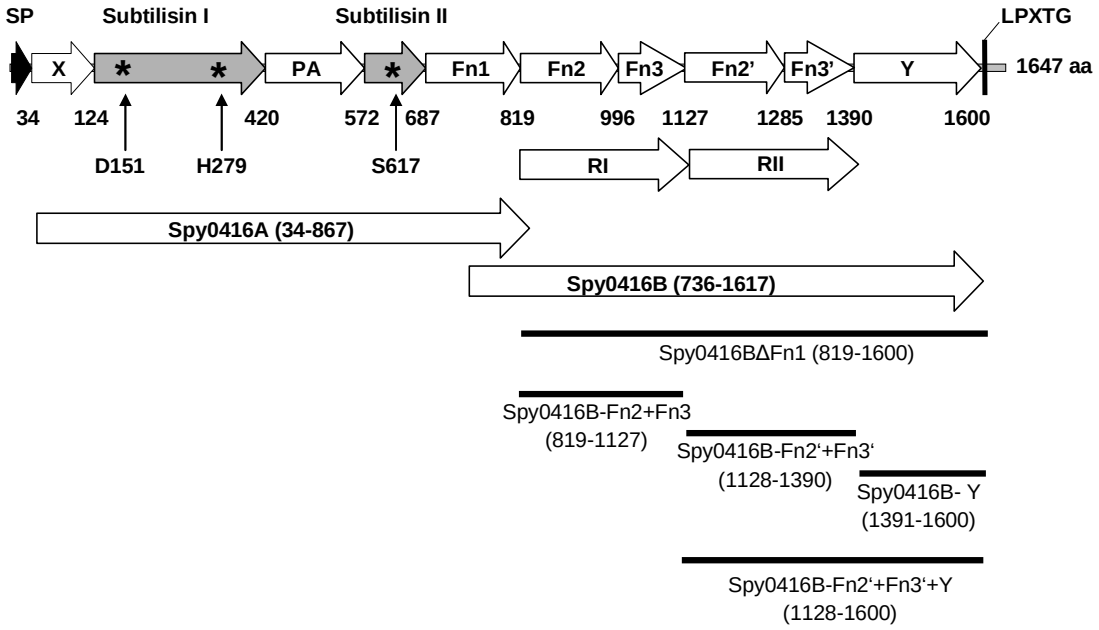

Figure 4: Schematic representation of the domain architecture of serine proteinase Spy0416 and Spy0416B subconstructs

SP, signal peptide; X, Y, low complexity regions; PA, protease associated domain; RI, RII, repeat regions; Fn1-3, Fn2', Fn3', Fibronectin type III (Fn) domains; LPXTG, cell wall anchor motif. Predicted active site residues within Spy0416 are depicted by asterisk. Spy0416A and Spy0416B show a sequence overlap of 132 amino acids located in the Fn1 domain. Spy0416B subconstructs Spy0416B$_{\Delta Fn1}$ (Phe^{819}-Ala^{1600}), Spy0416B$_{Fn2Fn3}$ (Phe^{819}-Thr^{1127}), Spy0416B$_{Fn2`Fn3`}$ (Leu^{1128}-Asp^{1390}), Spy0416B$_{Y}$ (Leu^{1391}-Ala^{1600}) and Spy0416B$_{Fn2`Fn3`Y}$ (Leu^{1128}-Ala^{1600}) have also been generated.

As shown in Figure 5A, after approximately 1.5 hours 50% of the chemokine was degraded by Spy0416A and Spy0416B. Approximately 80% of IL-8 was degraded after 2.5 hours which was only barely detectable on the immunoblot. Degradation of IL-8 was not only dependent on the time of incubation with the cell envelope serine proteinase, but also depending on the enzyme concentration (see Figure 5B). At a concentration of 44 µg/ml Spy0416A and Spy0416B approximately 40% of IL-8 was degraded. At an enzyme concentration of 110 µg/ml, inactivation of 100% of IL-8 could be observed already after 30 minutes of incubation. These data confirm the dependence of IL-8 degradation on incubation time and Spy0416 concentration.

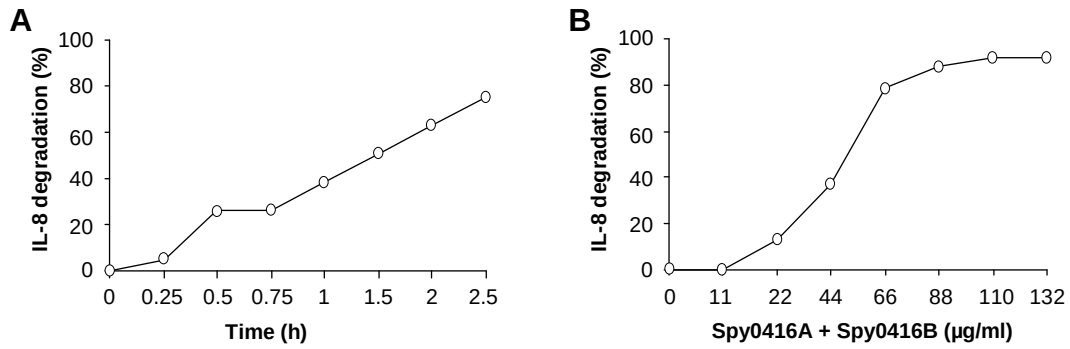

Figure 5: Time and concentration dependent degradation of IL-8 by Spy0416A and Spy0416B
(**A)** Time dependent IL-8 degradation using each 22 µg/ml Spy0416A and Spy0416B incubating at various time intervals from 0 – 2.5 hours and constant amounts of IL-8 (6.6 µg/ml). IL-8 was quantified by the measurement of chemiluminescence using the FluorChem SP Imaging System. (**B)** Concentration dependent IL-8 degradation after 30 minutes at 37°C using increasing amounts of Spy0416A and Spy0416B (0 – 132 µg/ml) and constant amounts of IL-8 (6.6 µg/ml). IL-8 was quantified as described under A.

6.3.2 Binding of IL-8 by Spy0416 is Mainly Mediated by the N-terminal Domain

In order to further evaluate the essential contribution of Spy0416B to the enzymatic activity of Spy0416A, we assessed whether IL-8 binding occurs via the catalytic Spy0416A domain or the catalytically inert Spy0416B domain. Our immunoprecipitation studies with IL-8 and IL-8 antibodies failed for technical reasons (non-specific binding of IL-8 to Protein G beads used for IgG binding). Therefore, in collaboration with Andreas Kungl from the Karl-Franzens University in Graz, Austria, we applied isothermal fluorescence using soluble proteins and the fluorophore bis-ANS in order to evaluate binding of IL-8 to Spy0416A and B proteins. Titration of IL-8 to the samples containing Spy0416A or B and bis-ANS, should induce displacement of the fluorophore upon binding of IL-8 to *S. pyogenes* proteins, resulting in fluorescence quenching of bis-ANS emission. These analyses showed that both proteins bind IL-8, however Spy0416A had an approximately 20 times higher affinity for IL-8 (K_D = 2.7x 10^{-7} M) compared to Spy0416B (K_D = 1.2x 10^{-6} M) (see Figure 6A). In order to confirm the large differences in affinities of Spy0416A and Spy0416B towards its IL-8 ligand, we performed Surface Plasmon Resonance (SPR) experiments in which IL-8 was immobilised via amine coupling to a BIAcore C1 chip. By this means, a kinetically-derived K_D value of 13.1 nM was determined for the interaction of Spy0416A with IL-8, but no interaction of IL-8 with Spy0416B was detected in this set-up. We also evaluated the binding of the Spy0416A1 mutant protein to IL-8 and detected a 70-fold lower, but still significant K_D value of 926 nM for the interaction of the chemokine with Spy0416A1 (see Figure 6B).

The difference in K_D values for Spy0416A interacting with IL-8 derived by the two different methods (Isothermal fluorescence titration and Surface plasmon resonance) relates to the fact that potentially induced oligomerisation and/or cooperative structural effects induced by protein-protein binding lead to different results, if one of the interacting partners is immobilised.

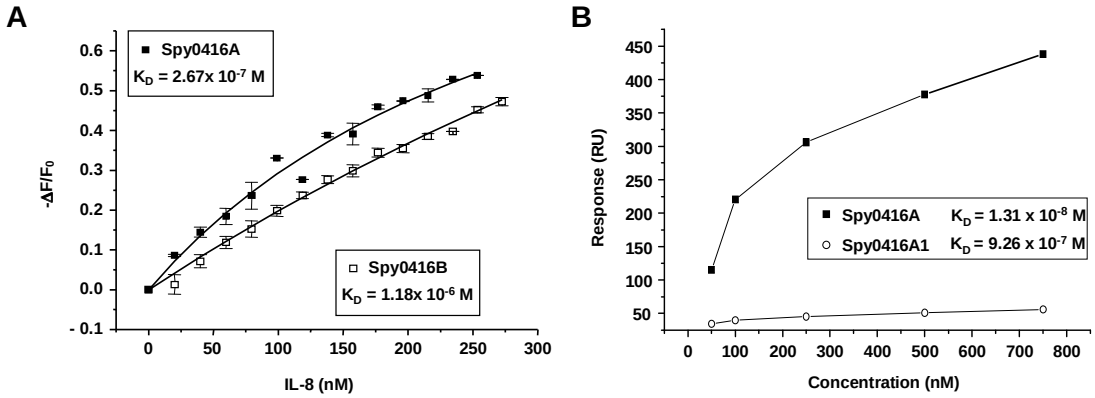

Figure 6: Binding of IL-8 to Spy0416 as measured by Isothermal Fluorescence Titration and Surface Plasmon Resonance (SPR) analysis
(**A**) Isothermal fluorescence titrations of 39 nM Spy0416A (■) or 39 nM Spy0416B (□) was measured in the presence of 1 μM bis-ANS. Excitation was set at 395 nm and fluorescence emission spectra were collected between 450 nm and 550 nm. The fluorescence quenching of bis-ANS emission upon addition of increasing IL-8 concentrations was background corrected and normalised for generation of the binding isotherms. K_D values were fitted by non linear regression as described in Materials and Methods. **(B)** Sensorgram overlay of Spy0416 variants binding to immobilized IL-8. Spy0416A (■) or Spy0416A1 (○) were injected over an IL-8 sensorchip at 0, 50, 100, 250, 500, 750, 1000 nM concentration for 3 min at 30 μl/min flow rate. The RU (resonance unit) response was recorded as a function of time and kinetic rates were calculated from both association and dissociation phases.

6.3.3 The Interaction of Spy0416A and Spy0416B Competes with Binding of Spy0416A to IL-8

Since the isothermal fluorescence experiment was performed by an indirect displacement using a non-covalently attached fluorophore, we were not able to measure the interaction of three proteins – Spy0416A, Spy0416B and IL-8 – in one assay. As both, Spy0416A and Spy0416B were required for proteolytic activity we applied again in collaboration with Andreas Kungl a gel depletion assay to investigate how the two fragments of Spy0416 influence each other's binding to IL-8. In this assay, binding of the Spy0416 protein to IL-8 would shift the resulting complex towards the opposite charge-flow direction as compared to IL-8 alone, resulting in depletion of IL-8 from the gel. Despite the intermediate binding affinity between IL-8 and the Spy0416 domains (low micromolar range), we were able to detect significant shifts due to the chemokine's quaternary structure that tends to increase from a dimeric to an oligomeric structure

upon ligand binding. A 1:10 molar ratio of the Spy0416 proteins and IL-8 used in the gel depletion assays led to chemokine oligomerisation and thus to a complete depletion of IL-8 by the stronger binding to Spy0416A (see Figure 7, lane 1), whereas Spy0416B alone was not capable of fully depleting the chemokine (see Figure 7, lane 2). The Spy0416A$_{D151A}$ protein, which was shown to be devoid of catalytic activity [162], was also able to deplete IL-8, indicating significant binding to the chemokine even in the absence of proteolytic activity (see Figure 7, lanes 3 & 4). Interestingly, in the presence of Spy0416B, Spy0416A and Spy0416A$_{D151A}$ were unable to completely deplete IL-8 from the gel (see Figure 7, lanes 5 & 6) suggesting that the interaction between the N- and C-terminal parts of Spy0416 modulates substrate binding.

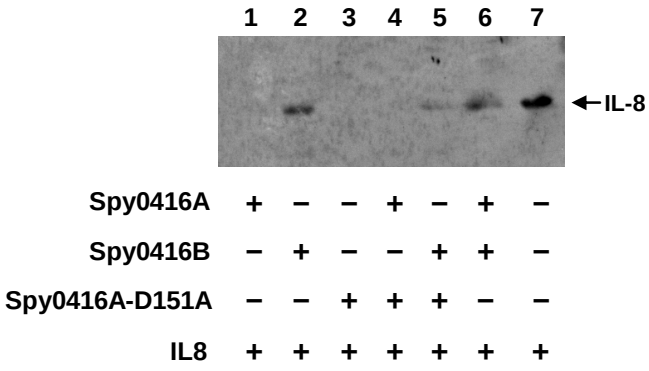

Figure 7: Interaction of Spy0416 variants and IL-8 evaluated by gel depletion assay
Western blot analysis of IL-8/ Spy0416-protein complexes after 15% native-PAGE (3 hrs, 100 V). **lane 1**: 5.2 μM Spy0416A + 54 μM IL-8; **lane 2**: 4.9 μM Spy0416B + 54 μM IL-8; **lane 3**: 5.2 μM Spy0416A-D151A + 54 μM IL-8; **lane 4**: 2.6 μM Spy0416A + 3.5 μM Spy0416A-D151A + 54 μM IL-8; **lane 5**: 4.9 μM Spy0416B + 3.5 μM Spy0416A-D151A + 54 μM IL-8; **lane 6**: 2.6 μM Spy0416A + 4.9 μM Spy0416B + 54 μM IL-8; **lane 7:** 54 μM IL-8.

6.3.4 Influence of IL-8 Degradation by Spy0416A/B on Ca^{2+}-Release of Neutrophils

It was shown recently that cleavage of IL-8 by Spy0416 reduces the migration of neutrophils in response to the cytokine [89]. As it was previously also described that one can distinguish between IL-8 mediated activation and migration of neutrophils [174, 175] we investigated in collaboration with Andreas Kungl whether IL-8 cleavage by Spy0416 also abrogates human neutrophil activation. We thus assessed the Ca^{2+}-release of neutrophils before and after pre-incubation of the chemokine with Spy0416A/B. Neutrophils showed the expected Ca^{2+}-release after injection of native IL-8 (see Figure 8A). However, prior incubation of IL-8 with Spy0416A/B had no effect on neutrophilic Ca^{2+}-release (see Figure 8B). A signal of similar intensity could even be detected after an incubation time of IL-8 with Spy0416A/B of 3.5 hours, indicating that the degradation

product of IL-8 (devoid of the C-terminal 13 amino acids) is able to stimulate neutrophils, most probably due to the ELR motif at N terminus of the protein which is mainly responsible for receptor recognition and activation via CXCR1 (CXC chemokine receptor 1) and CXCR2 (CXC chemokine receptor 2).

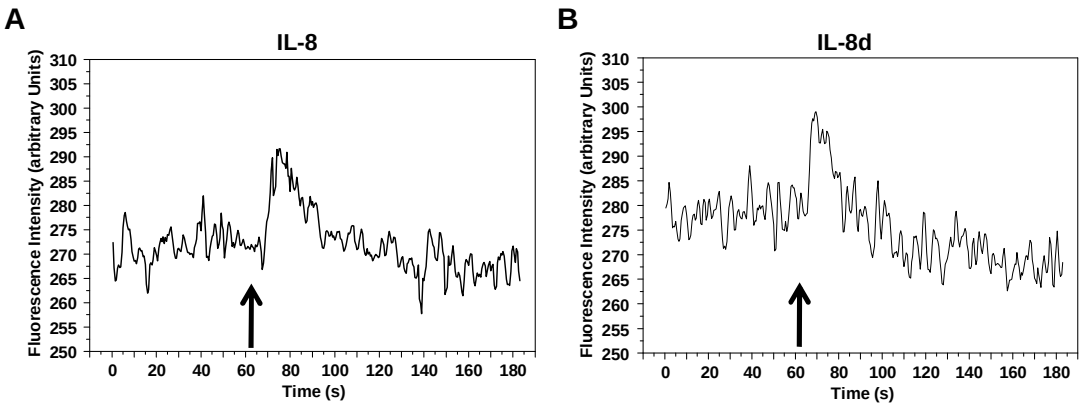

Figure 8: Ca^{2+}- release by neutrophils
Neutrophils were isolated as described under Materials and Methods. IL-8 was incubated with Spy0416A and Spy0416B in PBS at 37°C and degradation was stopped every 30 minutes for 3.5 hours by freezing the sample at -20°C. Immediately before measurement, 1 ml aliquots were thawed, centrifuged for 3 minutes at 500 g and resuspended in 1 ml HBSS(+). Fluorescence was monitored for app. 3 minutes and cells were stimulated with 100 nM protein 60 seconds after pre-equilibration, and maximum and minimum fluorescence were determined. **(A)** Calcium-release by neutrophils following injection of undigested IL-8 (840 ng) after 60 seconds (arrow). **(B)** Calcium-release of neutrophils after IL-8 (840 ng) injection previously incubated for 3.5 hours with Spy0416A/B (described as IL-8d in this Figure).

6.3.5 Mutation of Single Residues of IL-8 Reduces Degradation by Spy0416

The G protein-coupled (GPC) receptor binding activity of the chemokine IL-8 has been assigned to the N-terminal amino acid residues Glu^4-Leu-Arg^6 [165, 174], whereas the cleavage by Spy0416 was reported to occur between residues Gln^{59} and Arg^{60} of mature IL-8 (see Figure 9A) [89]. It was thus of interest to determine whether the cleavage activity of Spy0416 could be influenced by mutations within IL-8 and whether the specificity could be assigned to the cleavage residues itself or residues outside of the cleavage site. Recently, Skelton et al. have shown that the interaction of IL-8 with the GPC receptor peptide derived from CXCR1 involves the phenylalanine residues at position 17 and 21 of the chemokine [176]. Interestingly, the degradation of the Interleukin 8 mutant $IL\text{-}8_{F17K,F21K}$ is abolished as compared to wild type IL-8 (see Figure 9B). Other IL-8 mutants containing amino acid substitutions at the Phe^{17} and Phe^{21} positions other than Lys residues (for example Arg residues) exhibited a similar

reduction in cleavage by Spy0416 (data not shown), indicating that changes within IL-8 distinct from the cleavage site strongly influence the interaction with Spy0416.

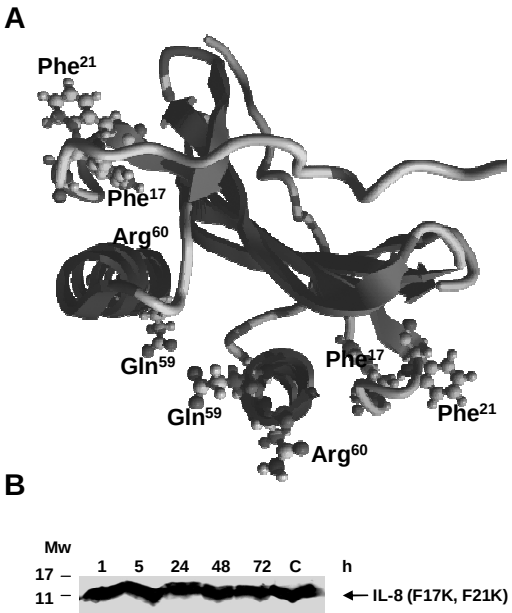

Figure 9: Degradation of the IL-8 mutant IL-8$_{F17K,F21K}$
(A) Ribbon drawing of the IL-8 backbone structure; shown in ball and stick representation are the amino acids targeted by the Spy0416 enzyme as well as the sites which are, in addition, required for processing by Spy0416. (**B)** The mutant form of IL-8, IL-8$_{F17K,F21K,}$ was used in a concentration of 6.6 µg/ml and tested for degradation by Spy0416A and Spy0416B (22 µg/ml each) at 37°C from one up to 72 hours. The control (C) represents chemokine without Spy0416A and Spy0416B incubated at 37°C for 72 hours. IL-8$_{F17K,F21K}$ was visualized by Western blot analysis as described in materials and methods.

6.3.6 Deletion of *spy0416* Delayed Virulence in an Intraperitoneal Murine Challenge Model

The *spy0416* gene deletion mutant, ΔSpy0416 was generated in collaboration with Bernd Kreikemeyer (Rostock, Germany). In order to evaluate the role of Spy0416 in streptococcal pathogenesis we investigated the effect of *spy0416* gene deletion in an intraperitoneal murine challenge model and survival of mice was monitored over a period of 2 weeks. As it is shown in Figure 10 ΔSpy0416 showed a reduced or delayed virulence compared to the M49591 wild type strain which confirmed the recently published reduced virulence of the Spy0416 mutant [91]. In an attempt to confirm the *emm* type of the ΔSpy0416 mutant, it was later found that it showed a deviation in *emm* type. Instead of the expected M49 serotype, the M gene sequencing revealed an *emm* type 102. For this reason no further experiments were performed with the mutant.

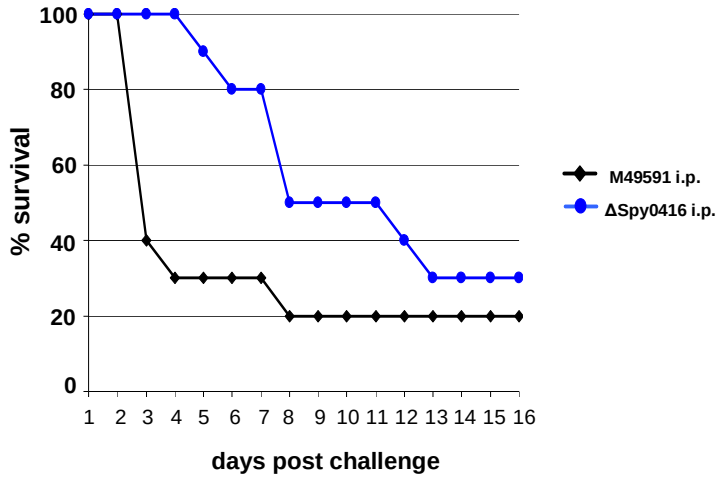

Figure 10: Virulence of ΔSpy0416 in an intraperitoneal murine challenge model
CD-1 mice were challenged with 5x 10^8 cfu of *S. pyogenes* strains M49591 wild type and ΔSpy0416 applied intraperitoneally. Survival of mice was monitored over a period of 16 days.

6.3.7 Generation of Spy0416 Subconstructs

The *spy0416* gene comprises 4944 base pairs and encodes a protein with a calculated molecular weight of 181 kDa. The generation of the two subfragments, Spy0416A and Spy0416B, was already described previously [162, 163]. The Spy0416A protein (Mw = 94.52 kDa), encompasses the Subtilisin-like protease domains intervened by a protease associated domain (see Figure 4, page 62). The Spy0416B protein (Mw = 103.11 kDa) encompasses the entire C-terminal fragment of Spy0416, devoid of 50 amino acids cleaved off due to anchoring in the cell wall by the LPXTG motif. I could show that the N-terminal domain Spy0416A alone, did not reveal any IL-8 degrading activity [162, 163]. IL-8 degradation could only be reconstituted by the addition of Spy0416B as separate recombinant protein. Therefore I wanted to continue my work on the evaluation of distinct domains contributing to IL-8 degradation.

The C-terminal region Spy0416B, entails two repetitive regions of unknown function, RI and RII, each of which consists of two domains identified in C5A peptidase as Fn2/Fn3 and Fn2'/Fn3', respectively, postulated to contribute to regulation of enzymatic activity [177]. The Y region located at the C terminus between Fn3'and the LPXTG motif shows homology to similar regions in proteinases such as CspA of *S. agalactiae* [177] and to sigma A factors of Flavobacteriaceae (app. 30% identity with the N-terminal 100 amino acids). Based on this I generated the following Spy0416 subconstructs: Spy0416B$_{Fn2Fn3}$ (Phe^{819}-Thr^{1127}), Spy0416B$_{Fn2'Fn3'}$ (Leu^{1128}-Asp^{1390}), Spy0416B$_Y$ (Leu^{1391}-Ala^{1600}),

Spy0416B$_{Fn2'Fn3'Y}$ (Leu^{1128}-Ala^{1600}), Spy0416A$_{\Delta Fn2}$ (Ala^{34}-Gln^{818}) and Spy0416B$_{\Delta Fn1}$ (Leu^{1128}-Ala^{1600}). The latter ones lack the Fn2 and the Fn1 domains, respectively, which led to the elimination of the overlap between recombinant Spy0416A and Spy0416B (see Figure 4, page 62). Sequences were amplified from SF370 (primers listed in Table 4, Appendix I) since this was the *S. pyogenes* strain used for the generation of genomic libraries. All proteins were expressed by *E. coli* from expression vector pET28b(+) with a tag of six histidines at their C-terminus for purification except for Spy0416B$_{\Delta Fn1}$. Its expression could only be observed in pET28bn-His resulting in a N-terminal His-tag. The hexa-His-tagged fusion proteins were isolated from the soluble fraction only since insoluble recombinant proteins are usually purified using 8M urea which might result in loss of their original protein conformation and thus in enzymatic activity.

The expressed and purified recombinant Spy0416 proteins were assessed for purity by SDS-PAGE as presented in Figure 11.

Spy0416B$_{Fn2'Fn3}$ (Mw 28.9 kDa), Spy0416B$_{Y}$ (Mw 23.1 kDa) and Spy0416B$_{Fn2'Fn3'Y}$ (Mw 52 kDa) recombinant proteins do not show any major degradation products. By the use of BSA as quantitative standard a final protein amount of ~32.5 mg, 15 mg and 30 mg was calculated as product obtained from a shaking *E. coli* culture of 1 litre, respectively. Spy0416B$_{\Delta Fn1}$ (85.9 kDa) shows increased purity and less degradation than the recombinant Spy0416B full length protein which was prone to degradation [162, 163].

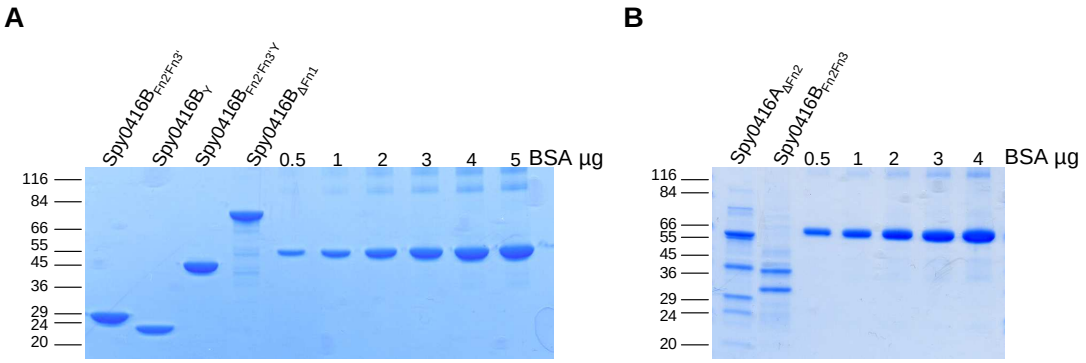

Figure 11: Analysis of recombinant Spy0416B subconstructs by SDS-PAGE
Recombinant proteins Spy0416A$_{\Delta Fn1}$, Spy0416B$_{Fn2Fn3}$, Spy0416B$_{Fn2'Fn3'}$, Spy0416B$_{Y}$ and Spy0416B$_{Fn2'Fn3'Y}$ were expressed in BL21-CodonPlus® (DE3)-RIPL *E. coli* cells using pET28b(+) and Spy0416B $_{\Delta Fn1}$ was expressed using pET28bn-His. **(A)** SDS-PAGE of 1 µl Spy0416B$_{Fn2'Fn3'}$, of 1µl Spy0416B$_{Y}$ and Spy0416B$_{Fn2'Fn3'Y}$ and 5 µl of Spy0416B $_{\Delta Fn1}$ deriving from elution using 15 ml of 500 mM imidazole, 20 mM NaH_2PO_4, 0.5 mM NaCl in 50 mM Tris/HCl pH 8.0 after dialysis against PBS. BSA served as a quantitative standard using 0.5, 1, 2, 3, 4 and 5 µg for comparison. **(B)** SDS-PAGE of 1 µl Spy0416A$_{\Delta Fn2}$ and Spy0416B$_{Fn2Fn2}$ deriving from elution using 15 ml of 500 mM imidazole, 20 mM NaH_2PO_4, 0.5 mM NaCl in 50 mM Tris/HCl pH 8.0 after dialysis against PBS. BSA served as a quantitative standard using 0.5, 1, 2, 3, and 4 µg.

The final protein amount from a 1 litre *E. coli* culture was ~3 mg. In contrast, Spy0416B$_{Fn2Fn3}$ (34.8 kDa) could not be purified with the same purity and quantity as the other Spy0416 subconstructs. For this recombinant protein only 0.188 mg could be purified (see Figure 11B). Spy0416A$_{\Delta Fn2}$ with a calculated molecular weight of 87.3 kDa could not be expressed in full length (see Figure 11B).

6.3.8 The Entire Spy0416 C-terminal Region is Required for IL-8 Degradation

The entire C-terminal domain was shown to be required for IL-8 degradation which indicated an essential function of Spy0416B possibly modulating and/or contributing to the N-terminal protease activity. In order to find out whether any of the domains can be deliniated, distinct domains generated as separate recombinant proteins were tested for their contribution to IL-8 degradation in combination with Spy0416A. As it is shown in Figure 12A Spy0416B$_{\Delta Fn1}$ combined with Spy0416A degraded IL-8 with the same efficiency as seen with the full length Spy0416B protein. In contrast, for none of the three proteins Spy0416B$_{Fn2Fn3,}$ Spy0416B$_{Fn2'Fn3'}$ and Spy0416B$_{Fn2'Fn3'Y}$ in combination with Spy0416A we could observe IL-8 degradation (see Figure 12B). Also Spy0416A combined with Spy0416B$_{Fn2Fn3,}$ and Spy0416B$_{Fn2'Fn3'}$ or Spy0416B$_{Fn2Fn3}$ and Spy0416B$_{Y}$ or Spy0416B$_{Fn2Fn3}$ and Spy0416B$_{Fn2'Fn3'Y}$ (see Figure 12C) did not reveal cleavage of IL-8.
These results suggested that all the domains of Spy0416 need to be present for enzymatic activity.

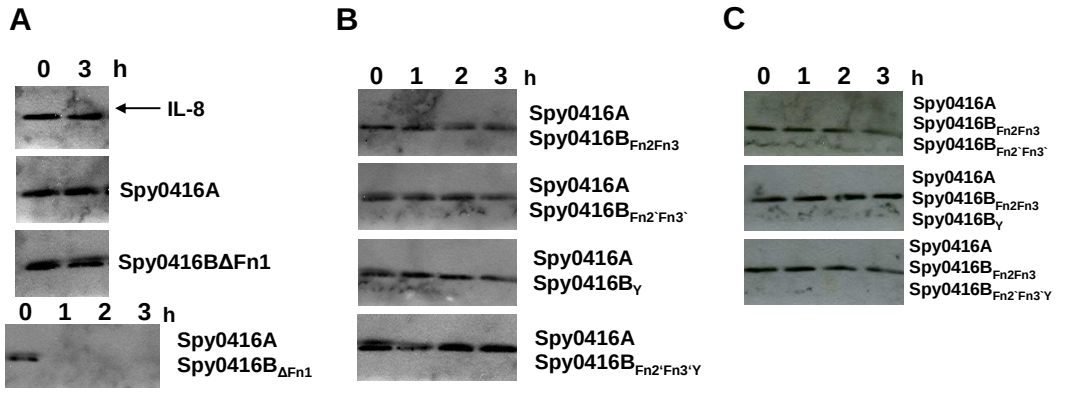

Figure 12: Degradation of IL-8 by Spy0416A combined Spy0416B subconstructs
Western blot analysis of active human IL-8 using goat anti-human CXCL8/IL-8 specific antibodies (1:1000 dilution) and secondary rabbit anti-goat IgG/HRP (1:5,000) for detection. **(A)** Spy0416A and Spy0416B$_{\Delta Fn1}$, were individually (44 µg/ml) and in combination (22 µg/ml) incubated with IL-8 (6.6 µg/ml) at 37°C for up to 3 hours. **(B)** IL-8 (6.6 µg/ml) was incubated with 22 µg/ml Spy0416A combined with 22 µg/ml Spy0416B$_{Fn2Fn3}$ or Spy0416B$_{Fn2`Fn3`}$ or Spy0416B$_{Y}$ or Spy0416B$_{Fn2'Fn3'Y}$. **(C)** IL-8 (6.6 µg/ml) was incubated with 14.5 µg/ml Spy0416A combined with 14.5 µg/ml Spy0416B$_{Fn2Fn3}$ and 14.5 µg/ml Spy0416B$_{Fn2'Fn3'}$ or Spy0416B$_{Fn2Fn3}$ and Spy0416B$_{Y}$ or Spy0416B$_{Fn2Fn3}$ and Spy0416B$_{Fn2'Fn3'Y}$.

6.3.9 IP-10, MCP-1 and i-TAC are Not Degraded by Spy0416 *In Vitro*

So far human IL-8, GCP-2 (granulocyte chemoattractant protein 2) and GRO-α (growth-related oncogene alpha) as well as murine KC (keratinocyte-derived chemokine) and murine MIP-2 (macrophage inflammatory protein 2) were the cytokines that have been reported to be degraded by ScpC (Spy0416) from culture supernatants [91, 178]. These CXC chemokines are responsible for acute neutrophil priming and/or mobilization and migration in their respective species. In contrast, RANTES, which is responsible for T-cell and monocyte chemotaxis, was not degraded at all [91]. Therefore, we wanted to analyse further human chemokines of the CXC family for activity exerted by the recombinantly produced Spy0416. As GRO-α was also degraded by Spy0416 *in vitro* [162], the chemokines IP-10 (Interferon-inducible protein 10) and I-TAC (IFN-inducible T-cell alpha-chemoattractant) were selected to evaluate whether they can also be degraded by Spy0416. Although they are involved in the recruitment of activated T-cells they were selected for the degradation assay as they belong to the same chemokine (CXC) family and display very similar overall three-dimensional folds as seen in Gro-α. Furthermore MCP-1 was selected from the CC-chemokine family. The conditions applied during the degradation assay were the same as used for IL-8 degradation, except that the concentration of IP-10, I-TAC and MCP-1 was increased to 8.8 μg/ ml. As can be seen in Figure 13 the combined Spy0416A & B domains were not capable of readily degrading the human CXC chemokines IP-10 and I-TAC or the CC chemokine MCP-1 tested, despite their similar three-dimensional structure.

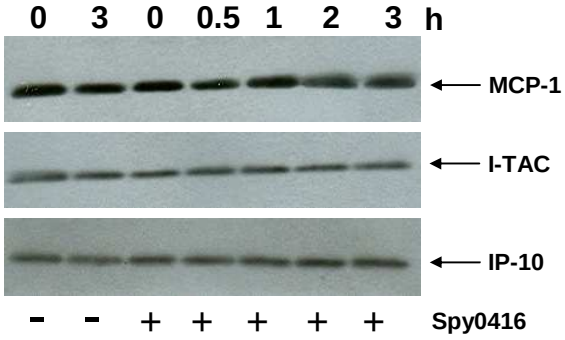

Figure 13: Degradation of MCP-1, I-TAC and IP-10 by Spy0416A combined Spy0416B
Western blot analysis of active human MCP-1, I-TAC and IP-10 using respective polyclonal goat anti-human antibodies diluted 1:1000 and secondary rabbit anti-goat IgG/HRP diluted 1:5,000 for detection. Spy0416A and Spy0416B were in combination (22 μg/ml) incubated with MCP-1, I-TAC and IP-10, utilized at a final concentration of 8.8 μg/ ml at 37°C for up to 3 hours.

6.4 Generation of *spy0895* and *spy1536* Gene Deletion Mutants

The two genes encoding Spy0895 and Spy1536 were targeted for the generation of gene deletion mutants. Spy0895 is annotated as putative histidine protein kinase and Spy1536 is a conserved hypothetical protein with a C-terminal Lon protease domain. Both proteins showed consistent protection in animal experiments. No function or possible contribution to pathogenesis of *S.pyogenes* was reported and they do not show a high degree of identity to other known proteins allowing to predict a function. The *spy0895* and *spy0894* genes (*spy0894* encodes a putative purine nucleoside phosphorylase) constitute a two gene operon with overlapping open reading frames of 8 bp. The *spy1536* gene is located with *spy1537*, encoding a putative 3-deoxy-D-manno-octulosonic-acid transferase, and with a conserved hypothetical protein encoded by *spy1538* in a three gene operon (14B, page 74).

The purpose of the generation of gene deletion mutants was to assess the function by analyzing their phenotype in terms of growth defects and virulence in mice as well as by *in vitro* assays, transcriptome analyses and interaction with host proteins.

Initially, generation of gene deletions was attempted in *S. pyogenes* AP-1 since this was the strain used for protection studies in the mouse model. However, all attempts to transform this strain with pGhost5, our shuttle vector of choice failed. Therefore, we decided to use strain M49591 that was kindly provided by Bernd Kreikemeyer (Rostock, Germany) and which had successfully been transformed previously [166].

6.4.1 Design of Gene Deletion Constructs

The first step for generation of gene deletion mutants was the generation of knock out constructs consisting of N- and C-terminal flanking regions of *spy0895* and *spy1536* ligated to one another. The purpose of the knock out constructs is to serve as recombination sites for vector integration into the chromosome which in the ideal case leads to gene deletion after excision. The design of knock out constructs also defines the size of the generated deletion within *spy0895* and *spy1536*. The fact that *spy0895* and *spy1536* are located in a two and a three gene operon, respectively, complicates gene deletion since the flanking genes must not be affected by the deletion in terms of

transcription. This was taken into consideration for the design of the knock out constructs.

A 3-step PCR strategy was employed for the generation of knock out constructs (see Figure 14A) using primers listed in Table 5 (Appendix I).

For *spy0895*, first a 662 bp fragment was amplified using primers 5412 and 5413 containing 120 bp of *spy0895* gene specific sequence. The second 621 bp fragment was amplified using primers 5414 and 5415 containing 66 bp of the *spy0895* gene. In a third PCR the two fragments were ligated using primers 5412 and 5415 resulting in a 1273 bp fragment. This design of the knock out construct would allow a deletion of 606 bp from the 792 bp full length *spy0895* (see Figure 14B), leaving *spy0894* gene untouched. In case of transcription of the remaining *spy0895* gene sequence, a transcript of 132 bp due to stop codons within the ORF could be expected which could result in the expression of a mutant protein with a calculated molecular weight of 5 kDa.

For the *spy1536* gene an N-terminal fragment of 589 bp was amplified using primers 5416 and 5417 and the C-terminal 284 bp fragment was amplified using primers 5418 and 5419. The ligated fragments constitute the *spy1536* knock out construct and would result in a deletion of 501 bp (see Figure 14C) in the M49591 strain. The design of this construct was also based on the GC content, thermodynamic properties and palindromic sequences of the primers. For this reason, 537 bp of the *spy1536* gene would be still found on the chromosome of the respective mutant strain. Due to the generation of stop codons within the ORF of *spy1536,* a transcript of 138 bp could result in a mutant protein of 5.5 kDa. The *spy0895* and *spy1536* knock out constructs were finally transferred into the pGhost5 vector resulting in vectors pGhost5-*0895* and pGhost5-*1536*, respectively.

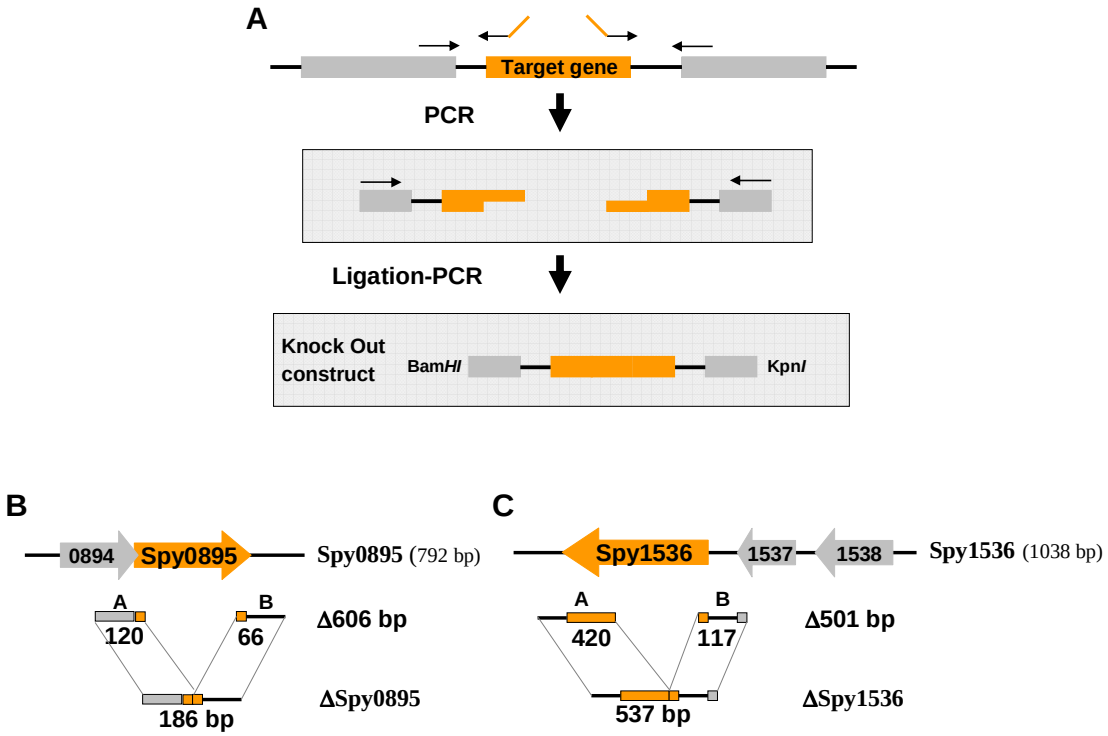

Figure 14: Design and generation of *spy0895* and *spy1536* gene deletion constructs
(**A)** Schematic representation of 3 step-PCR strategy for generation of knock out constructs. N- and C-terminal regions of the gene of interest were amplified first. In a third PCR the two flanking regions were ligated giving the final knock out construct for cloning into pGhost5. **(B)** Design of knock out construct for *spy0895*: 120 bp and 66 bp of the *spy0895* gene were included into N- and C-terminal flanking regions, respectively resulting in a knock out construct of 1273 bp carrying 186 bp of the *spy0895* sequence. This would lead to a deletion in ΔSpy0895 of 606 bp **(C)** Design of knock out construct for *spy1536*: 420 bp and 117 bp of the N- and C-terminal region, respectively, resulting in a knock out construct of 873 bp carrying 537 bp of the *spy1536* sequence. This would lead to a deletion of 501 bp in ΔSpy1536.

6.4.2 Generation of Gene Deletion Mutants ΔSpy0895 and ΔSpy1536 in Three Steps

Three steps were required to delete the sequences in *spy0895* and *spy1536*, defined by the designed knock out constructs using the temperature sensitive shuttle vector pGhost5. The first step included the transformation of pGhost5-*0895* and pGhost5-*1536* into the M49591 parental strain. The resulting erythromycin resistant strains M49*pGhost5-0895exc* and M49*pGhost5-1536exc*, harboured pGhost5-*0895* and pGhost5-*1536* extrachromosomally, respectively. In the second step a temperature shift to 37°C induced homologous recombination either via the N- or C-terminal fragment contained within the knock out constructs in vectors pGhost5-*0895* and pGhost5-*1536* (see Figures 15A and 16A). This led to the integration of pGhost5-*0895* and pGhost5-*1536* into the operon regions of *spy0895* and *spy1536* resulting in strains M49*pGhost5-0895int* and M49*pGhost5-1536int*, respectively.

Before continuing with step 3, the excision of the vector from the chromosome, proper integration of pGhost5 in strains M49*pGhost5-0895int* and M49*pGhost5-1536int* had to be confirmed by PCR using oligos (see Table 5, Appendix I) that flanked the insertion sites of the vector. For this confirmation two scenarios had to be taken into consideration.

As shown in Figures 15A and 16A pGhost5 integration can either occur via the N- or the C-terminal regions flanking the *spy0895* and *spy1536* genes. This results in two different scenarios of vector integration in the respective operons presented in Figures 15B and 16B. Consequently for each scenario caused by either recombination via the N- or the C-terminal flanking regions different PCR fragments are expected to be amplified.

Analysis of different erythromycin resistant clones of M49*pGhost5-0895int* using oligos (see Table 5, Appendix I) flanking the insertion site of pGhost5-*0895* showed that two different recombination scenarios occurred. In strain M49*pGhost5-0895int-N* recombination occurred via the N-terminal fragment as presented in Figure 15B. The use of primers 6005 and 6007 as well as primers 6006 and 6008 (see Table 5, Appendix I), designed to amplify sequences across the borders of pGhost5-*0895* integration, indeed showed the expected size in PCR. As shown in Figure 15C, primers 6005 and 6007 resulted in a fragment of 1863 bp and primers 6006 and 6008 amplified the expected 2374 bp. In strain M49*pGhost5-0895int-C* on the other hand the recombination occurred via the C-terminal region of *spy0895* which resulted in the generation of a 2647 bp and a 1767 bp product using the same set of primers as above (see Figure 15B and 15C). For the two strains M49*pGhost5-0895int*-N *and* M49*pGhost5-0895int*-C correct integration of pGhost5-*0895* in the *spy0895* gene region could be confirmed.

Different clones of M49*pGhost5-1536int* also showed that integration of pGhost5-*1536* construct either occured via the N- or C-terminal flanking regions (Figure 16A). This resulted in strains M49*pGhost5-1536int*-N and M49*pGhost5-1536int*-C. In strain M49*pGhost5-1536int-N* recombination occurred via the N-terminal fragment as presented in Figure 16B. The use of primers 6007 and 6009 as well as primers 6006 and 6010 (see Table 5, Appendix I), designed to amplify sequences across the borders of pGhost5 integration, indeed showed the expected size in PCR. As shown in Figure 16C primers 6007 and 6009 resulted in a fragment of 1794 bp and primers 6006 and 6010 amplified the expected 1198 bp. In strain M49*pGhost5-1536int-C* the recombination occured via the C-terminal region of *spy1536* which resulted in the generation of 1294 bp

and 1698 bp using the same set of primers as above (see Figure 16B). Also for the two strains M49*pGhost5-1536int*-N *and* M49*pGhost5-1536int*-C correct integration of pGhost5-*1536* in the *spy1536* operon was confirmed by PCR (see Figure 16C).

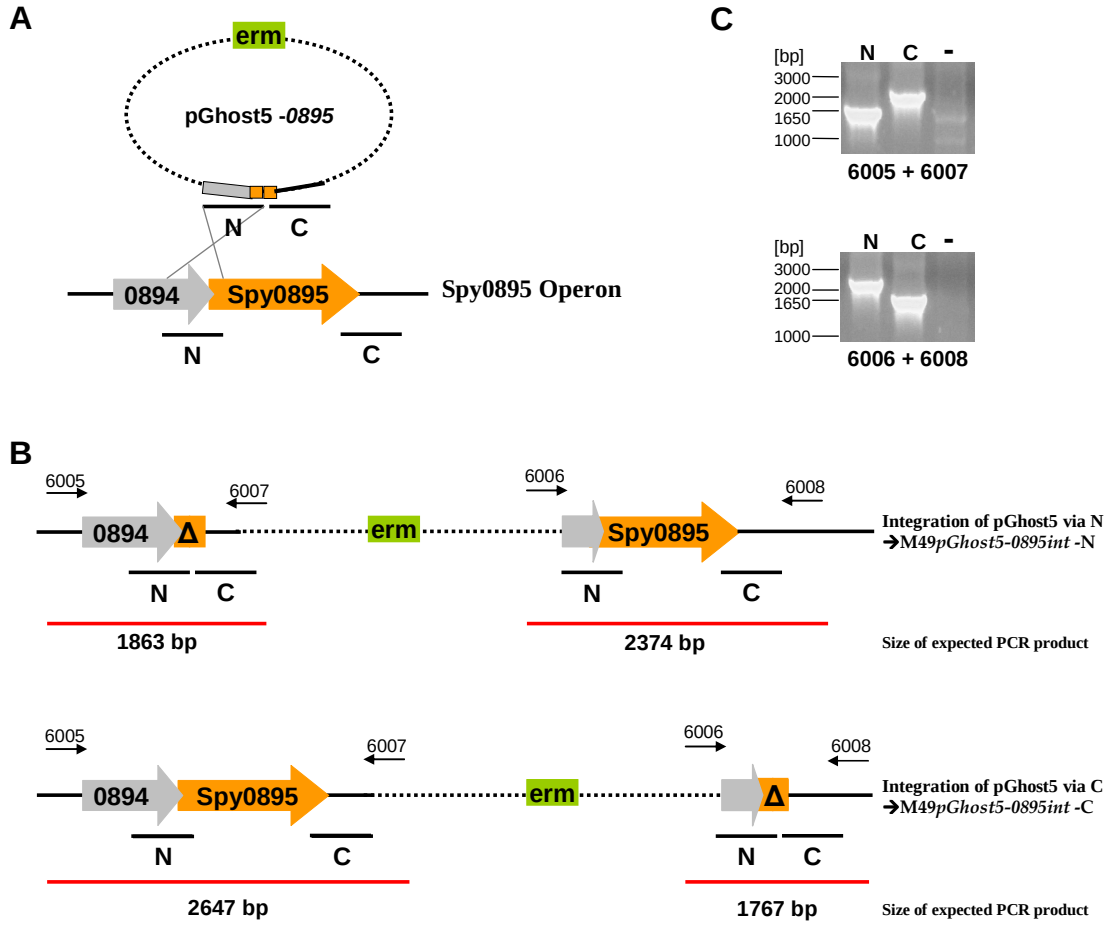

Figure 15: pGhost5-*0895* integration into the *spy0895* operon
(A) Schematic representation of pGhost5-*0895* that recombines with the N-terminal region of the *spy0895* gene. **(B)** Schematic representation of pGhost5-*0895* integrated into the *spy0895* operon by recombination via the N-terminal and C-terminal *spy0895* regions resulting in strains M49*pGhost5-0895int*-N *and* M49*pGhost5-0895int*-C, respectively. Primer binding sites are indicated as arrows and corresponding PCR products are indicated in red. **(C)** PCR analysis of M49*pGhost5-0895int*-N *and* M49*pGhost5-0895int*-C with primer pairs 6005 and 6007, 6006 and 6008.

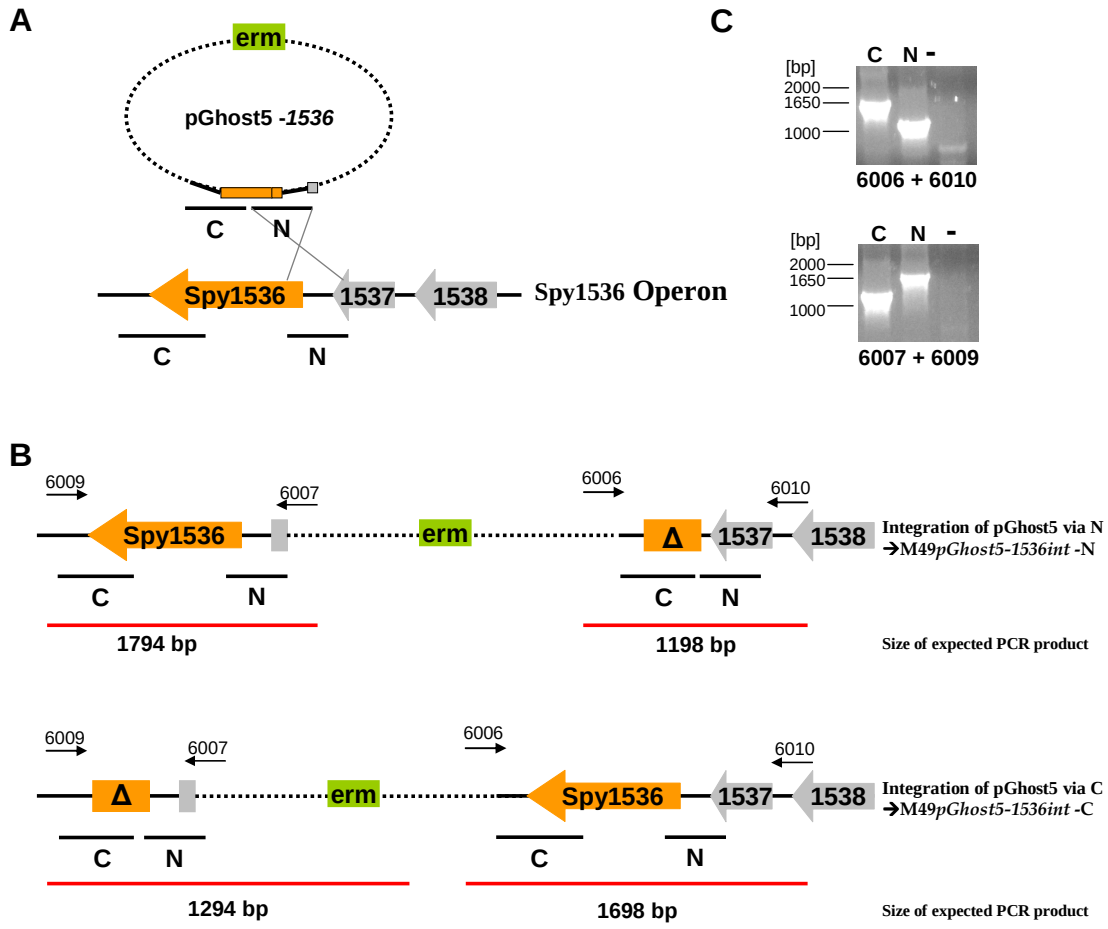

Figure 16: pGhost5-*1536* integration into the *spy1536* operon
(A) Schematic representation of pGhost5-*1536* that recombines with the N-terminal region of the *spy1536* gene. **(B)** Schematic representation of pGhost5-*1536* integrated into the *spy1536* operon by recombination via the N-terminal and C-terminal *spy1536* regions resulting in strains M49*pGhost5-1536int*-N *and* M49*pGhost5-1536int*-C, respectively. Primer binding sites are indicated as arrows and corresponding PCR products are indicated in red. **(C)** PCR analysis of M49*pGhost5-15365int*-N *and* M49*pGhost5-1536int*-C with primer pairs 6007 and 6009 and 6006 with 6010.

After confirmation of correct insertion of pGhost5-*0895* and pGhost5-*1536*, the third step, excision of the vector from the *S. pyogenes* chromosome, was carried out. In this last step pGhost5 excision was induced by a temperature shift back to 28°C. For this I continued for the *spy0895* and *spy1536* gene deletion with both strains that had been generated, M49*pGhost5-0895int*-N and M49*pGhost5-0895int*-C as well as M49*pGhost5-1536int*-N and M49*pGhost5-1536int*-C, respectively. This was due to the probability that in one of the two strains, where integration happened either via the N- or the C-terminal flanking region, the excision might be favored to lead to the desired recombination event - the deletion of *spy0895* and *spy1536*. Excision of pGhost5 results in an erythromycin sensitive *S. pyogenes* phenotype which can either be wild type M49591 or a gene deletion mutant. PCR was used to identify those erythromycin sensitive clones which harboured the desired gene deletion.

As shown in Figure 17A by the example of strain M49*pGhost5-0895int*-N, the excision of pGhost5-*0895* is explained. Recombination via the N-terminal regions of *spy0895* results in the parental wild type strain while recombination via the C-terminal regions in ΔSpy0895. Approximately 550 erythromycin sensitive clones were randomly picked from both strains, M49*pGhost5-0895int*-N and M49*pGhost5-0895int*-C subjected to the temperature shift, which then have been analysed by PCR. Primers 6005 and 6008 (see Figure 17A and Table 5 in Appendix I) in strains converted back to wild types amplified 2772 bp, while in strains where gene deletion occurred a PCR fragment 606 bp smaller in size was obtained (see Figure 17B). A difference between excision of pGhost5 in strains M49*pGhost5-0895int*-N and M49*pGhost5-0895int*-C resulting in gene deletion could not be observed. Among ~550 erythromycin sensitive clones analyzed ~ 300 clones were identified as *spy0895* deletion mutants. The success rate was approximately 55%

In case of *spy1536* gene deletion the example of pGhost5-*1536* excision using strain M49*pGhost5-1536int*-C is presented (see Figure 18A). In this case recombination via the C-terminal regions of *spy1536* results in the parental wild type strain, recombination via the N-terminal regions of *spy1536* leads to gene deletion, resulting in strain ΔSpy1536. Approximately 500 erythromycin sensitive clones of M49*pGhost5-1536int*-N and M49*pGhost5-1536int*-C were subjected to PCR analysis. Indeed PCR analysis with primers 6009 and 6010 (see Figure 18A and Table 5 in Appendix I) identified one clone which showed the 1461 bp PCR product instead of the 1962 bp fragment found in the M49591 wild type strain (Figure 18B). The success rate for *spy1536* gene deletion was about 0.2%. The gene deletion mutant ΔSpy1536 was among the erythromycin sensitive clones deriving from strain M49*pGhost5-1536int*-C.

To sum up, the three steps of gene deletion, transformation of pGhost5-*0895* and pGhost5-*1536*, integration into the *S. pyogenes spy0895* and *spy1536* operons as well as excision, successfully led to the generation of ΔSpy0895 and ΔSpy1536.

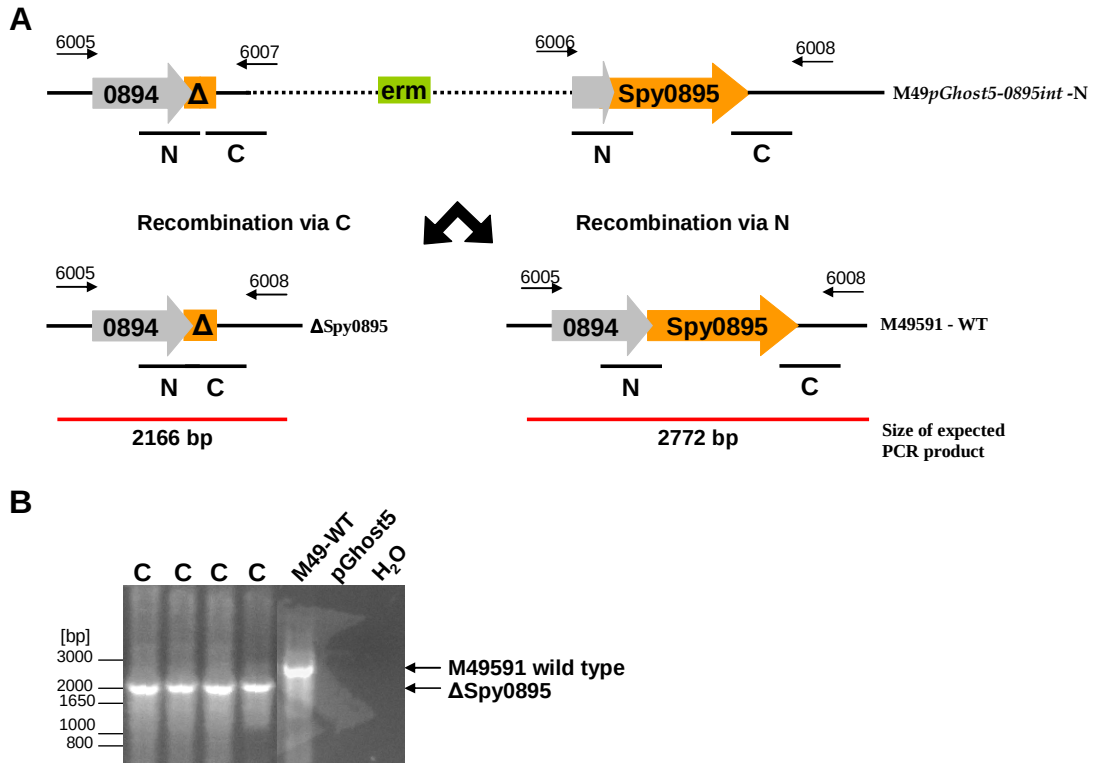

Figure 17: Gene deletion by excision of pGhost5 from the *spy0895* operon region
(A) Schematic representation of pGhost5 excision in strain M49*pGhost5-0895int*-N. Primer binding sites are indicated as arrows and corresponding PCR products are indicated in red. Recombination via the N-terminal *spy0895* region leads to the parental wild type strain, recombination via the C-terminal region results in gene deletion. **(B)** PCR results of erythromycin sensitive *S. pyogenes* phenotypes after pGhost5-*0895* excision from the *spy0895* operon obtained from amplification with primers 6005 and 6008.

6.5 Confirmation of *spy0895* and *spy1536* Gene Deletion

In order to confirm *spy0895* and *spy1536* gene deletions, sequencing of the regions where the deletion occurred was carried out with oligos that flanked the insertion site. Sequencing successfully confirmed *spy0895* and *spy1536* deletions as presented in the Appendix III. Additionally Southern blot analysis and Northern blot analysis were carried out to verify complete removal of pGhost5 from the *S. pyogenes* genome and evaluate whether a transcriptional influence of *spy0895* and *spy1536* gene deletion on the genes located within the same operon occurred (see Figure 14B and 14C, page 74).

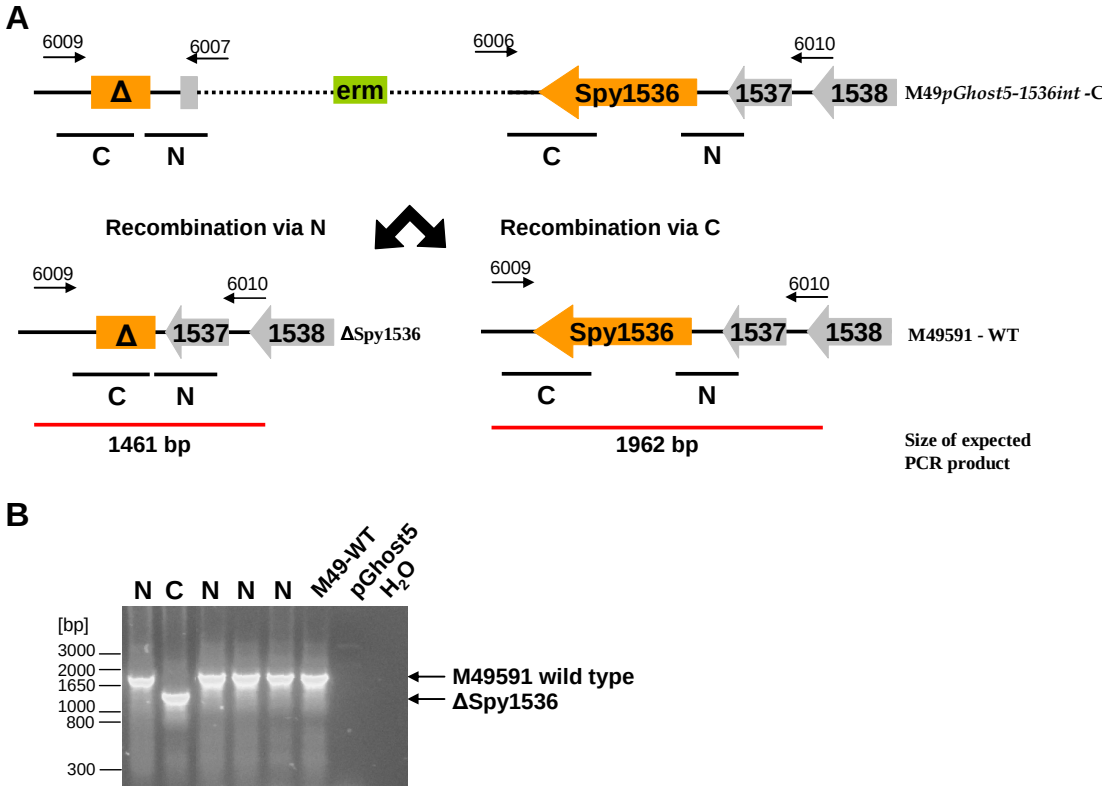

Figure 18: Gene deletion by excision of pGhost5 from the *spy1536* operon region
(A) Schematic representation of pGhost5 excision in strain M49*pGhost5-1536int*-C. Primer binding sites are indicated as arrows and corresponding PCR products are indicated in red. Recombination via the C-terminal *spy1536* region leads to the parental wild type strain, recombination via the N-terminal region results in *spy1536* gene deletion. **(B)** PCR results of erythromycin sensitive *S. pyogenes* phenotypes after pGhost5-*1536* excision from the *spy1536* operon obtained from amplification with primers 6009 and 6010.

6.5.1 Confirmation of *spy0895* and *spy1536* Gene Deletion by Southern Blot Analysis

Although gene deletion was verified by sequencing, we considered it indispensable to be sure that the pGhost5 vector was completely removed from the genome and hence Southern blot analysis was performed. Therefore genomic DNA was isolated from the M49591 wild type strain and from all strains generated during the three steps of gene deletion, M49*pGhost5-0895exc,* M49*pGhost5-1536exc,* M49*pGhost5-0895int*-N, and M49*pGhost5-1536int*-C as well as of the identified gene deletion mutants ΔSpy0895 and ΔSpy1536.

In case of *spy0895* gene deletion genomic DNA of the M49591 wild type, M49*pGhost5-0895exc,* M49*pGhost5-0895int*-N and ΔSpy0895 was digested with Eco*RI*. DIG-labeled probes have been generated for specific detection of pGhost5, *spy1536* and of the deleted sequence in ΔSpy0895. As presented in Figure 19A pGhost5 could only be detected with

the specific DIG labeled probe in the *S. pyogenes* strains M49*pGhost5-0895exc* and M49*pGhost5-0895int*-N at 9997 bp and a very strong signal was obtained when purified pGhost5 was detected. In ΔSpy0895 elimination of pGhost5 from the chromosome was confirmed by the absence of a signal. The *spy0895* DIG labeled probe specific for the sequence deleted in ΔSpy0895 resulted in a signal of 4748 bp in the wild type strain as well as in strain M49*pGhost5-0895exc.* In strain M49*pGhost5-0895int*-N the fragment size increased corresponding to the size of integrated pGhost5-*0895*. Successful *spy0895* gene deletion was confirmed with the *spy0895* specific probe since no signal was detected. Furthermore the *spy1536* DIG labeled probe was used which resulted in the wild type strain as well as in strains M49*pGhost5-0895exc,* M49*pGhost5-0895int*-N and ΔSpy0895 in a signal of the same size.

In case of *spy1536* gene deletion genomic DNA from the wild type strain and strains M49*pGhost5-1536exc,* M49*pGhost5-1536int*-C and ΔSpy1536 was digested with Eco*RV* and pGhost5, *spy1536* and *spy0895* specific probes were used for detection of the digested DNA fragments. As it is shown in Figure 19B use of the pGhost5 specific DIG-labeled probe resulted in a very strong signal in strain M49*pGhost5-1536exc* and in strain M49*pGhost5-1536int*-C a signal at 7359 bp was detected. In ΔSpy1536 like its parental wild type strain no pGhost5 detection was observed which confirmed successful excision and removal of pGhost5 from the ΔSpy1536 chromosome. For detection of the *spy1536* sequence deleted in ΔSpy1536 the same specific signal in strain M49591 and M49*pGhost5-1536exc* was detected and an increase of fragment size corresponding to the size of integrated pGhost5-*1536* was observed in M49*pGhost5-1536int*-C. In ΔSpy1536 the lack of a specific *spy1536* signal confirmed successful deletion of this sequence. In contrast with the *spy0895* specific probe in both the wild type strain and ΔSpy1536 a fragment size of ~9400 bp was detected.

By the use of Southern blot it was confirmed that *spy0895* and *spy1536* were successfully deleted in strains ΔSpy0895 and ΔSpy1536, respectively. Furthermore complete removal of shuttle vector pGhost5 was shown.

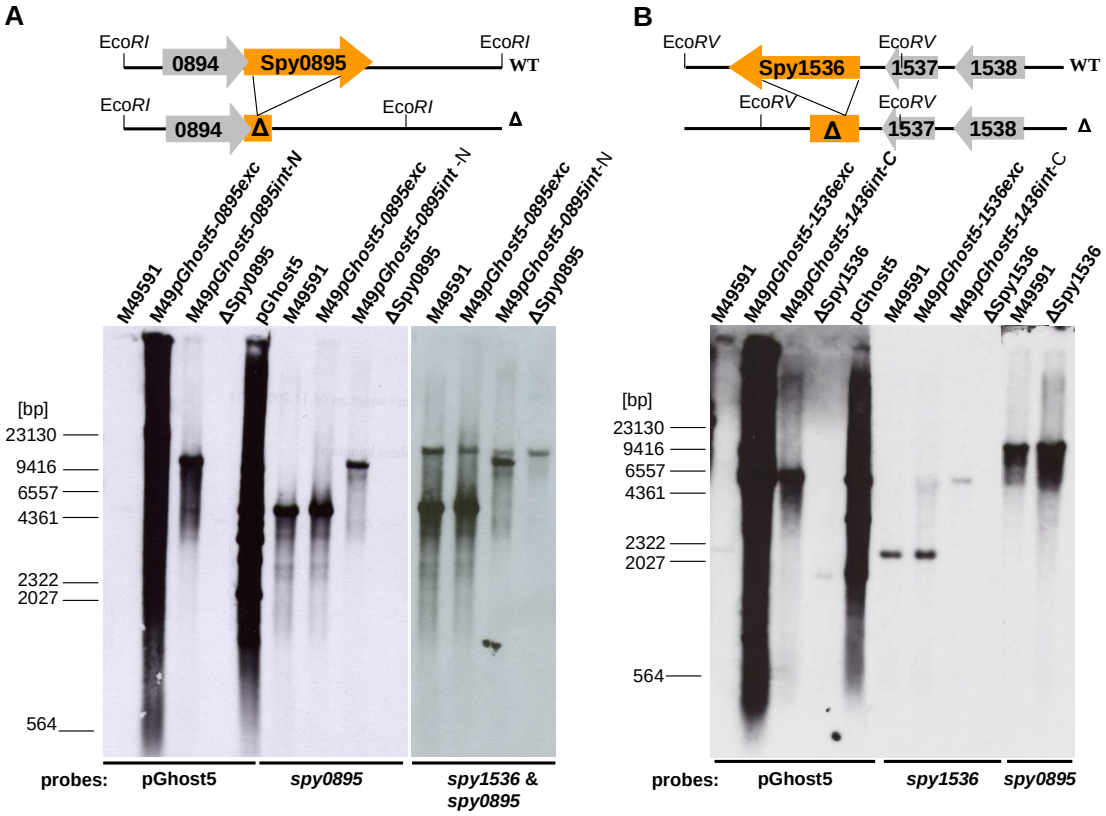

Figure 19: Southern blot analysis of ΔSpy0895 and ΔSpy1536
(A) Schematic representation of the *spy0895* operon region in M49591 wild type and ΔSpy0895 showing Eco*RI* restriction enzyme recognition sites. Southern blot analysis of 0.5 µg Eco*RI* digested genomic DNA deriving from strains M49591 wild type, M49*pGhost5-0895exc,* M49*pGhost5-0895int-N* and ΔSpy0895. Detection with DIG labeled probes specific for pGhost5, *spy0895* gene sequence deleted in strain ΔSpy0895 and for *spy1536*. **(B)** Schematic representation of the *spy1536* operon region in M49591 wild type and ΔSpy1536 showing Eco*RV* restriction enzyme recognition sites. Southern blot analysis of 0.5 µg Eco*RV* digested genomic DNA deriving from strains M49591 wild type, M49*pGhost5-1536exc,* M49*pGhost5-1536int-C* and ΔSpy1536. Detection with DIG labeled probes specific for pGhost5, *spy1536* gene sequence deleted in strain ΔSpy1536 and for *spy0895*.

6.5.2 Confirmation of *spy0895* and *spy1536* Gene Deletion by Northern Blot Analysis

Spy0895 and Spy1536 are located on the streptococcal chromosome as part of an operon. In order to verify that deletion of the *spy0895* and *spy1536* gene sequences did not affect transcription of *spy0894*, *spy1537* and *spy1538* Northern blot analysis was carried out.

As it is presented in Figure 20 DIG-labeled probes specific for *spy0895* and *spy0894* did not give any signal at the expected 1506 bp in the M49591 wild type strain and 900 bp in the gene deletion mutant (see Figure 14, page 74) which leads to the assumption that *spy0895* is not expressed under the *in vitro* growth condition. This result was somehow expected as Western blot analysis had previously shown that Spy0895 protein is not expressed *in vitro* [163]. However, DIG labeled probes being specific for *spy1536*, *spy1537*

and *spy1538* detected the wild type transcript at the expected 2070 bp (see Figure 14, page 74). In the ΔSpy1536 mutant the transcript decreased in size corresponding to the 501 bp deletion resulting in a signal at 1569 bp with *spy1537* and *spy1538* specific probes. No signal was observed with the *spy1536* specific probe.

To sum up, Northern blot analysis indicates lack of *in vitro* transcription of the *spy0895* operon, but did confirm lack of *spy1536* gene transcription without an effect on transcription of *spy1537* and *spy1538*.

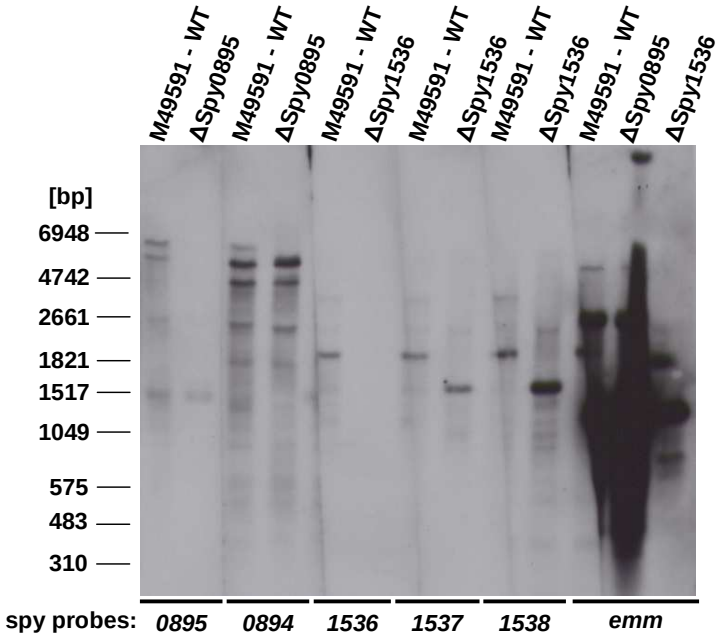

Figure 20: Northern blot analysis of ΔSpy0895 and ΔSpy1536
Five microgram of RNA derived from a mid-exponential growth phase (OD_{600nm} ~0.5) separated by agarose gel electrophoresis were used for detection of *spy0895, spy0894, spy1536, spy1537 and spy1538* transcripts with respective DIG labeled probes. A probe specific for the transcript of the *emm* gene encoding the M protein served as control.

6.6 Characterization of *spy0895* and *spy1536* Gene Deletion Mutants

In order to identify a potential function of Spy0895 and Spy1536 for streptococcal virulence or pathogenesis the gene deletion mutants were examined for a specific phenotype which might help leading the way to a more precise characterization of the two vaccine candidates. As their annotation did not really indicate where to start searching for a phenotype, first a more general characterization including the examination of growth at different environmental conditions as well as virulence in CD-1 mice and the bacteria's shape were performed. A more specific characterization by evaluating the effects of gene deletion in terms of transcription was possible within the

collaboration with Bernd Kreikemeyer where I could perform microarray analysis. Furthermore since the process of interaction with and adherence to epithelial cells during the course of streptococcal infection represents an important step in the initiation of disease the binding of ΔSpy0895 and ΔSpy1536 to human proteins was analyzed as well.

6.6.1 ΔSpy0895 and ΔSpy1536 Cells Have No Major Growth Defects under *In Vitro* Growth Conditions

During an infection bacteria are subjected to different environmental conditions in their human host such as the environment of the throat or the blood. Although growth of bacteria under varying *in vitro* conditions will differ from *in vivo* growth conditions due to the highly artificial environment of growth, the current study was done to analyze the mutants for potential growth defects. Therefore growth of ΔSpy0895 and ΔSpy1536 cells was compared to wild type cells at different temperatures, glucose levels, NaCl levels in medium ranging from 3 mM to 1 M and different iron levels from 1 μM up to 40 μM. Additionally, they were subjected to growth in acidic (pH 5.1 and 6) and basic (pH 8.5) medium.

Growth under standard conditions in THY medium at 37°C with 5% CO_2 as well as decreased and increased temperatures of 28°C and 42°C did not show a difference between M49591 wild type and the two mutants, ΔSpy0895 and ΔSpy1536. The growth of bacteria in NaCl which increases stress and changes hyper or hypotonicity in the medium did not reveal either altered growth of ΔSpy0895 and ΔSpy1536 when compared to wild type cells. ΔSpy0895 and ΔSpy1536 subjected to an excess of iron which can be toxic for the bacteria also showed the same growth as their parental wild type strain. The same was the case for growth in acidic pH (5.1 and 6.0) as well as basic pH (8.5). To evaluate the effect of glucose which on the one hand represents a source of energy and on the other hand can be a growth retarding component due to increase of osmolarity, the three streptococcal strains were grown in THB medium containing 1%, 2% and 3% glucose. In large scale and shaking (80 rpm), the bacteria did not show a difference in growth behavior. Interestingly, when grown in 96-well plates the ΔSpy1536 cells showed a reproducible altered growth behavior (see Figure 21). While the M49591 wild type and ΔSpy0895 cells reached OD_{600nm} of 0.7 after approximately 7 hours of

growth and stopped growing at that time maybe due to the small amount of medium and used up nutrients, ΔSpy1536 increased its optical density from 0.5 to 1.3 within 1 hour (see Figure 21).

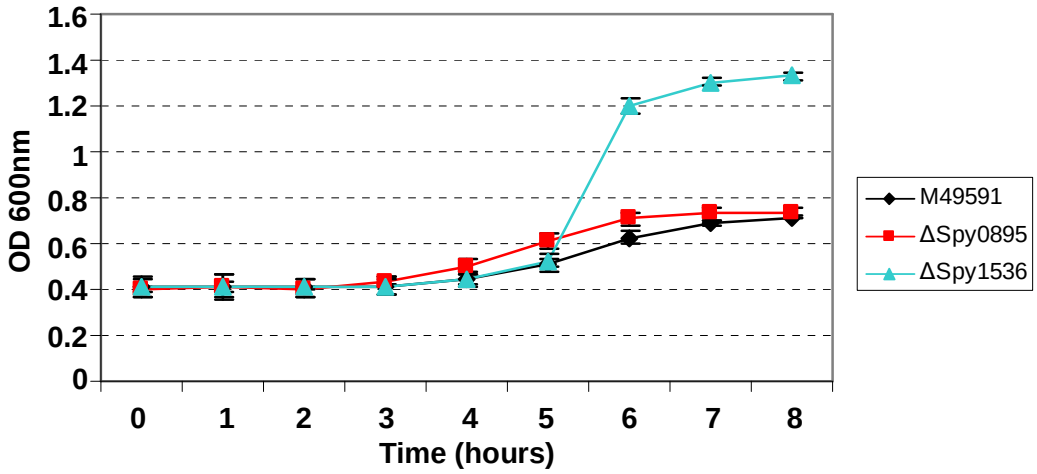

Figure 21: Growth of M49591, ΔSpy0895 and ΔSpy1536 in THB supplemented with 2% glucose
Optical density at 600 nm was measured in 96-well plates using Synergy 2 ELISA reader. Results of four independent experiments represented.

6.6.2 Deletion of *spy0895* and *spy1536* Did Not Decrease Virulence in Mice

The effect of *spy0895* and *spy1536* gene deletion was also investigated *in vivo* in an intraperitoneal murine challenge model. Although in case of GAS there are no models available in mice that entirely mimick human disease and only a few strains can establish infection in mice, the mouse model still has been extensively used for studies in streptococcal pathogenesis [47, 179-181]. As the M49591 wild type strain was found not to be very virulent in mice 5x 10^8 cfu/100 µl had to be applied intraperitoneally to see an effect on the survival of the mice which was monitored over a period of 2 weeks. As it is shown in Figure 22 a decrease of virulence could be observed neither for ΔSpy0895 nor for ΔSpy1536 in this mouse model. It is possible that an effect on virulence as consequence of gene deletion might be observed in a different mouse model for instance an intranasal or a subcutaneous challenge model.

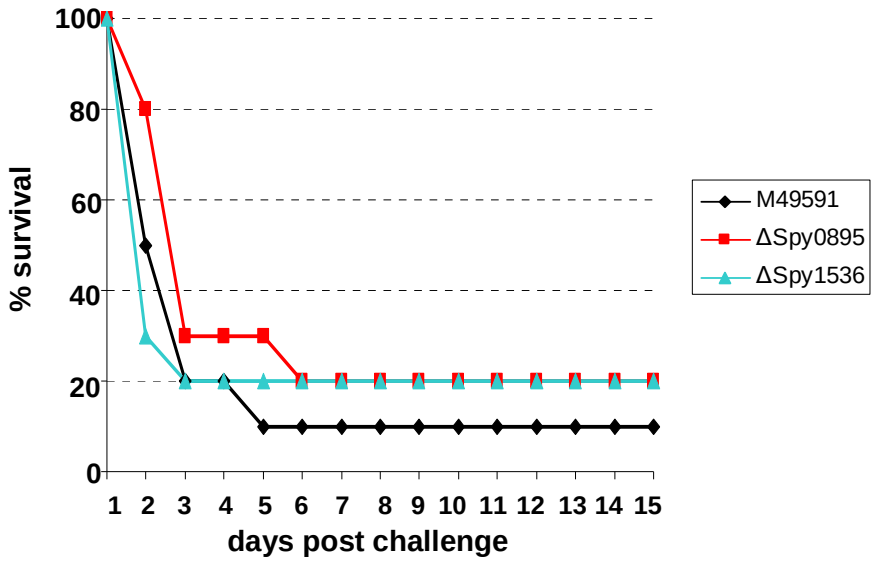

Figure 22: Virulence of M49591, ΔSpy0895 and ΔSpy1536 in an intraperitoneal murine challenge model
CD-1 mice were challenged with 5x 10^8 cfu of *S. pyogenes* strains M49591 wild type, ΔSpy0895 and ΔSpy1536 applied intraperitoneally. Survival of mice was monitored over a period of 15 days.

6.6.3 Electron Microscopy Did Not Reveal a Conspicuous Change of Bacterial Shape in the Gene Deletion Mutants

In order to examine whether *spy0895* or *spy1536* gene deletion affects the overall shape or potentially the surface of the streptococcal chains, the cells were visualized by scanning electron microscopy. From the pictures represented in Figure 23 it is not possible to determine whether the gene deletion mutants differ in their phenotype compared to the wild type, since even the shape of the wild type cells seems to be damaged. The reason might be the preparation of bacterial cells before the pictures had been taken. The fixed bacteria were dried with vacuum which might have been a too aggressive method as even the remnants of the fixation solution are visible surrounding the bacteria.

Observation of the ΔSpy0895 and ΔSpy1536 cells compared to wild type cells did not reveal a change of bacterial surface or shape.

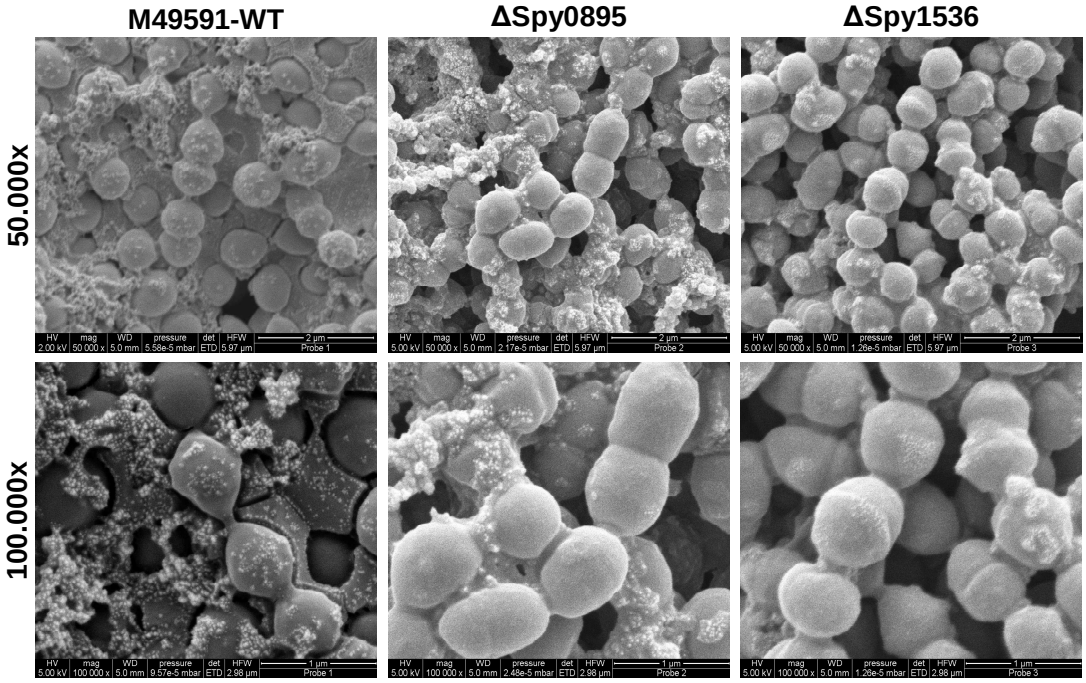

Figure 23: Scanning electron microscopy of wild type, ΔSpy0895 and ΔSpy1536 cells
S. pyogenes wild type and mutant cells were fixed and visualized with an analytical high-resolution scanning electron microscope of the type FEI Quanta 200F. GAS cells are shown with 50,000 and 100,000 fold magnification.

6.6.4 ΔSpy1536 Showed Significantly Reduced Binding to Human Plasma and Extracellular Matrix Proteins

As we could neither observe any reduced virulence for ΔSpy0895 and ΔSpy1536 nor was any significant *in vitro* growth defect determined I assessed the possible role of both proteins for the interaction with host proteins. Since GAS possesses many proteins involved in interaction with human host proteins, deletion of *spy0895* or *spy1536* could influence this interaction. Both proteins, Spy0895 and Spy1536, were identified with human sera. This means that they are recognized by the human immune system and are accessible for antibodies. Their lack on the bacterial surface could possibly influence human protein interactions because Spy0895 or Spy1536 themselves interact with human components or their deletion influences other surface proteins involved in host protein binding.

Binding of FITC-labeled M49591 wild type, ΔSpy0895 and ΔSpy1536 cells to selected human plasma- and extracellular matrix-proteins was therefore analyzed *in vitro* by an ELISA based assay. Using the fluorescent dye FITC, that unspecifically stains bacterial cell surfaces, bacteria can easily be labeled and their binding behaviour to immobilized

proteins can be evaluated by the measurement of fluorescence emitted by the bacteria bound to the immobilized proteins.

As it is shown in Figure 24, ΔSpy1536 cells showed significantly (p-value < 0.005, 2 clones tested in 3 independent experiments) reduced binding to human fibronectin, fibrinogen, laminin, plasminogen and collagen type I coated on ELISA plates compared to wild type and *spy0895* gene deletion mutant cells. In contrast, wild type cells and both gene deletion mutants were not able to bind to haptoglobin and collagen type IV. The reduced binding of ΔSpy1536 cells was accompanied by a 2 to 3-fold increase in fluorescent light emission of FITC labeled ΔSpy1536 cells measured at 560 nm in comparison to M49591 wild type and ΔSpy0895 cells, indicating changes in the composition of the cell surface of the mutant ΔSpy1536 GAS cells. As ΔSpy1536 could no longer bind to the listed human plasma and extracellular matrix proteins, I found it interesting to evaluate whether this reduced ability to adhere to human proteins would also result in a reduced binding to Detroit 562 cells, which are pharynx carcinoma cells. These experiments unfortunately did not show any consistency as already adherence of wild type GAS to Detroit 562 cells fluctuated. An imprecise use of bacterial cfu per experiment could be excluded as the relation between bacteria used for infection of the Detroit 562 cells and the controls was always correct. Nevertheless it was not possible to prove that ΔSpy1536 was consistently adhering less to the Detroit 562 cells than the wild type strain. The same results were obtained with Chang cells, which derive from normal conjunctiva and are similar to human pharynx cells. Such binding assays were previously performed [182, 183]. For sure optimization of the adhesion and internalization assays would have been required but unfortunately time was restricted in the end. In literature human cells have been treated differently for lysis. Manetti and colleagues used saponin but also Triton X-100 [184] was used to lyse cells. In my experiments water was used which might have led to partial lysis and resulted in the inconsistent results.

The significantly reduced binding of ΔSpy1536 cells to human proteins indicated a change on the bacterial surface possibly due to a defect in peptidoglycan synthesis or in surface protein anchoring.

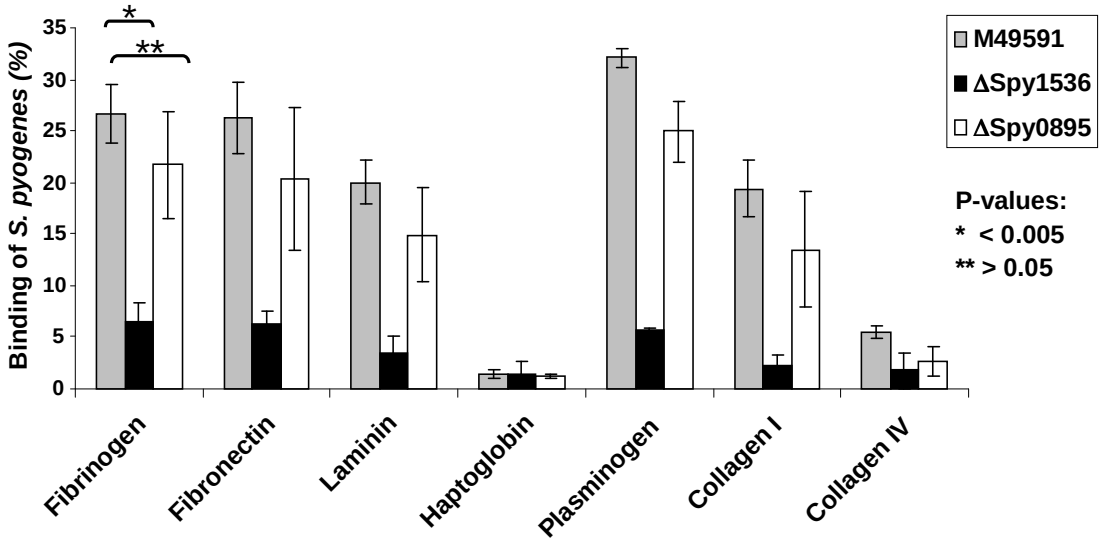

Figure 24: M49591 wild type, ΔSpy0895 and ΔSpy1536 binding to human plasma and extracellular matrix proteins *in vitro*

Binding ability of M49591 wild type and *spy0895* and *spy1536* gene deletion strains was tested using an ELISA based assay. Human fibrinogen, fibronectin, laminin, haptoglobin, plasminogen, collagen type I and IV were coated in an amount of 10 µg on 96-well plates. The FITC-labeled *S. pyogenes* strains were tested for their adherence to the human proteins by measuring their emission of fluorescent light using BioTek Synergy2 ELISA reader with the following settings: Excitation at 485/20 nm, Emission 560/60 nm, Gain: 72, 80, 75, 77.

6.6.5 In Absence of *spy1536* Surface Expression of M protein and Spy0269 is Reduced on *S. pyogenes* Cells

The reduced binding of ΔSpy1536 cells to selected human proteins prompted me to investigate whether this reduction is due to changes of the exposure of group A streptococcal proteins on the bacterial surface. Therefore the surface localization of the major virulence factor of *S. pyogenes*, M protein, and of two candidate antigens, Spy0269 and Spy1666, was assessed by immunostaining.

First M protein surface localization was assessed. For the visualization of M protein on the bacterial surface M1 and M23 specific polyclonal mouse antibodies were used to specifically detect M protein on the bacterial surface as there was no access to M49 specific polyclonal mouse serum. Due to the fact that polyclonal antibodies are known to be specific for both, the variable and the conserved region of the M protein, we expected a M protein specific signal also with antibodies other than M49 specificity. M1 and M23 specific mouse antibodies resulted in the same surface staining and therefore results generated using M23 specific antibodies are not demonstrated here.

As can be seen in Figure 25 wild type and ΔSpy0895 cells showed localization of M protein at the streptococcal cell septum. In contrast, ΔSpy1536 cells displayed no M

protein specific staining when visualized under the same conditions as performed for wild type and ΔSpy0895 cells. Similarly, we could observe a strong reduction in surface localization of the Spy0269 protein on the surface of ΔSpy1536 cells while surface localization of the Spy1666 protein was not affected by mutation of the *spy1536* gene (see Figure 26).

These results indicate that Spy1536 affects surface expression of distinct GAS proteins.

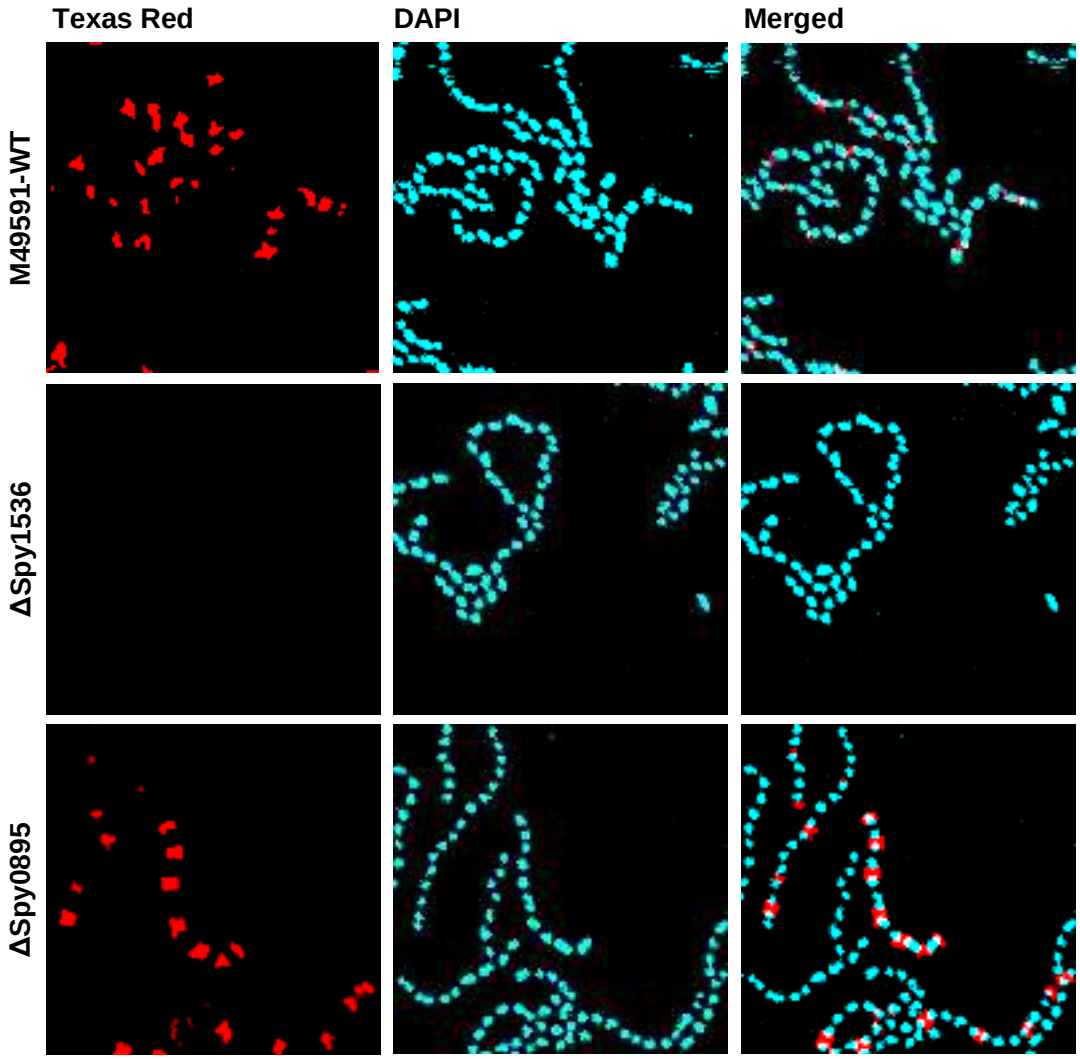

Figure 25: Localization of M protein on the surface of M49591 wild type, ΔSpy1536 and ΔSpy0895
S. pyogenes cells of an overnight culture of wild type, ΔSpy1536 and ΔSpy0895 cells were subjected to immunofluorescence analysis with M23 specific polyclonal mouse antibodies (1:25), Texas Red-dye conjugated goat anti-mouse antibodies (1:100) and with DAPI, following microscopic visualization with a Zeiss Axiovert 200M microscope equipped with a Zeiss LSM510META confocal laser-scanning unit and a Plan-Apochromat 63x1.40 oil DIC MC27 (aperture, 0.19 mm) objective (Zeiss).

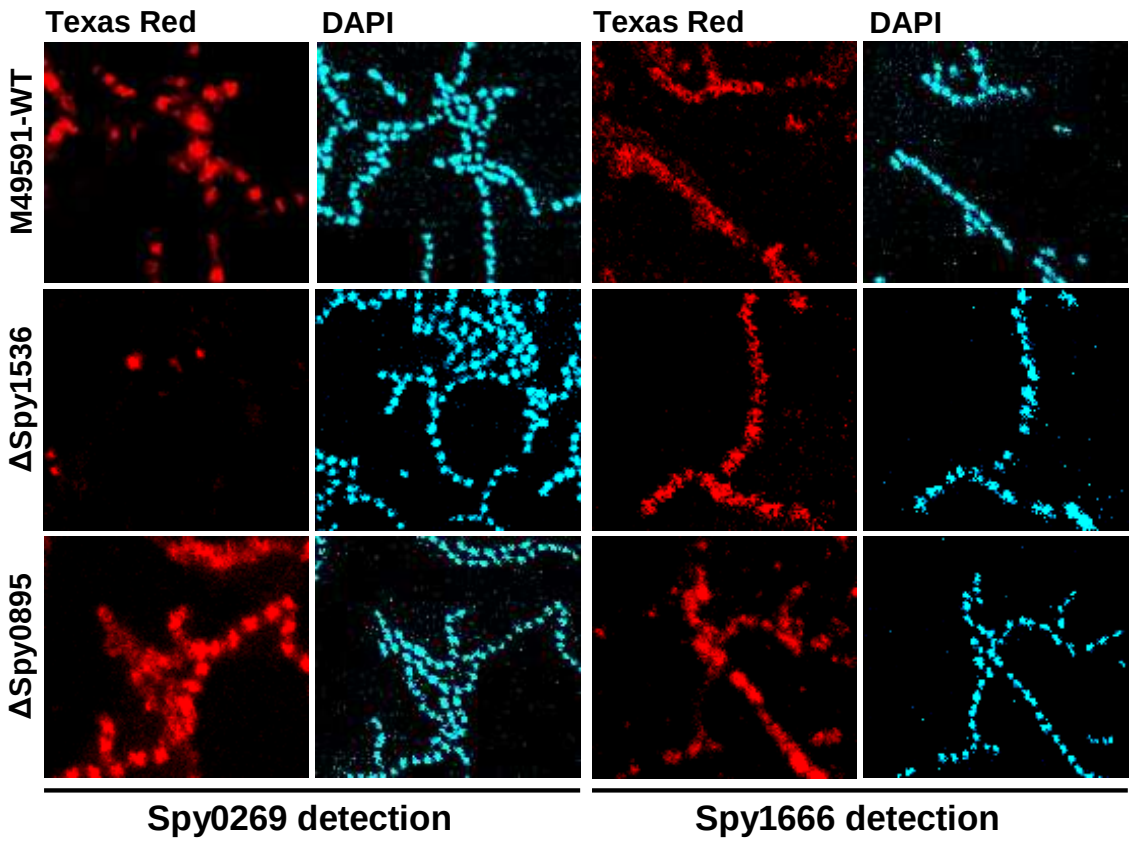

Figure 26: Localization of Spy0269 and Spy1666 on the surface of M49591 wild type, ΔSpy1536 and ΔSpy0895 cells
S. pyogenes cells of an overnight culture of M49591, ΔSpy1536 and ΔSpy0895 gene deletion mutants were subjected to immunofluorescence analysis. Primary Spy0269 and Spy1666 specific polyclonal mouse antibodies and Texas Red-dye conjugated goat anti-mouse antibodies were used in 1:25 and 1:100 dilutions, respectively. DAPI was used for staining DNA. Microscopic visualization was carried out with a Zeiss Axiovert 200M microscope equipped with a Zeiss LSM510META confocal laser-scanning unit and a Plan-Apochromat 63x1.40 oil DIC MC27 (aperture, 0.19 mm) objective (Zeiss).

6.6.6 Deletion of *spy1536* Does Not Affect e*mm*-Gene Transcription

Since M protein and Spy0269 were no longer detected on the surface of ΔSpy1536 cells the next logical step was to determine whether this interesting observation was the result of a transcriptional or translational modulation caused by *spy1536* deletion.

Therefore cell fractions generated from the wild type strain and the two gene deletion mutants, ΔSpy0895 and ΔSpy1536 were subjected to Western blot analysis for the detection of M protein, Spy0269 and Spy1666. Interestingly, this showed that M protein can be detected in the bacterial lysates of M49591, ΔSpy0895 and ΔSpy1536 and it is still expressed at similar levels in the cytoplasm of all cells (see Figure 27A), indicating that the lack of M protein on the bacterial surface is not due to any influence of the *spy1536* deletion on the transcription or translation of the M protein encoding gene. Also in the

cell wall fractions of wild type and ΔSpy0895 cells M protein is still detectable. In contrast, the cell wall fraction for ΔSpy1536 cells showed a reduced presence of M protein, in agreement with the immunofluorescence data. In contrast, for Spy0269 (see Figure 27B) and Spy1666 (see Figure 27C) no differential expression in lysates, cytoplasmic and cell wall fractions of wild type and ΔSpy0895 and ΔSpy1536 cells could be observed. Besides that, a high background was observed in case of Spy0269 and Spy1666 detection.

These results demonstrate that the reduced surface localization of GAS protein signal on ΔSpy1536 cells is not the consequence of a transcriptional or translational modulation. At least for the M protein there is an indication that the transport of the protein to the bacterial cell surface is affected by *spy1536* deletion, since it is no longer detectable in ΔSpy1536 cell wall fractions.

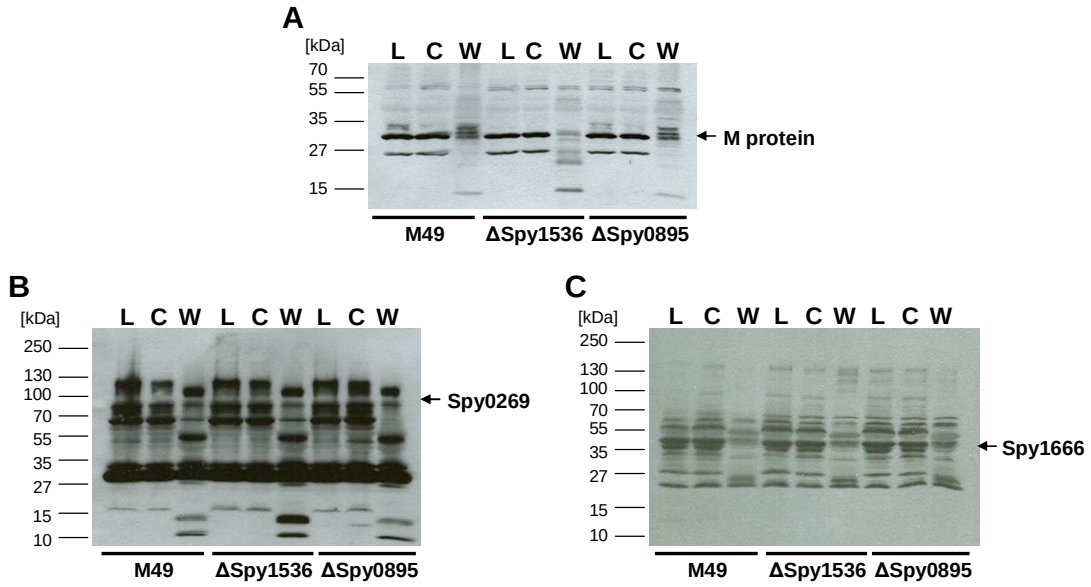

Figure 27: Detection of M protein, Spy0269 and Spy1666 in subcellular fractions of M49591, ΔSpy1536 and ΔSpy0895

Western blot analysis of M protein, Spy0269 and Spy1666 detected in cell fractions of M49591 wild type, ΔSpy1536 and ΔSpy0895 cells showing total lysate (L), cytoplasmic fraction (C) and cell wall fraction (W). For all fractions approximately 30 μg as measured by BCA assay were loaded. **(A)** Detection with M1 protein specific polyclonal mouse antibodies diluted 1:1000 and secondary goat anti-mouse IgG/HRP (1:5,000). **(B)** Detection with Spy0269 polyclonal mouse serum (1:1000) and secondary goat anti-mouse IgG/HRP (1:5,000). Spy0269 is expected at a size of 94.7 kDa. **(C)** Detection with Spy1666 polyclonal mouse serum (1:1000) and secondary goat anti- mouse IgG/HRP (1:5,000). Spy1666 is expected at a size of 37 kDa.

6.6.7 ΔSpy1536 Cells Secrete Mainly Inactive Pyrogenic Exotoxin B in their Culture Supernatant

Since M protein is still expressed by ΔSpy1536 cells and detectable in the cytoplasm but no longer on the bacterial surface I wanted to further evaluate the reasons for this observation. The question was whether the absence of surface expressed M protein is the result of a defect in transport or anchoring to the cell wall or might be the consequence of overexpression of proteases secreted by the mutant cells. SpeB, pyrogenic exotoxin B, was reported to release biologically active fragments from the streptococcal surface including M protein [185]. Therefore the expression of SpeB was analysed in the culture supernatant of wild type, ΔSpy0895 and ΔSpy1536 cells.

Western blot analysis revealed that in culture supernatant of wild type and ΔSpy0895 cells SpeB was mainly detected at a size of 28 kDa, the size of the active form of this enzyme (see Figure 28). Interestingly, we found out that in the culture supernatant of ΔSpy1536, SpeB was not overexpressed as we expected. SpeB could only be detected as the enzymatically inactive precursor (40 kDa) with the corresponding anti-SpeB antibody. However, the inactive 40 kDa SpeB secreted by ΔSpy1536 could be partly activated using 10 mM DTT. DTT did not have any effect on the active SpeB of M49591 wild type and ΔSpy0895 cells. Active SpeB deriving from the wild type supernatant in contrast did not activate inactive SpeB.

The detection of mainly inactive SpeB in ΔSpy1536 culture supernatant indicates that lack of M protein on the bacterial surface is not the result of degradation by SpeB.

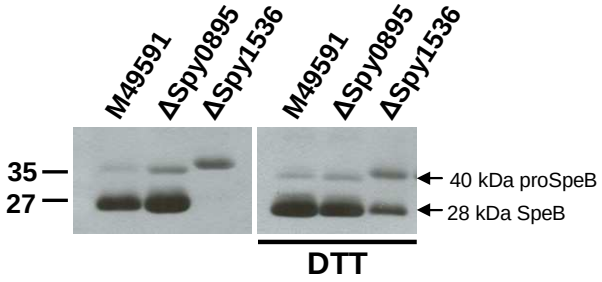

Figure 28: Detection of secreted SpeB of M49591, ΔSpy1536 and ΔSpy0895 cells
Western blot analysis of TCA precipitated supernatants deriving from M49591 wild type, ΔSpy1536 and ΔSpy0895 cells. Equal amounts of protein (7 μg) were loaded for each strain and SpeB was specifically detected with 1:1000 diluted polyclonal goat anti-*S. pyogenes* SpeB antibodies and secondary rabbit anti-goat IgG/HRP (1:5,000). For activation of inactive SpeB (40 kDa) culture supernatant proteins (7 μg) were treated with 10 mM DTT as reducing agent for 30 minutes at 37°C.

6.6.8 Deletion of *spy1536* Causes Transcriptional Upregulation of *hasA* and Downregulation of Adhesin Encoding Genes

In order to identify a potential effect of *spy0895* and *spy1536* gene deletion on the transcription of GAS genes, a genome wide analysis of the transcriptome was carried out using Microarray and Real Time RT-PCR.

6.6.8.1 Transcriptome Analyses by Microarray

In collaboration with Bernd Kreikemeyer (Rostock, Germany) it was possible to analyse the *spy0895* and *spy1536* gene deletion mutants for differential gene expression. In order to identify potentially up- or down-regulated genes as a consequence of *spy0895* or *spy1536* gene deletion I performed dual colour microarray hybridization analysis in Bernd Kreikemeyer's lab. The DNA microarrays that were used contained 50mer oligos and were designed from open reading frame sequences of a partial serotype M49 genome sequence (NZ_AAFV00000000) and complete serotypes M1, M3 and M18 sequences [168].

The microarray data was read from the Agilent Technology instrument from Affymetrix, normalized and analyzed using GeneSpring GX Software by Jana Normann (Rostock, Germany). From these data, the selection of genes for further analysis by Real Time RT-PCR was based on an at least two fold change of up- or down-regulation and identification as such in two of three independent experiments unless otherwise stated. An overview of the genes being selected the way mentioned above can be seen in Table 2 (page 97).

In case of the *spy0895* gene deletion mutant, 9 genes were found to be differentially expressed compared to the wild type M49591 in 2 of the 3 independent experiments. Among these candidates were 2 conserved hypothetical proteins, *spy0441* and *spy1691*, that showed according to the microarray data a 2.1-2.3 fold upregulation in the *spy0895* gene deletion mutant. Additionally, *spy0570*, a putative drug resistance protein, *spy1718*, a putative esterase, *spym49b0400*, a putative DNA-damage-inducible protein P and *spym49c0816* which is similar to *spy1364* (DNA polymerase iii subunits gamma) were all found to be upregulated between 2.3-2.5 fold. Downregulation was observed for the genes *spy1031*, a putative dihydrolipoamide dehydrogenase, *spy180822*, a hypothetical protein, *spym49b0627*, which is annotated as 50S ribosomal protein rpmF and

spym49c1025, a putative biotin carboxyl carrier protein. The gene *spym180822* in contrast to the other genes was also listed and selected for further analysis by Real Time RT-PCR not because it was identified in 2 of the 3 microarray experiments but because it showed a ~25 fold decreased transcription in ΔSpy0895 compared to the M49591 wild type strain.

In the ΔSpy1536 strain 12 genes showed a differential gene expression in 2 of the 3 independent experiments. Among these 12 candidates was for example *spy2010*, annotated as C5a peptidase precursor scpA, that was identified a second time as *spym31726*. This was possible since some genes were represented more than just once whenever it was not possible to design a common oligo for the four serotypes. Also spym49c1149 was detected a second time by a different oligo representing the same gene (spym49b0494). From the 12 candidates that were represented as up- or down-regulated genes in 2 independent experiments six were further analyzed by Real Time RT-PCR. First of all because twice the same genes with two different oligos on the microarray chip were identified, second because strain M49 derived gene sequences encoded proteins with unknown functions (e.g. *spym49b1334*, *spym49b0529* and *spym49b0495*) or they did not show homology to known GAS proteins. The genes would probably not contribute to identify a role for Spy1536. Oligos specific for *spy2010* amplified only unspecific PCR products from genomic DNA. Design of another primer pair did not change this which is the reason that this gene was not further analysed by Real Time RT-PCR. Therefore the following six genes were analyzed: *spym49b0963*, a conserved hypothetical protein similar to Spy0587, *spym49b1739*, encoding a collagen like-protein ScIB, *spym49s0005* similar to fcrA protein precursor and *spym49s0078*, encoding a protein similar to serum opacity factor as well as *spy0611*, which encodes a putative translation elongation factor tufA. All of the genes showed according to the microarray data a decreased transcription between 2.1-4.8 fold except *spy0611* which was 2.3 fold upregulated. Due to the fact that among the candidates the transcription of the two genes, *spym49b1739* and *spym49c1149*, encoding putative collagen-like proteins ScIB and ScIA, respectively, have been found, I searched among all 2 fold differentially regulated genes of the microarray data for other potential virulence factors being differentially expressed that are involved in *S. pyogenes* pathogenesis such as genes encoding proteins binding to human plasma and extracellular matrix proteins like the already identified

putative collagen-like proteins binding to collagen. This led to the selection of 4 additional genes to be tested in Real Time RT-PCR: *spy0738* encoding a streptolysin S associated protein, *spy1642* that encodes a protein similar to autoinducer-2 production protein LuxS, *spy2200* that encodes hyaluronate synthase for the production of hyaluronic acid capsule and *spym49s0079* encoding the fibronectin-binding protein SfbX49. Microarray analysis revealed that *spy1642* and *spy2200* were approximately 2 fold increased in their transcript level resulting probably from the *spy1536* gene deletion, while *spy0738* and *spym49s0079* showed a decrease in transcription of ~4 and ~2 fold, respectively.

6.6.8.2 Transcriptome Analyses by Real Time RT-PCR

The microarray analysis has identified several genes in the *spy0895* and *spy1536* gene deletion mutants that showed a change on the level of transcription compared to the M49591 wild type strain. To verify the data for the selected genes in the microarray experiments Real Time RT-PCR was performed.

First, gene specific primers (see Table 6, Appendix I) were designed to amplify 100-200 bp of the genes that have been selected based on the data obtained from microarray analysis. All primers were designed to have a melting temperature between 54.3-55.8°C and tested in PCR using genomic DNA of M49591 wild type strain. First experiments were carried out with different dilutions of cDNA ranging from 1:5 to 1:125. As the best dilution for amplification in 40 cycles was 1:25, for all PCR reactions 5 µl of this dilution was used. SYBR Green I served as fluorescent dye during the amplification of the cDNA binding to amplified double stranded DNA. Increase of the measured fluorescent signal corresponds to an increase of amplicons. The melting curve, a stepwise increase from 60-95°C, was performed for visualization of PCR product specificity because the temperature at which the amplified DNA is separated into single strands leading to the loss of fluorescent SYBR Green is characteristic for each PCR product. The housekeeping gene *mutS* (encoding DNA mismatch repair protein) and its mRNA expression was used as internal control as it produced more stable results than using *gki* (glucose kinase) or *recP* (transketolase) transcripts as internal controls.

The obtained Real Time data was analyzed using the 2-(ΔCTsample-ΔCTcalibrator) method [169] to reveal quantitative differences in transcript levels between wild type

and gene deletion mutants. Results in Table 2 and Figure 29 are presented as ratio between the mutant and the wild type strain from two independent experiments.

In the ΔSpy0895 mutant none of the genes showed a differential gene expression in Real Time RT-PCR as the ratio between ΔSpy0895 and M49591 wild type was in a range between ~0.5-1. Only *spy1718* showed a slight increase in transcript levels (see Table 2).

Table 2: List of genes being differentially expressed in ΔSpy0895 and ΔSpy1536 as assessed by Microarray analysis and Real Time RT-PCR

Genes that were identified to be differentially regulated in ΔSpy0895 and ΔSpy1536 by microarray analysis are listed. Microarray results are listed as fold change (F. c.) of mutant vs. wild type strain. Values derive from 2 independent experiments except for *spym180822* in the ΔSpy0895 mutant and *spy0738*, *spym49s0079*, *spy1642* and *spy2200* in the ΔSpy1536, where the values derive from one experiment only. Real Time RT-PCR results are presented as ratio between mutant and wild type transcript levels calculated using the 2-(ΔCTsample-ΔCTcalibrator) method. The ratio values derive from 2 independent experiments.

		Microarray ΔSpy0895		Real Time RT-PCR ΔSpy0895	
Genes	**Annotation**	**F. c.**	**Regulation**	**Ratio**	**Regulation**
spy0441	conserved hypothetical protein	2.131	up	0.532	-
spy0570	similar to Spy0570, putative drug resistance protein;	2.394	up	0.946	-
spy1031	similar to Spy1031, putative dihydrolipoamide dehydrogenase, component e3; acol	2.461	down	0.615	-
spy1691	Spy1691, conserved hypothetical protein	2.394	up	1.609	-
spy1718	putative esterase	2.133	up	0.994	-
spym180822	hypothetical protein, epuA	25.720	down	0.804	-
spym49b0400	putative DNA-damage-inducible protein P, dinP,	3.222	up	0.751	-
spym49b0627	50S ribosomal protein L32, rpmF	2.231	down	0.830	-
spym49c0816	similar to Spy1364 DNA polymerase iii subunits gamma/ tau; dnax	2.547	up	0.804	-
spym49c1025	putative biotoin carboxyl carrier protein, accB	2.306	down	0.729	-

		Microarray ΔSpy1536		Real Time RT-PCR ΔSpy1536	
Genes	**Annotation**	**F. c.**	**Regulation**	**Ratio**	**Regulation**
spy0611	putative translation elongation factor EF-Tu, *tufA*	2.345	up	0.751	-
spy0738	similar to Spy0738, streptolysin s associated protein; *sagA*	4.042	down	0.575	-
spy1642	similar to Spy1642, autoinducer-2 production protein; *luxS*	2.261	up	0.586	-
spy2200	hyaluronate synthase, *hasA*	2.068	up	1.755	up
spym49b0963	similar to Spy0587, conserved hypothetical protein;	2.519	down	0.664	-
spym49b1739	collagen-like protein SclB, *sclB*	3.264	down	0.140	down
spym49c1149	putative collagen-like protein, *sclA*	4.823	down	0.065	down
spym49s0005	similar to NF004459_41, *fcrA* protein precursor	2.682	down	0.004	down
spym49s0078	similar to NF004451_15, Serum opacity factor	2.143	down	0.831	-
spym49s0079	similar to NF004466_21, Fibronectin-binding protein, SfbX49	2.147	down	0.011	down

For ΔSpy0895 the Real Time RT-PCR could not verify the data obtained from microarray analysis.

In contrast, Real Time data generated with ΔSpy1536 looked very promising for some genes. As it is presented in Figure 29 among the 10 tested genes selected by microarray analysis 5 genes, *spy0611*, *spy0738*, *spy1642*, *spym49cb0963* and *spym49s0078* did not seem to be affected by *spy1536* gene deletion as detected by Real Time RT-PCR and therefore microarray results could not be confirmed. However, five genes showed a different gene expression, confirming at least for those genes the observations in the microarray analysis. One of the candidates, namely *spy2200* (*hasA*) is slightly upregulated (see Figure 29A). The gene *hasA*, part of the *hasABC* operon, encodes membrane associated hyaluronate synthase and adds alternating N-acetyl-D-glucosamine and D-glucuronic acid residues to form the linear hyaluronic acid polymer for hyaluronic acid capsule production [186]. The other 4 genes, also potential virulence factors, *spym49b1739* (*scIB*) and *spym49c1149* (*scIA*), both encoding putative collagen-like proteins, as well as *spym49s0005* and *spym49s0079* encoding an fcrA protein precursor and the fibronectin-binding protein SfbX49, respectively, showed significant downregulation (see Figure 29B).

The two genes encoding the collagen-like proteins ScIA and ScIB, which are reported to act as adhesins as a consequence of their extracellular location and collagen-like structure [49] showed a ratio of 0.065 and 0.140 between mutant and wild type strain (see Table 2) which equates with 5-10 fold decreased transcription. The genes that encode the fcrA protein precursor (*spym49s0005*) and SfbX49 (*spym49s0079*), encoding a fibronectin-binding function and linked to serum opacity factor in one operon [187], showed even a more decreased transcript level, resulting in a ratio between ΔSpy1536 and wild type of 0,004 and 0,011, which equates with ~100 fold downregulation of transcription. FcrA encodes an M protein-related Ig-binding protein and is usually clustered on the streptococcal chromosome with M protein and C5a peptidase [188-190].

In summary, the verification of the transcriptome analysis using Microarray chips by Real Time RT-PCR was successful for the ΔSpy1536 strain. The data strongly indicate that surface proteins binding to collagen (ScIA and ScIB) or fibronectin (SfbX49) are strongly downregulated which is in agreement with the protein binding studies *in vitro* where binding to human proteins including fibronectin and collagen was significantly

reduced by deletion of *spy1536* (see Figure 24, page 89) and with the immunofluorescence analysis (see Figures 25 and 26, pages 90 and 91) which revealed a reduced exposure of M protein and Spy0269 on the bacterial surface.

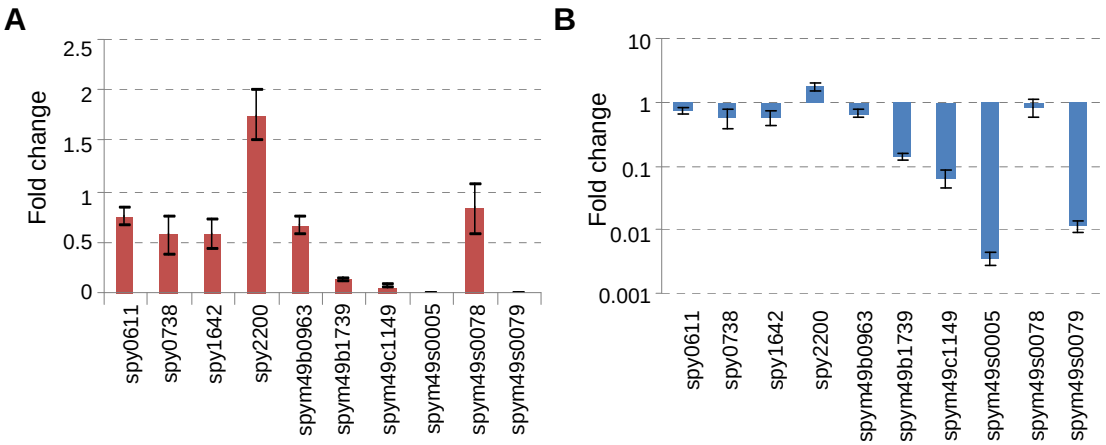

Figure 29: Differential gene expression in ΔSpy1536 as assessed by Real Time RT-PCR
Representation of up- and down-regulated genes from ΔSpy1536 cells of mid-log phase analysed by Real Time RT-PCR. Real Time data analysis was supported by the Eppendorf RealPlex software and quantitative differences for each sample between wild type and ΔSpy1536 were determined using the 2-(ΔCTsample-ΔCTcalibrator) method. The housekeeping gene *mutS* served as internal control. Data of two independent experiments is presented. **(A)** Fold change of transcript level between wild type and *spy1536* gene deletion mutant. **(B)** Logarithmic view of fold change analyzed in ΔSpy1536 giving a better view of downregulated genes.

7 DISCUSSION

The observation that M protein-derived GAS vaccines can induce cross-reactive antibodies in humans causing rheumatic fever [79] and the existence of more than one hundred different group A streptococcal serotypes have recently focused vaccine development efforts on novel protein antigens from *S. pyogenes*. While a number of individual antigens, such as C5A peptidase and SfbI, were identified by traditional technologies, large scale proteomic approaches building on the available genomic sequence information have recently identified novel candidates from *S. pyogenes* on a broader scale [154-156]. Severin and colleagues for example employed a method to identify surface antigens of *S. pyogenes* by generating peptides of surface exposed proteins using trypsin digestion and analyses of these peptides by multidimensional tandem mass spectrometry. This approach led to the identification of 79 proteins on the bacterial surface. Among them were 14 out of 17 predicted peptidoglycan-attached proteins, 12 out of 27 predicted lipoproteins, 9 secreted proteins, 22 membrane proteins, 1 bacteriophage-associated protein and 21 proteins commonly identified as cytoplasmic proteins, thus providing guidance in the development of a novel vaccine to prevent infections caused by *S. pyogenes*. Lei and his colleagues focused on a systematic analysis of culture supernatant proteins by two-dimensional Western blot analyses of GAS strains grown *in vitro* [155]. In order to identify supernatant proteins expressed in the course of host pathogen interactions, sera from mice with experimentally induced soft tissue infections and sera from humans with pharyngitis, acute rheumatic fever and severe invasive diseases were utilized. This approach identified 5 new candidates, Spy0385, Spy1245, Spy1274, Spy1390 and Spy1558. Active immunization of mice with the recombinant forms of these proteins stimulated the production of antibodies which significantly inhibited growth of GAS assessed by bactericidal activity assay. Nevertheless the mentioned strategies to identify new potential vaccine candidates may miss potential vaccine candidates due to limitations of *in vitro* expression of proteins. In order to avoid any potential shortcomings by lack of *in vitro* expression or proper bioinformatic annotation of protein function, we have applied the ANTIGENome

technology [157, 158, 172] to select novel protective GAS vaccine candidates on a genomic scale for the purpose of designing and developing subunit vaccines directed against GAS disease that cannot be properly mastered by present vaccination strategies [153]. Using this technology, 95 antigenic proteins have been identified from *S. pyogenes*, including most of the known protective antigens. Among the 8 most frequently selected antigens in our screens were thus 5 published protective proteins, confirming the strength of the technology to identify relevant candidates. Applying several *in vitro* assays and three different murine infection models, nine proteins capable of providing protection against GAS infection have been identified. The aim of my work was to further characterize some of these candidates to support their utilization as vaccine candidates. Based on their protectivity in the murine challenge models and the location on the bacterial chromosome as single genes or as part of an operon I focused on three of the nine candidates, namely on Spy0416, which was one of the most frequently selected antigens, as well as on Spy0895 and Spy1536. While Spy0416 was also identified recently by a proteomic approach and shown to provide protection against an M23 *S. pyogenes* strain in mice [154], Spy0895 and Spy1536 as well as the other 6 selected proteins have not previously been recognized to be capable of providing protection against GAS infection, nor have they been characterized in more detail in regard to their biochemical function or role in streptococcal pathogenesis.

7.1 Role of Spy0416 in Group A Streptococcal Pathogenesis

Our studies aiming at the identification of novel candidates for a Group A streptococcal vaccine have selected the cell envelope serine proteinase Spy0416 (ScpC) as one of several protective antigens. The evidence that IL-8 cleavage activity is mediated by Spy0416 [91] and that it is assumed to prevent recruitment of polymorphonuclear leukocytes to the site of infection as well as its selection as vaccine candidate by the ANTIGENome technology [153] warranted further characterization.

First, it was necessary to establish an *in vitro* system to measure IL-8 cleavage activity of Spy0416. Using recombinant Spy0416 protein, I could show in my previous work that it mediates indeed proteolysis of IL-8 *in vitro* and that the predicted active site residues are essential for enzymatic activity [162]. In the same study I also found out, that the

predicted catalytic domain alone (Spy0416A) was not sufficient to provide enzymatic activity. Instead activity could only be restored by supplying the C-terminal region of Spy0416 (Spy0416B) as separate recombinant protein. Therefore, I was interested in the possible contribution of the predicted distinct domains of Spy0416 to IL-8 degradation activity and in the interaction between the protease and IL-8, as well as in possible other substrates targeted for degradation.

7.1.1 Contribution of PA, Fn1 and Fn2 Domains to Spy0416 Catalytic Activity

Although many of the known cell envelope proteinases consist of multiple domains [191] for most of them the functions have not been elucidated so far. Therefore, we investigated the contribution of the PA, Fn1 and Fn2 domains to Spy0416 catalytic activity. We were already able to show that the PA domain, which in the C5a peptidase of *S. agalactiae* may be involved in recognition of a cellular receptor thus triggering enzymatic activity [177], and the Fn1 domain - both located in the N-terminal region of Spy0416 - contribute to enzymatic activity [162]. In our publication we were further able to show that upon deletion of the Fn1 domain within the Spy0416A protein, IL-8 degradation was abolished. In this regard the 131 bp region of the Fn1 domain represented the overlap between Spy0416A and Spy0416B. At that time we hypothesized that the Fn1 domain might be involved in mediating interactions with Spy0416B and therefore be essential for IL-8 cleavage activity. In this work I demonstrated that the Fn1 sequence overlap between Spy0416A and Spy0416B is not essential, because IL-8 degradation is exerted by both Spy0416A and Spy0416B$_{\Delta Fn1}$. Only a short 48 bp sequence overlap within the Fn2 domain remains between Spy0416A and Spy0416B$_{\Delta Fn1}$ that might be involved in mediating the interaction between Spy0416A and Spy0416B. The important role for the Fn2 and PA domain in ScpC may be explained by a model that was recently published for ScpA interacting with its substrate C5a. Since Spy0416 and ScpA show a high sequence homology, homologous domains may exert similar functions. Based on the crystal structure of ScpA from *S. pyogenes* it was postulated that the PA and Fn2 domains are involved in substrate selectivity and binding [95, 177]. It was suggested that the access to the ScpA catalytic site is restricted by the presence of PA and Fn2 domains and that electrostatic interaction is the potential driver in substrate recruitment by ScpA. Interaction of the in the PA domain located

RGD motif with integrins may stabilize conformational changes required for substrate binding [177]. The model of the C5a-ScpA complex presented by Kagawa *et al.*, places the core of C5a on the Fn2 domain and the C5a C-terminal tail making contact to the catalytic, Fn2 and PA domains. In this case the interaction between C5a and the Fn2 domain would be electrostatic in nature due to charge complementarity between Fn2 and C5a and thus predicts that both interactions in the active site, but also interactions on the Fn2 domain, would contribute to the selectivity of ScpA. A similar way of interaction between Spy0416 and its substrate might occur, but this is just speculation. To date we also do not know whether the sequence overlaps between Spy0416A and Spy0416B are indeed necessary for IL-8 degradation, because the generation of recombinant Spy0416$_{\Delta Fn2}$ failed due to lack of expression in *E. coli*.

7.1.2 Essential Function of Ancillary C-terminal Spy0416 Domains

We found out that the predicted catalytic domain alone (Spy0416A) was not sufficient to provide enzymatic activity but was dependent on the presence of the ancillary C-terminal domains. Therefore, we wanted to evaluate the essential function of Spy0416B for enzymatic activity in terms of interaction with Spy0416A and IL-8 but also the contribution of distinct Spy0416B domains to enzymatic activity.

First we addressed how Spy0416 interacts with IL-8 using isothermal fluorescence analyses. The data from isothermal fluorescence studies indicated that binding of IL-8 was mainly mediated by the proteinase domain of Spy0416, but it also showed that the Spy0416B protein could contribute to binding of IL-8. In agreement with its higher affinity, only Spy0416A but not Spy0416B was capable of significant binding to IL-8 as determined by a gel depletion and surface plasmon resonance assays. However, when both proteins, Spy0416A and Spy0416B, were supplied together in the gel depletion assay, it was observed that Spy416B reduced binding of Spy0416A to IL-8. Apparently, under these conditions, the interaction of Spy0416A and Spy0416B dominates over Spy0416A binding to IL-8. Since Spy0416A binds with significantly higher affinity to IL-8 (yet without exhibiting proteolytic activity), we hypothesise that in the full-length Spy0416 protein, IL-8 binds predominantly to the A domain which causes a conformational change leading the B domain to ´´lock´´ the IL-8 substrate in the catalytic site, which then triggers enzymatic activity. Under gel depletion conditions, however,

Spy0416B prevents binding of IL-8 to Spy0416A by a strong interdomain (A-B) interaction which is somehow leaky in the case of the Spy416A-D151A mutant. The reduced binding of IL-8 by Spy0416A in the presence of Spy0416B may also indicate Spy0416B-induced changes in structure or binding of Spy0416A resulting in the observed reduction in IL-8 binding.

The lack of proteolytic activity of the individual domains and participation of the non-catalytic domains of Spy0416B to CXC chemokine degradation *in vitro* also argues for the essential contribution of several, if not all of the domains of Spy0416 for enzymatic activity and thus for its contribution to virulence. This most likely also explains the high sequence conservation level that we determined for this large cell envelope proteinase throughout the entire amino acid sequence. PCR analyses showed that Spy0416 is present in at least 98% of more than 300 tested Group A streptococcal strains. Subsequent sequencing of the Spy0416 gene from more than 60 strains, including 26 distinct emm types, revealed that the protein shows more than 95% sequence identity within the analyzed Group A streptococcal strains [153].

7.1.3 Specificity of Spy0416 towards IL-8

In the next step we wanted to evaluate whether Spy0416 cleavage activity is also exerted towards the biological inactive IL-8^{F17K,F21K} mutant. The observed reduced activity of Spy0416 towards IL-8^{F17K,F21K} provided first evidence for the basis of the specificity of IL-8 cleavage activity by Spy0416. Since the IL-8 mutant is deficient in neutrophil chemotaxis, Spy0416 seems to have evolved in a very efficient way to proteolytically inactivate the biological active chemokine, whereas inactivation of IL-8 by mutation coincided with abolished cleavage activity by Spy0416. As can be seen from the molecular structure of the wild type IL-8 molecule (see Figure 9A, page 67), the proximal loop containing the amino acids Phe^{17} and Phe^{21} is in close proximity to the C-terminal helix containing the Spy0416 cleavage site Gln^{21}-Arg^{60}. Introducing basic amino acids like lysine into the proximal loop of the chemokine induced a structural change – as detected in far-UV CD spectroscopy (data not shown) – which prohibited interaction with the protease by sterical and/or electrostatic hindrance.

To evaluate the impact of Spy0416 on the biological activity of IL-8, neutrophils were tested for being activated by measuring the release of Ca^{2+}. While Spy0416-cleaved IL-8

was unable to promote neutrophil chemotaxis, our Ca^{2+}-release data suggests that the cleavage of IL-8 did not affect its capability to activate neutrophils. This is in line with previous observations showing that the ELR motif of IL-8 is mainly responsible for receptor recognition. However, the C-terminal domain, cleaved off by Spy0416, is required for inducing neutrophil migration also because it is responsible for glycosaminoglycan (GAG) binding [174, 175]. Chemokine binding to cell-surface GAG molecules like heparan sulphate (HS) is a major prerequisite (1) for establishing the solid state chemotactic gradient along an inflamed endothelial tissue and (2) for inducing conformational changes and higher oligomerisation states of the chemokine in order to fully activate the protein. The impact of Spy0416 on IL-8 biological activity is apparently to impair the chemokine's GAG binding activity, thereby interrupting the chemotactic response of neutrophils.

7.1.4 Spy0416 Contribution to GAS Virulence *In Vivo*

It was shown that Spy0416 represents an important virulence factor for GAS to avoid clearance by phagocytes at the infection sites. Therefore we wanted to evaluate the effect of *spy0416* gene deletion on virulence of the mutant strain. An important role of Spy0416 for GAS pathogenesis *in vivo* could be demonstrated although ΔSpy0416 showed deviation of M type from the parental M49591 strain. Nevertheless, we assume that our observations are the result of gene deletion and not of a change of *emm*-type. The delay of virulence observed for ΔSpy0416 over a period of two weeks in our intraperitoneal murine challenge models is in agreement with observations from other groups. Hidalgo-Grass *et al*., demonstrated that in a murine model of GAS necrotizing fasciitis, a *spy0416*/*scpA* double mutant strain was significantly less virulent than either the *scpA* single mutant or the parental M14 wild type GAS strain [91]. Zinkernagel and colleagues showed by a murine necrotic skin infection model that a M1 mutant strain deficient in Spy0416 activity caused lesions ~50% reduced in size compared to those produced by the wild type strain [192]. Interestingly, contrasting observations were made by Sumby et al., where an isogenic *spy0416* mutant strain generated significantly larger skin lesions than its parental strain in a mouse model of soft tissue infection [178]. In an experimental model of local severe soft tissue infection, Spy0416 augmented disease severity whereas in a systemic infection such as sepsis Spy0416 had a seemingly protective effect against

disease [193]. This indicates that Spy0416 might mediate different effects depending on the site of infection.

7.1.5 Other Contributions of Spy0416 to GAS Pathogenesis

The high level of sequence conservation of a large GAS protein gives reason to speculate that Spy0416 may exhibit additional functions besides its degradation of the CXC chemokines IL-8, GCP-2 and Gro-α.

The successful confirmation of IL-8 degradation in our established *in vitro* Spy0416 assay led us to examine whether other CXC chemokines or even CC chemokines are also targeted by the Spy0416 enzymatic activity. Therefore I analyzed IP-10 (Interferon-inducible protein 10) and I-TAC (IFN-inducible T-cell alpha-chemoattractant) belonging to the group of CXC-chemokines and MCP-1 (monocyte chemotactic protein 1), a CC chemokine. Interestingly, the analysis of IP-10 and I-TAC degradation, both involved in T-cell chemotaxis, revealed that they are not degraded by Spy0416 *in vitro* although they display very similar overall three-dimensional folds as seen in Gro-α [162]. The same was observed in case of MCP-1 involved in T-cell and monocyte recruitment from the group of CC chemokines. It was previously shown for the CC chemokine RANTES, also responsible for T cell and monocyte chemotaxis, that it could not be degraded by Spy0416 [91]. Maybe CC chemokines are not a target for Spy0416. On the other hand we could also show that the CXC chemokine NAP-2 (neutrophil activating peptide 2) was not degraded by Spy0416 *in vitro* which is involved in neutrophil activation [162]. We hypothesised in our previous work that the difference in degradability by Spy0416 is, in addition to sequence-specificity, dependent upon the different oligomerisation behaviour of the different chemokines, since both NAP-2 (neutrophil activating peptide 2) and PF-4 (platelet factor 4) have been shown not to be degraded by Spy0416 *in vitro* although they contain similar isofunctional sequence deviations from IL-8 as Gro-α [162]. Such selectivity on chemokines that are targeted by Spy0416 could be the result from GAS facing situations in which the elimination of IL-8, GCP-2 and Gro-α is important. The specific chemokines that are produced and their deployment patterns may depend upon the location and stage of infection and therefore it is sufficient for GAS survival to target selected chemokines. This might also explain the observed different effects of Spy0416 depending on the infection site [193]. Similarly, it was

recently described that the related C5A peptidase from *S. agalactiae*, CspA, also exerts distinct specificity for individual CXC chemokines by degrading NAP-2 and Gro-α, but it displays no activity for IL-8 [194].

The involvement of Spy0416 in other immune evasion mechanisms was not further investigated in this work. Other functions apart from its protease activity have not been reported but due to the high sequence conservation level it is possible that Spy0416 may contribute to GAS pathogenesis by multifunctional properties. The streptococcal serum opacity factor for example consists of two distinct functional domains. One domain exerts a fibronectin-binding activity while the N-terminal domain is responsible for the opacity reaction [56, 57]. In this regard it is of interest that the protease domain of Group B streptococcal C5a peptidase, ScpB, has been shown to contribute to adhesion of host cells by binding to immobilized fibronectin [195, 196] besides its ability to degrade C5a. Spy0416 may also act like ScpB as a bifunctional molecule serving as a protease and mediating binding to fibronectin since it shares ~30% sequence identity and ~50% sequence similarity with the GBS C5a peptidase. Binding of fibronectin has been implicated in attachment and invasion of eukaryotic cells by streptococci but Zinkernagel and colleagues showed that Spy0416 does not act as a GAS epithelial cell adhesion or invasion. An isogenic ΔcepA mutant was found to be ~3-fold more adherent and ~2-fold more invasive than the WT parental strain [192].

Interestingly, CspA involved in group B streptococcal pathogenesis was beside its chemokine degradation activity found to be capable of degrading fibrinogen [197]. Harris et al., suggested that the cleavage of fibrinogen by CspA may be involved in protecting *S. agalactiae* from opsonophagocytic killing since they could show that a cspA$^-$ mutant was significantly more sensitive to opsonophagocytic killing by human neutrophils than the wild type *in vitro*. It might be possible that Spy0416, similar to CspA, allows GAS to avoid innate immune clearance in the non-immune host not only by IL-8 degradation but also by cleavage of fibrinogen. The resulting fibrin-like substances consequently would reduce access of opsonins or neutrophils to GAS. Although a fibrinogen cleaving activity for Spy0416 was not reported so far to my knowledge further work could be done on evaluating such an activity.

A functional homolog of Spy0416 was found in *S. iniae*, an emerging zoonotic pathogen of humans [192]. The genomic position of *cepl* is consistent with *spy0416* in GAS. A *S.*

iniae Δcepl strain lost the ability to degrade IL-8 and showed increased sensitivity to human neutrophil killing. This functional homolog of Spy0416 found in *S. iniae* contributes to chemokine degradation, neutrophil resistance and disease pathogenesis as it does for GAS. In case of *Staphylococcus aureus,* for example, similar bacterial factors that comprise the host immune response have been identified. A secreted beta-hemolysin inhibits TNF-α induced IL-8 secretion in endothelial cells and hence neutrophil transmigration [198]. This interference with the ability of the host to induce an adequate inflammatory response in an *S. aureus* infection is similar as in *S. pyogenes* only targeting IL-8 on the transcriptional level.

It would be interesting for future work to further evaluate the role of the ancillary C-terminal domains of Spy0416 for enzymatic activity, concerning how they might interact with the catalytic domains or with IL-8. The identification of factors that determine substrate range, such as a common structural motif present on the chemokines which mediates binding or entry to the Spy0416 active site or possibly triggering of a conformational change within the protease, would be interesting, too. Furthermore, the examination whether it exerts a proteolytic activity towards fibrinogen and evaluate the susceptibility of a *spy0416* gene deletion mutant to opsonophagocytosis might be interesting.

To come to a conclusion, the generated data, the high sequence conservation together with our unpublished and the recently reported protection data [153, 154, 178, 192, 193] provide strong evidence that Spy0416 can contribute to a vaccine capable of preventing Group A streptococcal infections.

7.2 Targeting of *spy0895* and *spy1536* for Gene Deletion

Among the 9 vaccine candidates selected by the ANTIGENome technology, Spy0895 with its putative histidine protein kinase function and Spy1536, annotated as conserved hypothetical protein, were selected for further characterization. Like the other 6 selected proteins from the ANTIGENome technology, these two proteins have not been previously characterized or shown to protect against GAS infections.

In order to obtain information about their possible role in streptococcal virulence in terms of colonization, evasion strategies, establishment of infection and in turn to emphasize their value as vaccine candidates, strains, being devoid of functional Spy0895 and Spy1536 expression, were generated and analyzed.

The generation of gene deletion mutants was performed with pGhost5 which was described in the literature as an effective transformation system [166, 199-201]. The work with this shuttle vector turned out to be very difficult. It appeared to be very unstable, as during propagation in an *E.coli* culture larger than 5 ml (large scale), pGhost5 started to decrease in size by excising 1000 bp as assessed by digestion with restriction enzymes. Only in small scale growth (5 ml) it retained its original size. This was a problem for ligation with inserts and transformation into *S. pyogenes* since a higher amount of pGhost5 than obtained from small scale purification was needed for transformation. We could overcome these problems finally by pooling pGhost5 preparations from several purifications from *E. coli* which allowed us to generate a larger amount of pGhost5-*0895* and pGhost5-*1536* for transformation into competent *S. pyogenes* cells. Transformation was not easy either. First of all, one reason might have been that *S. pyogenes* is not a naturally competent bacterium like for example, *S. pneumoniae,* and efficiencies in the generation of electrocompetent GAS cells for plasmid uptake may vary from strain to strain depending on capsule thickness. This might have been also the reason why we had to use strain M49591 kindly provided by Bernd Kreikemeyer (Rostock, Germany) instead of strain AP1. However, the problems with the transformation could also be attributed to the observed instability of pGhost5 as other vectors could be transformed into *S. pyogenes*.

7.2.1 Efficiency of *spy0895* and *spy1536* Gene Deletion in Strain M49591

Deletion of *spy0895* was very efficient, as 50-60% of the analyzed erythromycin sensitive colonies showed the desired recombination event resulting in gene deletion. In contrast, deletion of *spy1536* was only found in 1 of all analyzed clones. In order to lead to *spy1536* deletion, excision of pGhost5 occurred by recombination via the N-terminal and not the C-terminal fragment of *spy1536*. An explanation for the rare desired recombination event leading to *spy1536* gene deletion might be the choice of knock out construct constituted by *spy1536* N- and C-terminal regions. The C-terminal fragment for recombination was

296 bp larger than the N-terminal fragment which might have favored recombination resulting in the wild type. In contrast, the sizes of N- and C-terminal regions of *spy0895* for recombination differed only in 7 bp, and recombination to obtain the desired gene deletion occurred in 50-60% of the analyzed clones independent of the prior pGhost5 integration scenario.

7.3 Role of Spy0895 in Group A Streptococcal Pathogenesis

In order to identify a role for Spy0895 in GAS pathogenesis a gene deletion mutant, ΔSpy0895, was generated. The analysis of ΔSpy0895 did not lead to the identification of a potential function or contribution of Spy0895 to GAS pathogenesis. The main reason for that might already have been explained by the observed lack of *in vitro* expression. It is evident that the lack of *spy0895* expression represents a major obstacle for uncovering any effect of the respective gene deletion under *in vitro* conditions and consequently identifying a possible contribution to GAS pathogenesis. *In vitro* expression of such proteins is often very difficult because pathogenicity is a multifactorial property that is only fully manifested *in vivo*. It is necessary to simulate *in vivo* conditions in order to induce expression of certain proteins which is influenced by for example nutrient availability, pH, temperature, oxygen tension, osmolarity and growth phase [202] but an *in vitro* condition for Spy0895 expression could not be found to date for the strain M49591.

Since all effects of *spy0895* gene deletion can only be linked to Spy0895, the lack of *Spy0895 in vitro* expression in the wild type strain let us already expect the results for Spy0895 characterization. The *spy0895* gene deletion mutant cells did not show a different behaviour compared to wild type cells in growth under different environmental conditions or in the intraperitoneal murine challenge model. Furthermore, they did not show an aberrant behaviour in their ability to bind human plasma- and extracellular matrix-proteins *in vitro* or a difference in surface protein localization in comparison to the wild type cells.

The same was observed for the transcriptome analyses. It would have been especially interesting to know which genes are affected in terms of transcription by the lack of Spy0895. As putative histidine kinase it could be involved in a two-component system.

Lack of the sensor kinase would for sure reveal target genes regulated by the two component system in response to a certain environmental change. At least the genes that have been examined in this experiment due to the previous selection by microarray are not affected by *spy0895* gene deletion. Under *in vitro* conditions, as already mentioned before, some virulence factors are expressed only in response to certain environmental changes. Under the applied conditions Spy0895 seems not to be needed to adapt to its environment. In their host the bacteria are subjected to mostly different environments that can rapidly change and therefore it might adapt to this milieu by the help of Spy0895 expression. The function of Spy0895 in streptococcal pathogenesis and whether it really acts as a virulence factor remains to date completely unknown.

7.3.1 Attempts to Overcome Lack of *spy0895 In Vitro* Expression

Since no *in vitro* expression for Spy0895 was observed in the wild type, overexpression of Spy0895 in the M49591 was attempted. The overexpression of Spy0895 unfortunately failed due to several reasons:

First, Spy0895 is encoded by a gene that is part of a two-gene operon. The promoter region of the operon from which transcription is regulated is not directly located upstream of *spy0895* but upstream of *spy0894*. Therefore, after having identified a potential promoter region upstream by bioinformatic analyses, the potential promoter sequence was fused to the *spy0895* gene sequence and ligated into an expression vector lacking an inducible promoter. This means that the plasmid-driven expression of Spy0895 was dependent on this putative promoter region identified by bioinformatic analysis. Plasmid-driven expression of Spy0895 in M49591 analyzed by Western blot showed a signal which was twice the size as expected. Attempts to confirm specificity of the signal by blocking the Western blot signal with recombinant Spy0895 proteins failed. The fact that the promoter region was not directly located upstream of *spy0895* besides the possibility of regulators acting additionally at more proximal sites reduces the guarantee of successful expression from a vector not containing an inducible promoter. The use of a constitutive promoter might have led to the overexpression of Spy0895, but there was no time left for this.

7.4 Role of Spy1536 in Group A Streptococcal Pathogenesis

7.4.1 *In Vitro* Expression of Spy1536

In contrast to Spy0895, characterization of Spy1536 was not hindered by the lack of expression under *in vitro* conditions. The analysis of transcription of *spy1536* in wild type GAS cells by Northern blot verified first of all transcription of *spy1536 in vitro.* It further confirmed for ΔSpy1536 that *spy1537* and *spy1538* are not affected in transcription by the *spy1536* deletion, since the polycistronic RNA was still detected. In view of this, under the applied *in vitro* conditions, Spy1536 is expected to be expressed in the wild type strain and thus some influence of *spy1536* gene deletion on the mutant strain could be expected.

7.4.2 *In Vitro* Growth, Bacterial Shape and *In Vivo* Virulence were Not Affected in ΔSpy1536

Initially, the characterization of ΔSpy1536 was focused on the evaluation of growth under different environmental conditions, virulence in mice and bacterial shape by electron microscopy.

Growth under different environmental conditions did not reveal a major growth defect. The only exception was the observation of growth in medium supplemented with 2% glucose. ΔSpy1536 doubled its optical density within one hour while the wild type strain already reached stationary phase. Bacteria coordinately control gene expression and virulence factor production by complex regulatory circuits in response to alterations in the host milieu [202, 203]. They encounter multiple microenvironments during the infectious process and must interact with numerous types of host cells and therefore *S. pyogenes* regulate virulence determinants in response. Transcription of the genes encoding M protein and C5a peptidase for example is stimulated by growth in elevated CO_2 and slightly decreased in elevated O_2 [204]. Other environmental factors such as temperature, osmolarity and iron limitations have also been shown to influence expression of the M protein [205]. For protein F, too, it could be shown that expression is influenced by alterations in atmosphere, and conditions that favour M protein expression tend to repress protein F expression [206]. The lack of certain virulence factors therefore may affect growth of the bacteria which might not be able to adapt to

the growth conditions applied. The reason for the observed rapid increase of optical density of ΔSpy1536 compared to wild type cells remains unclear. We can only speculate that the increased optical density is caused by aggregates of mutant cells resulting from changes of the bacterial surface. A decreased capsule expression may lead to increased clumping and aggregation or expression of pili that influence interaction between the cells. In regard to this it could be shown that expression of pili led to cellular aggregates of GAS cells in liquid culture, while GAS mutant cells characterized by mutation of genes encoding either the pilus backbone structural protein or the sortase C1 were much less efficient in forming such aggregates [182]. Another possibility might be the secretion of certain components similarly seen in biofilm formation. Nevertheless, this behaviour was not further investigated as it was only reproducible in a small scale 200 µl culture.
In order to reveal a possible change on the surface of ΔSpy1536 cells or a change of their shape as a consequence of gene deletion, the mutant GAS cells were visualized with an analytical high-resolution scanning electron microscope. This analysis did not show any obvious alteration on the bacterial surface. It is known that scanning electron microscopy has not provided nearly as much information about cell walls as transmission electron microscopy, since it can only reveal the surface topography. The wall surface was "gold sputtered" to provide a thin conductive layer that prevents charging by the electron beam and this gold layer can reduce the resolution as well as compact polymeric filaments extending from the wall. Transmission electron microscopy was planned examining thin sections of the bacterial surface which might have given an idea of the thickness of the cell wall or capsule.

To investigate the effect of gene deletion not only under *in vitro* but also under *in vivo* conditions, we analysed the virulence of ΔSpy1536 in an intraperitoneal murine challenge model. Our results did not show a reduction of virulence in ΔSpy1536. In this regard it should be mentioned that the mouse environment does not fulfil the requirements of human disease entirely although it has still been extensively used for studies in streptococcal pathogenesis [47, 179-181]. In addition, it was shown that bacterial virulence factors may trigger different disease symptoms and outcomes depending on the site of infection [193]. Therefore a different mouse model might have revealed an effect of *spy1536* deletion on virulence. The use of a different mouse strain

might have differed markedly in its susceptibility to group A streptococcal infection. BALB/c mice, for example, have shown to be much more resistant than C3H/HeN mice [207], not only due to their impaired capacity of bacterial clearance but also due to a genetic predisposition to produce inflammatory mediators in response to streptococcal products [208]. The use of another infection model would have also been a possibility to test ΔSpy1536 virulence because M49591 wild type strain can cause both pharyngeal and skin infections. Subcutaneous or intranasal challenge might have given a better insight on virulence of ΔSpy1536.

As we could not observe an obvious growth defect or reduction of virulence in mice for ΔSpy1536 as compared to wild type cells, we started with a more detailed analysis using functional assays.

7.4.3 Spy1536 is Crucial for the Binding of Streptococci to Human Plasma and Extracellular Matrix Proteins

The success of pathogens depends on their ability to colonize host tissue and persist by ensuring their long-term survival. Accordingly, bacterial pathogenesis is a complex process that can involve attachment, colonization as well as internalization by epithelial and endothelial cells, local tissue destruction and dissemination to distant anatomic sites. *S. pyogenes* expresses virulence factors that mediate one or more of these processes including cell surface adhesins that participate in attachment to host cells [209]. This is the reason why the interaction with and adherence to epithelial cells of *S. pyogenes* is generally accepted as a crucial mechanism to colonize and invade the human host and consequently cause diseases. Many different surface molecules (adhesins) mediate the attachment of GAS to human extracellular matrix components. Therefore, we wanted to evaluate whether deletion of *spy1536* had an effect on the potential to bind selected human proteins *in vitro* in terms of adherence.

Surprisingly, our data revealed that the deletion of the *spy1536* gene in *S. pyogenes* M49591 showed an almost complete loss of binding to all of the assessed human plasma- and extracellular matrix proteins, fibrinogen, fibronectin, laminin, collagen type I and plasminogen, while the wild type adhered to these proteins *in vitro*.

It was previously shown that insertional inactivation of for example the gene *prtF* encoding protein F in *S. pyogenes* had resulted in the loss of fibronectin binding, and mutant cells had a much lower capacity to adhere to respiratory epithelial cells [50, 54]. Due to the fact that we did not only observe lack of fibronectin-binding but observed a broad effect on binding to several human proteins (fibrinogen, fibronectin, collagen type I, laminin and plasminogen) led to the question whether the strongly reduced binding of *spy1536* gene deletion mutant was the result of an unspecific change on the bacterial surface. For instance, it was shown that group A streptococci expressing M protein have a more hydrophobic and a more negatively charged surface than M-negative strains attaching better to pharyngeal epithelial cells [210]. Apart from the possibility that the *spy1536* gene deletion affected the charge of the bacteria, Spy1536 could be involved in a more general process relevant for proper surface expression of streptococcal proteins or the consequence of a modulated gene expression of surface proteins.

7.4.4 Spy1536 Influences the Surface Expression of Streptococcal Proteins

The significantly reduced binding of *spy1536* mutant cells to human plasma and extracellular matrix proteins indicated a change on the surface of ΔSpy1536. By immunostaining of selected surface proteins we wanted to evaluate whether there is indeed a change on the surface by reduced expression of adhesins such as the M protein. M protein specific surface staining showed on wild type cells a distribution to both the closing mother cell septum and the forming daughter cell septa, as it was also seen before [211, 212]. M protein is anchored concomitantly with peptidoglycan synthesis at the septum and is therefore distributed throughout the cell periphery [211]. One might expect an equal distribution of M protein over the wild type surface, but the observed pattern of M protein could be the result of taking a culture in late stationary phase of growth for immunofluorescence analysis. The use of M protein specific antibodies deriving from an M1 and not M49 serotype might also influence detection of M protein on the surface, meaning that the conserved M protein regions might only be limitedly accessible at these observed bacterial sites. It was shown previously that M protein is localized on the bacterial surface to sites of Lipid II export. There, newly anchored M protein was often detected at the closing primary division septum and the mature equatorial rings/daughter cell division sites [212].

In contrast to the wild type cells, ΔSpy1536 did not show any M protein specific staining when visualized under the same conditions. This is in accordance with the assumption of a change on the bacterial surface as a result of *spy1536* gene deletion.

Apart from the fact that the M protein is the most prominent molecule on the surface of all pathogenic group A streptococci and an important mediator of streptococcal adherence [213], lack of M protein on the bacterial surface would make ΔSpy1536 cells more prone to phagocytic killing as compared to the wild type.

In the virulence of GAS the M protein confers resistance to complement mediated killing by polymorphonuclear leukocytes and macrophages [46], thus protecting the bacteria from phagocytosis. By binding fibrinogen it interferes with complement deposition contributing to the known anti-opsonic property of M protein [214]. It could be previously shown that surface localization of M protein affected by the deletion of *sagA* increased the strains susceptibility to phagocytosis due to the lacking M protein on the bacterial surface [215]. In regard to this it might be very interesting to evaluate whether the *spy1536* mutant cells would be more efficiently killed in an opsonophagocytosis assay than the parental wild type cells.

Similar observations were made in case of Spy0269 specific staining, which also lacked on ΔSpy1536 cells. The Spy0269 protein was identified like Spy1536 using the ANTIGENome technology. It is annotated as a putative surface exclusion protein and possesses a short region showing similarity with the EzrA protein in *B. subtilis* that interacts with the cell-division protein FtsZ [170]. The observed lack of Spy0269 on the bacterial surface of ΔSpy1536 cells might indicate an effect of *spy1536* gene deletion on selected surface proteins, since Spy1666 was still detectable on the surface. It is unknown how Spy0269 surface expression is affected. The Western blot analysis showed signals in cytoplasmic and cell wall fraction, also seen for the recombinant Spy0269 protein. It remains unclear why this protein is no longer detectable in immunofluorescent analysis. The probability that antibodies targeted to the recombinant Spy0269 protein do not bind surface exposed Spy0269 is excluded since the recombinant protein also provides protection against a lethal *S. pyogenes* challenge in mice [153].

7.4.5 Spy1536 is Involved in Regulation of Surface Protein Localization Independent of Transcription

Since we could partly explain the reduced binding of ΔSpy1536 cells to human proteins caused by the lack of M protein on the mutant's surface, we wanted to investigate whether this was the consequence of a transcriptional or translational modulation. The detection of M protein in the cytoplasm at similar levels in wild type and ΔSpy1536 cells, but not in the supernatant, suggests that Spy1536 does not influence the transcription of the *emm* gene. This was further supported by microarray analysis according to which the *emm* gene was not differentially expressed in ΔSpy1536 compared to the wild type strain.

Since we could exclude a change on the transcriptional level, we hypothesized that Spy1536 might be involved in a process of protein surface localization or anchoring.

In order to assess how Spy1536 could be involved in surface localization of M protein we tested whether recombinant Spy1536 added to ΔSpy1536 cells during overnight growth would be able to restore M protein surface localization. If it would be directly involved in the process of M protein anchoring, recombinant Spy1536 might be able to restore the wild type situation. However, the addition of recombinant Spy1536 did not affect surface expression of M protein.

It is possible that Spy1536 not directly affects M protein localization. An indication for the involvement of Spy1536 might give its protein structure. Although Spy1536 is annotated in the SF370 genome as conserved hypothetical protein, it shows similarity to Lon-proteases, including a Lon protease PDZ domain, for which reason it was annotated in subsequently published GAS genomes as ATP-dependent endopeptidase Lon. Lon proteases are multi-domain enzymes [216-218] found in all living organisms and are involved in *E. coli* in the degradation of naturally unstable and misfolded proteins [218, 219] as well as in targeting regulatory proteins for degradation. Spy1536 might act in similar ways as it was shown for the *sagA* gene product, which is involved in surface localization of M protein without directly affecting transcription of the M protein gene, but rather by indirectly affecting its proteolysis, thus preventing peptidoglycan anchoring [215]. Similarly, Spy1536 may affect the surface localization of other proteins such as Spy0269 using the same or yet another mechanism.

The obtained data, the lack of immunofluorescence staining of M protein and Spy0269 on ΔSpy1536 cells in comparison to wild type M49591 cells, is consistent with the assumption that the reduced binding of ΔSpy1536 cells to human proteins is due to the diminished surface localization of several streptococcal proteins.

7.4.6 Spy1536 Affects SpeB Activation in Culture Supernatant

The immunofluorescence data confirmed the absence of at least two virulence factors on the bacterial surface. We could exclude repression of transcription of the corresponding genes by microarray and Western blot analysis. Still, it remained unclear whether this is caused by a defect in surface localization/ anchoring or yet another mechanism. Therefore, we wanted to address the possibility of an overproduction of proteases which degrade surface exposed M protein. Since it was reported that SpeB releases biologically active fragments from the bacterial surface including M protein [185, 220], we wanted to find out whether the lack of M protein on the bacterial surface is attributed to an increased expression of active SpeB. Surprisingly some kind of the opposite was observed in Western blot analysis. Our results revealed that the *spy1536* gene deletion mutant showed only the inactive 40 kDa form of SpeB in culture supernatant. Despite the fact that this did not explain the lack of M protein on the ΔSpy1536 bacterial surface, inability of producing active SpeB in the culture supernatant attracted our attention.

SpeB, which has been shown to cleave proteins including ECM components [221] and plasma proteins such as fibrinogen [222], is secreted as an inactive precursor of 40 kDa zymogen form and is auto-catalytically cleaved into an active enzyme of 28 kDa mainly in the beginning of stationary phase after 10 hours of growth [223]. Since reducing conditions accelerate cleavage of the pro-region which results in mature cysteine proteinase [224] we evaluated the effect of DTT to see whether SpeB could be activated *in vitro*. Upon addition of a reducing agent, DTT, SpeB could be activated *in vitro*. This might be an indication that SpeB maturation is not a matter of conformation. It is not known what could influence SpeB and prevents it from being activated. Interestingly, it was postulated that the generation of mature streptococcal cysteine proteinase is dependent on cell wall-anchored M1 protein [223]. Collin and colleagues suggested that M protein, when anchored to the cell wall is involved in the unfolding of the zymogen and generation of mature cysteine proteinase that can be activated under reducing

conditions. This would have been a logical explanation why in the *spy1536* gene deletion mutant lacking cell wall anchored M protein, SpeB was only detected as inactive zymogen. On the contrary, according to Zimmerlein and his colleagues this hypothesis was not confirmed [225] when they showed that deletion of M49 and M-like proteins had no effect on protease maturation. This seems not to be a proper explanation for the inactive SpeB.

A connection how Spy1536 might be involved in surface localization of both the M protein and SpeB cannot be established, since M protein surface localization is associated with peptidoglycan synthesis and Sortase A function [212]. In contrast, SpeB is exported at a distinct location of the bacteria - at the ExPortal [226] - and proper targeting and maturation of nascent SpeB was shown to require the ribosome-associated Trigger factor [227, 228] and the cell membrane associated chaperone HtrA [229, 230].

Spy1536 might exert a similar function like Trigger factor or HtrA have, or influence their activity directly or indirectly. Interestingly, it was reported that HtrA is indirectly involved in the conversion of proSpeB into its active form [231]. In *S. mutans*, HtrA plays a role in the regulation of genetic competence, biofilm formation, and the biogenesis of cell wall-associated and secreted proteins [232, 233]. The involvement of HtrA in the degradation of unfolded or misfolded proteins destined for secretion across the cytoplasmic membrane [234] resembles an activity attributed to the Lon-protease in *E. coli* [218, 219]. Spy1536 containing a Lon-protease domain on its C-terminus might exert a similar function.

A role for transcription, secretion and even more importantly processing of SpeB was also attributed to ropA, an rgg-like transcriptional regulator and homologue of Trigger factor which is essential for SpeB maturation exerting a PPIase activity. It was shown that RopA acts both to assist in targeting SpeB to the secretory pathway and to promote the ability of the pro-protein to establish an active conformation upon secretion [228]. Site specific mutagenesis in *ropA*, the gene encoding trigger factor in *S. pyogenes*, produced a mutant protein deficient in PPIase activity. As a result, although the protease was secreted efficiently it did not mature to an active form [227]. The model presented by Lyon and colleagues suggests two functions for RopA, one which participates in targeting the SpeB-polypeptide for secretion and a second one which participates in folding, both functions located at different domains. Spy1536 could

interact with the domain involved in SpeB folding. Therefore, in the *spy1536* gene deletion mutant, lack of *spy1536* could result in a misfolded SpeB protein that cannot be auto-catalytically activated.

According to this, there is an indication that Spy1536 is involved in proper surface protein localization of M protein and Spy0269 independent of transcription. Furthermore, deletion of functional Spy1536 results in the inability of the mutant for proper SpeB activation in culture supernatant. SpeB makes important contributions to the pathogenesis of streptococcal infections. It could be shown that insertional inactivation of *speB* can reduce virulence of *S. pyogenes* following intraperitoneal challenge of mice [235, 236]. This supports the notion that Spy1536 is directly involved in *S. pyogenes* pathogenesis which even reflects the value of selecting this protein as vaccine candidate.

7.4.6.1 Complementation of *spy1536* Gene Deletion

Attempts to complement the gene deletion in order to attribute the observed phenotype to the lack of Spy1536 expression, failed due to several reasons. Like it was already explained for *spy0895*, the Spy1536 encoding gene is part of an operon which complicates complementation. For *spy1536*, too, a potential promoter region had to be identified by bioinformatic analysis since it is not directly located upstream of the gene but upstream of *spy1538*. Although the expression vector harboring *spy1536* with its potential promoter was successfully transformed into *S. pyogenes*, expression of Spy1536 could not be observed in Western blot. Unfortunately, time elapsed to complement ΔSpy1536 by expressing *spy1536* from an expression vector with a constitutive promoter. This would be necessary for the verification that the observed phenotype could indeed be attributed to *spy1536* deletion.

7.4.6.2 Spy1536 is Involved in Transcriptional Regulation of Adhesin Encoding Genes

I got the opportunity to perform microarray analysis in Rostock in Bernd Kreikemeyer's laboratory to evaluate the effect of gene deletion directly on the transcriptional level. Such quantitative whole genome transcript measurements, to name only a few, have repeatedly been used to compare regulatory gene mutant bacteria to their corresponding wild type strains [237, 238], or specifically stressed bacteria have been compared to their

non-stressed isogenic ones [239]. Biofilm associated bacteria, too, could be compared to their planktonic counterparts using microarray analysis [240]. In GAS serotypes M1 and M49 strains defined mutants have been employed to examine the impact of the CsrRS and Rgg regulators based on the whole genome gene expression levels [241, 242].

Microarray analysis of ΔSpy1536 has resulted in the selection of 10 genes showing differential expression compared to the wild type cells at least 2 fold. Among those candidates tested in Real Time RT-PCR this observation was confirmed for 5 genes. Previous experiments indicated an important role for Spy1536 in streptococcal virulence concerning its adhesion to human plasma- and extracellular matrix proteins and in the surface localization of M protein and Spy0269. Interestingly, among the genes that have been selected based on microarray analysis and furthermore confirmed to be differentially expressed by Real Time RT-PCR, some of them encode virulence factors involved in adherence and invasion of *S. pyogenes*. The results substantiate the reduced binding to human plasma and extracellular matrix proteins observed for the ΔSpy1536 strain.

The *spy1536* gene deletion mutant was characterized by a ~100 fold decreased transcription of *spym49s0079*, which encodes the fibronectin binding protein SfbX49. The observation of a decrease in *sfbX49* transcription is in agreement with the reduced binding of ΔSpy1536 to human fibronectin *in vitro*. SfbX49 has been identified by Jeng and his colleagues in 2003 [187] where the respective gene, *sfbX49*, was shown to encode a fibronectin-binding function. A *sfbX* mutant was characterized by a marked decrease in fibronectin binding which could be restored by complementation. Additionally it was demonstrated that *sfbX49* is located together with serum opacity factor (*sof49*) within a two-gene operon, under positive control of the GAS multiple gene regulator Mga [243]. According to the microarray data analysis, serum opacity factor expression was also affected by the *spy1536* gene deletion but this could not be confirmed by Real Time RT-PCR according to which the gene *spym49s0078* showed no significant change in transcription. However, studies on *sof* and *sfbX49* have shown that each gene contributes strongly and independently to the ability of log-phase GAS to bind to immobilized fibronectin [187].

The *spy1536* gene deletion mutant showed also reduced binding to collagen type I *in vitro*. This is again in accordance with the Microarray analyses and Real Time RT-PCR

which revealed ~5-10 fold downregulation of *spym49b1739* (*scIB*) and *spym49c1149* (*scIA*), both encoding putative collagen-like proteins, SclA and SclB, being structurally related to one another [244]. Isogenic strains in which the *scl* gene was inactivated were significantly reduced in their ability to adhere to human epithelial cells grown in culture and were significantly less pathogenic in a mouse model of soft tissue infection [49]. The downregulation of these two collagen-binding proteins may not have only an effect in terms of adherence but also in evasion of host immune mechanisms. For instance, it could be shown that *S. pyogenes* counteracts host attacks by recruiting host proteinases to its surface. SclA and SclB might be involved in modulating inflammatory reactions in the host. By binding TAFI, a Thrombin-activable Fibrinolysis Inhibitor [245] which has been shown to inactivate anaphylatoxins C3a and C5a [246], SclA and SclB could contribute to the inhibition of phagocyte recruitment to the infection site. Similar mechanisms for adhesins binding human proteinases have been described. The best studied human proteinase in this respect is plasminogen, which apart from *S. pyogenes* also interacts with many other species, including *Borrelia burgdorferi*, *E. coli*, *Salmonella enteritidis*, *Yersinia pestis* and the *Neisseria* species *gonorrhoeae* and *meningitides* [247]. Once plasminogen is bound to the bacterial surface, the zymogen can be activated and enhance bacterial dissemination. Plasminogen binding to the streptococcal surface is mediated by M and M-like proteins [61, 248] as well as α-enolase [63]. Some M proteins bind plasminogen indirectly via fibrinogen [248], and other strains that express PAM (Plasminogen-binding group A streptococcal M-like protein) bind plasminogen directly with high affinity [61].

Furthermore, SclA from an M6-type group A streptococcus has recently been shown to be involved in inhibiting the alternative pathway of complement by binding factor H [249], an important mechanism that has also been described for M and M-like proteins. This indicates that apart from the observed lack of M protein, ΔSpy1536 might be more prone to phagocytosis in its host.

The approximately 100-fold downregulation of *fcrA* (*spym49s0005)* in ΔSpy1536, too, speaks for the hypothesis of an increased susceptibility to phagocytosis. *FcrA*, encoding an M-related Ig-binding protein (precursor), has been identified in serotype M49 to be expressed under the control of the *vir49* regulon clustered on the streptococcal chromosome with *emm49*, *enn49* as well as *scpA49* [188-190]. A mutant devoid of *vir49*

function did not express Ig-binding proteins and became sensitive to phagocytosis by human leukocytes [250].

The only slightly upregulated gene identified in ΔSpy1536 was *hasA* (*spy2200*), encoding the membrane associated hyaluronate synthase, which is part of the *hasABC* operon. This effect of gene deletion is quite interesting because the question is whether this contributes to a reduced virulence or even supports adherence/ invasion of the streptococci apart from the fact of other adhesins not being expressed on the surface. There are contrasting reports regarding the role of hyaluronic acid capsule in GAS internalization. In one study the presence of capsule did not affect internalization [251], while other studies reported both augmentation [9] and inhibition of bacterial entry [252, 253]. The inhibitory effect of hyaluronic acid capsule concerning invasion was attributed to masking the bacterial surface molecules required for interactions of the host cells [254, 255].

7.4.7 Regulation of Transcription: Is Spy1536 Involved in a Regulatory Network of *S. pyogenes*?

In order to colonize and cause disease at distinct anatomical sites, bacterial pathogens must tailor gene expression in a microenvironment-specific manner. Interactions between regulatory systems contribute to the control of tissue tropism and infection. The main question that remains unanswered here is the role Spy1536 might play in all the observations described. An influence of the *spy1536* gene deletion was not only seen on the transcriptional level (upregulation of *hasA* and downregulation of *fcrA*, *sclA*, *sclB*, and *fsbX49*), also a defect in surface localization of the major virulence factor, the M protein, and Spy0269 could be observed. Besides that, an inability of producing an active form of SpeB, that is usually secreted as inactive zymogen and is mainly activated by its autocatalytic activity, was found for ΔSpy1536. Many regulatory networks have been described in Group A streptococci involving several transcriptional regulators that influence the expression of secreted and surface proteins. Mga for instance, coordinates expression of genes encoding proteins involved in adherence and proteins that confer ability to resist phagocytosis. Besides C5a peptidase (*scpA*) and serum opacity factor (*sof*), M (*emm*) and M related proteins (e.g. *fcrA*), SclA (*sclA*) as well as SclB (*sclB*) have been reported to be positively regulated by Mga [49, 79, 243, 256]. One could say that

activation of Mga might be mediated by Spy1536 which leads in ΔSpy1536 to lack of *sfbX49*, *sclA* and *sclB* transcription, but the M protein in contrast is not affected on the transcriptional level but merely not targeted to the cell surface. Similar it is seen for *speB* and *hasA*. SpeB expression and hyaluronic acid capsule synthesis are under the negative control of the *covR*/*covS* (control of virulence factors) two-component system [257]. Here, too, one could say Spy1536 might inhibit CovR activity to increase capsule expression, but this would at the same time also affect *speB* transcription. Whereas in ΔSpy1536 SpeB expression is not increased, the protease is just not detectable in its active form.

It is difficult to determine a common pattern of regulation maybe due to the fact that regulation of virulence in group A streptococcus is extremely complex. An example that virulence factor regulation is exerted from many different regulatory systems and not enough is also strain dependent was shown for Spy0416. The *spy0416* gene is one of the most highly differentially expressed upon comparison of the transcriptional profile of invasive M1T1 GAS isolates versus similar clonal strains isolated from the pharyngeal mucosa [178]. In simple colonization, results generated by Zinkernagel and colleagues indicate that Spy0416 may decrease epithelial cell adherence and therefore, its expression would not be favoured. Furthermore, it was found that its transcription is negatively regulated by CovRS where mutations in CovS in emm 49 clinical isolates led to a significant change of virulence factor transcription, increasing Spy0416 (ScpC), hyaluronic acid capsule, C5a-peptidase expression while SpeB was downregulated [258]. On the other hand it was reported by Hidalgo-Grass and colleagues that in an M14 strain SilCR pheromone downregulates transcription of *scpC* via the two component system SilA/B [91]. This again points out that an effective vaccine should target more than just one or two protective candidates.

The gene deletion studies have furthermore provided evidence for an important role of Spy1536. To my knowledge, all these observations in the *spy1536* gene deletion mutant have not been previously described for Spy1536. The significantly reduced binding to human plasma and extracellular matrix proteins is further substantiated by the reduced surface localization of M protein and Spy0269 and the reduced transcription of adhesin encoding genes. The data strongly indicates an important role for Spy1536 in regulation of virulence factors and hence streptococcal pathogenicity.

7.5 Conclusion

The presented data further strengthens the selection of Spy0416 and Spy1536 as potential vaccine candidates. Spy0416 was one of the most frequently selected protective antigens by the ANTIGENome Technology. Its ability to protect against lethal infection in mice was shown in three different mouse models that try to mimic human disease. In addition, Spy0416 (ScpC) was shown to mediate cleavage of IL-8, thereby impairing clearance from infected tissues and promoting resistance to neutrophil killing [91, 192]. We provided further evidence that this activity can be exerted by the recombinant Spy0416 protein and that it relied on the concerted activity of the enzymatic as well as ancillary domains of the large ScpC protein [162]. These studies strengthen the selection of Spy0416 as candidate for a GAS vaccine.

Furthermore, the results from the gene deletion mutant experiments indicated an important function for Spy1536 adherence to human plasma- and extracellular matrix proteins. This is a very interesting finding as Spy1536 was previously not characterized and a contribution to virulence has to my knowledge not been shown before. Adherence and invasion are the important disease-causing mechanisms of gram-positive organisms. Since interaction of these pathogens with the components of host ECM is involved in adherence and invasion, the underlying mechanism of this interaction are of utmost importance for designing novel therapeutic strategies. The exact mechanism how Spy1536 acts on gene transcription encoding adhesins (*sfbX49*, *hasA*, *sclA*, *sclB* and *fcrA*) still remains unanswered. Lon proteases have the ability to target regulatory proteins for degradation, so it might be possible that Spy1536 degrades general regulators such as Mga or CovR/S to control virulence factor expression according to the environmental conditions it is subjected to. Furthermore, Spy1536 seems to be directly or indirectly involved in the surface localization of M protein, Spy0269 and potentially other selected surface proteins as well as in the maturation of SpeB. A detailed understanding of these mechanisms is crucial, since interference with microbial surface components recognizing adhesive matrix molecules functioning alone or in conjunction with specific manipulations of regulators is an attractive goal for novel anti-infective strategies. So far we could not detect any proteolytic activity for Spy1536. Although the exact contribution and mechanism of how Spy1536 is involved in the transcriptional

regulation of adhesins and surface localization of proteins is unknown the data clearly emphasizes its important role for GAS pathogenesis.

In summary, the described GAS vaccine candidates are highly conserved not only in the published 13 *S. pyogenes* genomes, but also in up to 51 further analyzed clinical isolates. Thus it can be assumed that an immune response against any of these candidates will be able to recognize the pathogen if the respective protein is surface accessible during the infection. The observed high level of sequence conservation and their ability to individually induce protection in mice [153] argues that these proteins provide an important function to the pathogen. As *S. pyogenes* has evolved many mechanisms to evade the immune system of its human host, targeting not only a single protein but several surface proteins or virulence factors in a vaccine will decrease the likelihood that the pathogen will be capable of evading the host immune response and cause disease in an immunized individual. Studies are therefore currently ongoing to evaluate whether a combination of several proteins will be able to achieve a high level of protection against various serotypes of group A streptococcus. The described work will thus strongly support the endeavour to develop vaccines to lower the large burden of disease caused by group A streptococcus throughout the developed and developing world.

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9 ABBREVIATIONS

ABTS	2,2'-Azinobis-(3-ethylbenzthiazolin-6-sulfonsäure)
AEBSF	4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride
Ala	Alanine
Asp	Aspartate
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
EDTA	Ethylenediaminetetracetic Acid
CFA	Complete Freund's Adjuvant
CFU	Colony Forming Unit
DEPC	Diethylpyrocarbonate
DNA	Desoxyribonucleic Acid
DMEM	Dulbecco´s Modified Eagle Medium
ELISA	Enzyme-linked Immunosorbant Assay
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FITC	Fluoresceinisothiocyanat
GAS	Group A streptococcus
GBS	Group B streptococcus
Gln	Glutamine
GRO-α	Growth-Related-Oncogene α
HBSS	Hanks' Balanced Salt Solution
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
His	Histidine
His-tag	Histidine-tag
IFN	Interferon
IFA	Incomplete Freund's Adjuvant
IL-8	Interleukin 8
IP-10	Interferon-inducible protein 10
I-TAC	IFN-inducible T-cell alpha-chemoattractant

IPTG	Isopropyl β-D-Thiogalacto-Pyranoside
KC	Keratinocyte-derived Chemokine
Leu	Leucine
LB	Luria Bertani
MACS	Magnetic Cell Sorting
mid-log	mid-logarithmic
MIP-2	Macrophage Inflammatory Protein 2
MHB	Mueller-Hinton Broth
Mw	Molecular weight
MWM	Molecular Weight Marker
OD	Optical Density
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with 0.1% Tween-20
Phe	Phenylalanine
PMSF	Phenylmethylsulfonyl fluoride
PEG	Polyethylen-glycol
RHD	Rheumatic Heart Disease
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Ser	Serine
SLO	Streptolysin O
SLS	Streptolysin S
SPR	Surface Plasmon Resonance
STSS	Streptococcal Toxic Shock Syndrome
TCA	Trichloric acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
THB	Todd Hewitt Broth
Thr	Threonine
Tris	TRIZMA®base
Tx100	Triton

10 APPENDIX

10.1 Appendix I - Primers

Table 3: Primers for generation of gene specific probes used in Southern and Northern blot analysis
Primer names and respective sequences (forward and reverse, 5′-3′). Specificities indicate gene regions to which primers bind.

Name	Primer sequence (forward, reverse)	Specificity
6074	Fw: 5'-GAATTGAACCTTGCCTTTCTAGCG-3'	*spy0895*
6075	Rev: 5'-CCTCGTGATCAGATTCCAACATG-3'	
6076	Fw: 5'-GCATCGCTGTATCCGCCTGT-3'	*spy1536*
6077	Rev: 5'-CCAGTTTGTTGCAGTGGGCATT-3'	
6333	Fw: 5'-CCTCTTTAATTTGGTTATATGAATTTTGC-3'	pGhost5
6334	Rev: 5'-CTTGCTCTTTTGTCAGAGAAATCATAAC-3'	
7268	Fw: 5'-TGCCTTCGATCTCTATCTATGCTCG-3'	*spy0894*
7269	Rev: 5'-GTTATCTGAGATCGTCATGATTCCAAG-3'	
7270	Fw: 5'-CAGAGCTTGAGTAAGCATTGCTTTAC-3'	*spy1537*
7271	Rev: 5'-GGTCATTTGATCCTGTTACAAACG-3'	
7272	Fw: 5'-CCAGTCAAGCAATCAATAGCTCG-3'	*spy1538*
7273	Rev: 5'-GACGCTCGATGGAAAGATAACAC-3'	

Table 4: Primers used for generation of Spy0416 subconstructs
Open reading frames (ORF) with primer names and respective sequences (forward and reverse, 5′-3′) are presented. Amplification of PCR products (bp) carried out under standard conditions. Restriction enzyme recognition sites (underlined) for cloning are Nco*I* and Xho*I* except for Spy0416A and Spy0416B cloning where Eco*RI* and Xho*I* as well as Spy0416$_{\Delta Fn1}$ where Afl*II* and Xho*I* were utilized.

ORF	Name	Primer (forward, reverse)	Product
Spy0416A	2246	FW: 5'-TAGTAGGAATTCGGCAGATGAGCTAAGCACAATG-3	2502
	2247	REV: 5'-TAGTAGCTCGAGCTCTGAACCAAGAGTGACAAG-3'	
Spy0416B	2248	FW: 5'-TAGTAGGAATTCGCATGTAGACCCACAAAAGGGC-3	2646
	2249	REV: 5'-TAGTAGCTCGAGCGTTGATGGTAGGGCTTTTGC-3'	
Spy0416A	6408	FW: 5′-TAGTAGACATGTGCAGATGAGCTAAGCACAATGAG-3′	2382
ΔFn2	6409	REV: 5'-GTGGTGCTCGAGTTGCCCTTTAAAACCAACAAAAG-3'	
Spy0416B	6410	FW: 5'-GATATACCATGGCCTTTGAAAACTTAGCAGTTGCAGAAG-3'	950
Fn2Fn3	6411	REV: 5'-GTGGTGCTCGAGTGTTTGTGGTAAGTGATCTCCTAAC-3'	
Spy0416B	6412	FW: 5'-GATATACCATGGCCTTAGGTAAAACACCAATTAAACTTAAGC-3'	789
Fn2´Fn3´	6413	REV: 5'-GTGGTGCTCGAGGTCACGCAAGGTAGCAAAAGAC-3'	
Spy0416B	6414	FW: 5'-GATATACCATGGCCCTAAAAGCGGTCGGAAAAGAC-3'	630
Y	6415	REV: 5'-GTGGTGCTCGAGGGCAGAGGCTGTCAAAGCC-3'	
Spy0416B	6412	FW: 5'-GATATACCATGGCCTTAGGTAAAACACCAATTAAACTTAAGC-3'	1419
Fn2´Fn3´Y	6415	REV: 5'-GTGGTGCTCGAGGGCAGAGGCTGTCAAAGCC-3'	
Spy0416B	6410	FW: 5'-GATATACCATGGCCTTTGAAAACTTAGCAGTTGCAGAAG-3'	2343
ΔFn1	6415	REV: 5'-GTGGTGCTCGAGGGCAGAGGCTGTCAAAGCC-3'	

Table 5: Primers used for the generation of *spy0895* and *spy1536* knock out constructs and for confirmation of pGhost5 integration

Primer names and respective forward and reverse (5′-3′) sequences are listed. Restriction enzyme recognition sites Kpn*I* and Bam*HI* are underlined. Primer specificities indicate genome regions to which primers anneal. Standard PCR conditions were used for all primer pairs listed. Ligation of *spy0895* N- and C-terminal fragments required the following optimized conditions: 2.25 mM $MgCl_2$, 5 cycles with an annealing temperature of 47°C followed by 35 cycles with an increased annealing temperature of 50°C. Ligation of *spy1536* N- and C-terminal fragments required 2.25 mM $MgCl_2$, 5 cycles with an annealing temperature of 53°C followed by 35 cycles with an increased annealing temperature of 55°C

Name	Primer (forward, reverse)	Specificity
5412	Fw: 5′-ATATAGGTACCTGCCTTCGATCTCTATCTATGCTCG-3′	*spy0895*, N-term
5413	Rev: 5′-TGGCATTCATGTCATCAAAGTTAACATCTCTAGTAAATAGAGGGCAG-3′	*spy0895*, N-term
5414	Fw: 5′-CTGCCCTCTATTTACTAGAGATGTTAACTTTGATGACATGAATGCCA-3′	*spy0895*, C-term
5415	Rev: 5′-TATATGGATCCTCTCTTTATCAACGACTATAACCGAGAT-3′	*spy0895*, C-term
5416	Fw: 5′-ATATAGGTACCCGCTGTCAAGTTAGATATGTTTTATGTA-3′	*Spy1536*, N-term
5417	Rev: 5′-AAATGCCTGGAGGCGCTTACTGGGATTGGCATTGCCTTGAC-3′	*Spy1536*, N-term
5418	Fw: 5′-GTCAAGGCAATGCCAATCCCAGTAAGCGCCTCCAGGCATTT-3′	*Spy1536*, C-term
5419	Rev: 5′-TATATGGATCCTTGAAACCGTCTATTTGATATCAAG-3′	*Spy1536*, C-term
6005	Fw: 5′-ACATGGCGTTAGTTCCCTAAATTTCAGTC-3′	*spy0894*, upstream
6006	Fw: 5′-AGGGTTTTCCCAGTCACGACGTT-3′	pGhost5
6007	Rev: 5′-GAGCGGATAACAATTTCACACAGG-3′	pGhost5
6008	Rev: 5′-TGTACTTATGAGAATTCAACAACTGCATTA-3′	*spy0895*downstream
6009	Fw: 5′-TCCTTTTTGAGAAATATACCTGGAGACTGTT-3′	*spy1536*, upstream
6010	Rev: 5′-CAAACGGTCATCTCGATATTGTTAAAC-3′	*spy1536*,downstream

Table 6: Sequences of primers used for Real Time RT-PCR determinations

Genes that have been identified by microarray analysis as differentially expressed genes are listed with sense and antisense primers for the amplification of 100-150 bp gene specific sequences in Real Time RT-PCR.

ΔSpy0895

Gene	Sense (5'-3')	Antisense (5'-3')
spy0441	GTGCTTGGAGCAACAAATACCC	AAAGTCTCTTTGGTCAATCATGTCC
spy0570	CCACAGCTATTCGAGAATTGCC	GACACCGCTTGTGAATTCTGG
spy1031	GTGCTTTTGGCACTTCAAGTCC	AAGTTATCGGCTACATCGGTGC
spy1691	TGTCCTCCTTGGGCTAATATGG	AAAATAAGGAGCCGTATTGTGACC
spy1718	CTAAACAAGATGACCCAAGCTTCC	TCCAAGAGTAAACCTGCATGCC
spym180822	TTTCATAATTGCTGCCTTGGC	AATATGCCATGGACTCTGGCC
spym49b0400	CTTGAAAGAAGATGCCCTGTGC	AACAAATCCTGTCCTGTATAAATCCC
spym49b0627	TGACAGCTCCATCTGTACAATTCG	CTTCGTTAGCTTTAGCGATTTTACG
spym180822	CCGAGATATCACGAATGGAAGC	TTGTAAGTCGCACGACTTGGC
spym49b0627	TTGAAAGTCCTTTAGTCGGTGTAGC	ATGGCTTCGATAATCACAAGGG

ΔSpy1536

Gene	Sense (5'-3')	Antisense (5'-3')
spy0611	TATCCAAGGTTCAGCTCTTAAAGCTC	AGAATACATCTTCGACTGGAAGAAGC
spy0738	GCGTATAACTTCCGCTACCACC	AGTGTAGCTGAAACAACTCAAGTTGC
spy1642	AGGAACATCTTCCCAAGTAATCCC	ATGGGATGATTGATTGCTGTCC
spy2200	TTAGGAGTTCTTCTAAGGCATTTGG	AAACGTTATCGTTCACCGTTCC
spym49b0963	TTAACATCACTTGTCAATACAGAAATCG	CTCACTGATGATTATTGCACTCGG
spym49b1739	AACACAAGGTGGTAATCAGCTCG	CGTTTCGCAACAGCTACAACC
spym49c1149	CCTTTGGTATCATGCTCACGC	GGCAGAATGTGCTAAGAAAGCG
spym49s0005	TCCAATCTCTTGTTTATACTTGTCGC	TGTAAAAGCTGCTTTGGCAGG
spym49s0078	AACTTGCCGTTATCCACTTTAAGC	GCTTGAGCTGGTTTTCATCAGC
spym49s0079	TAAGCATGAGGAAATAGGTCTGGG	TAACGCTAGAACAGATCGCAACC

Housekeeping genes

Gene	Sense (5'-3')	Antisense (5'-3')
gki	TTTGCCATTGACAATGATGCC	TTACCATCAGCAATAATGCCTCC
mutS	AGTGATGACAATTGCAGTTAAATCG	AAGATTTCTAATGACAGCACGTTAGC
recP	CAGACGGTGTCGAAGCAACC	CAAAGCAAATGTGTAGTGATCAACG

10.2 Appendix II -Vectors

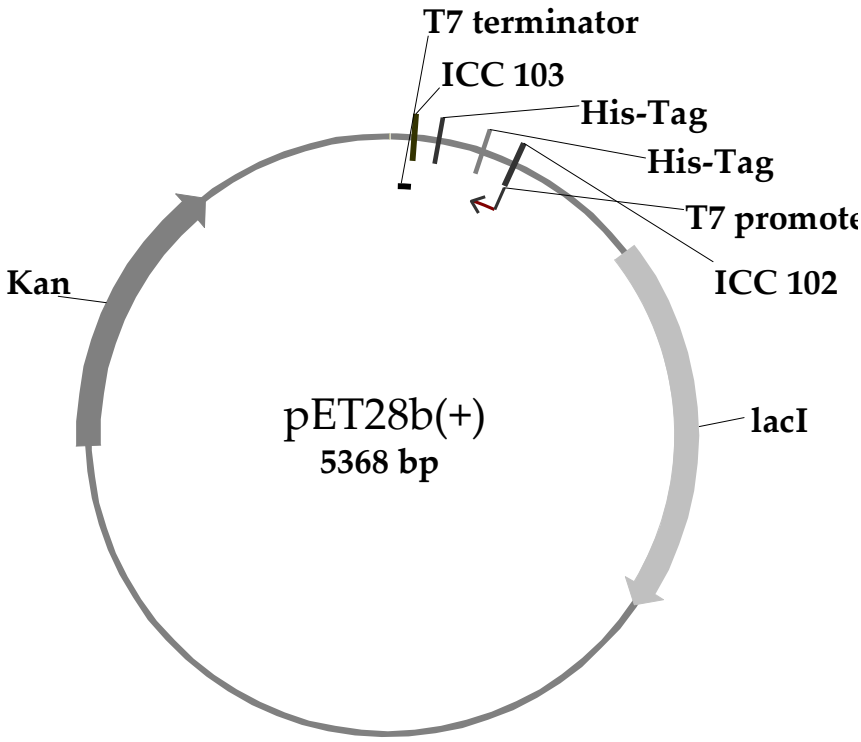

Figure: Expression vector pET28b(+)

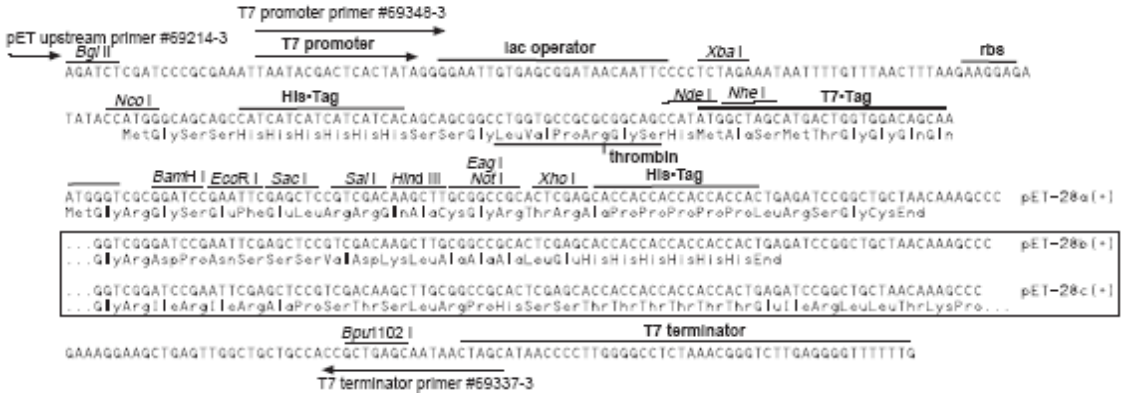

Figure: Cloning/Expression region of pET28b(+)

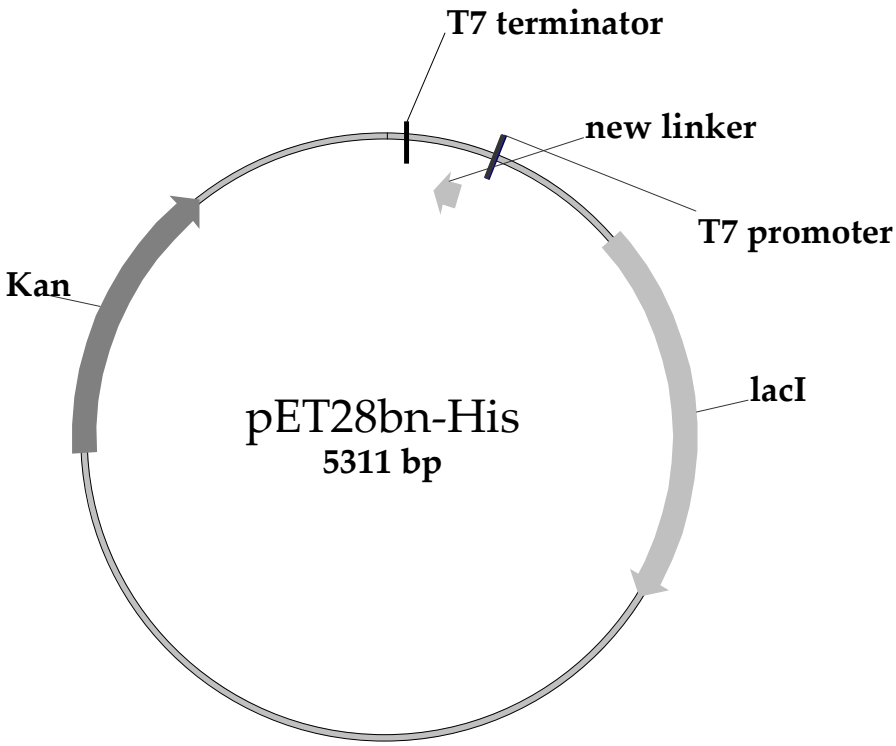

Figure: Expression vector pET28bn-His

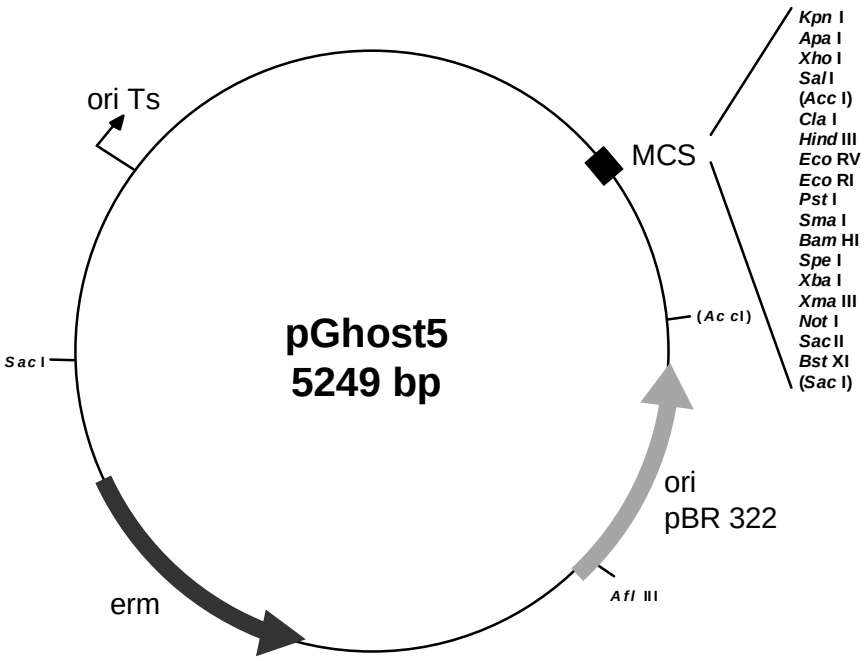

Figure 1: Shuttle vector pGhost5

10.3 Appendix III - Sequences

Spy0416A:

Protein sequence

```
ADELSTMSEPTITNHAQQQAQHLTNTELSSAESKSQDTSQITLKTNREKEQSQDLVSEPTTTELADTDAASMANTGSDA
TQKSASLPPVNTDVHDWVKTKGAWDKGYKGQGKVVAVIDTGIDPAHQSMRISDVSTAKVKSKEDMLARQKAAGINYGSW
INDKVVFAHNYVENSDNIKENQFEDFDEDWENFEFDAEAEPKAIKKHKIYRPQSTQAPKETVIKTEETDGSHDIDWTQT
DDDTKYESHGMHVTGIVAGNSKEAAATGERFLGIAPEAQVMFMRVFANDIMGSAESLFIKAIEDAVALGADVINLSLGT
ANGAQLSGSKPLMEAIEKAKKAGVSVVVAAGNERVYGSDHDDPLATNPDYGLVGSPSTGRTPTSVAAINSKWVIQRLMT
VKELENRADLNHGKAIYSESVDFKDIKDSLGYDKSHQFAYVKESTDAGYNAQDVKGKIALIERDPNKTYDEMIALAKKH
GALGVLIFNNKPGQSNRSMRLTANGMGIPSAFISHEFGKAMSQLNGNGTGSLEFDSVVSKAPSQKGNEMNHFSNWGLTS
DGYLKPDITAPGGDIYSTYNDNHYGSQTGTSMASPQIAGASLLVKQYLEKTQPNLPKEKIADIVKNLLMSNAQIHVNPE
TKTTTSPRQQGAGLLNIDGAVTSGLYVTGKDNYGSISLGNITDTMTFDVTVHNLSNKDKTLRYDTELLTDHVDPQKGRF
TLTSHSLKTYQGGEVTVPANGKVTVRVTMDVSQFTKELTKQMPNGYYLEGFVRFRDSQDDQLNRVNIPFVGFKGQFENL
AVAEESIYRLKSQGKTGFYFDESGPKDDIYVGKHFTGLVTLGSELEHHHHHH*
```

The protein has the HIS tag at both N & C termini and was purified from the soluble fraction over Ni-Agarose column. The theoretical molecular weight of SPy0416A is 94.52 kDa.

SPy0416B

Protein sequence

```
HVDPQKGRFTLTSHSLKTYQGGEVTVPANGKVTVRVTMDVSQFTKELTKQMPNGYYLEGFVRFRDSQDDQLNRVNIPFV
GFKGQFENLAVAEESIYRLKSQGKTGFYFDESGPKDDIYVGKHFTGLVTLGSETNVSTKTISDNGLHTLGTFKNADGKF
ILEKNAQGNPVLAISPNGDNNQDFAAFKGVFLRKYQGLKASVYHASDKEHKNPLWVSPESFKGDKNFNSDIRFAKSTTL
LGTAFSGKSLTGAELPDGHYHYVVSYYPDVVGAKRQEMTFDMILDRQKPVLSQATFDPETNRFKPEPLKDRGLAGVRKD
SVFYLERKDNKPYTVTINDSYKYVSVEDNKTFVERQADGSFILPLDKAKLGDFYYMVEDFAGNVAIAKLGDHLPQTLGK
TPIKLKLTDGNYQTKETLKDNLEMTQSDTGLVTNQAQLAVVHRNQPQSQLTKMNQDFFISPNEDGNKDFVAFKGLKNNV
YNDLTVNVYAKDDHQKQTPIWSSQAGASVSAIESTAWYGITARGSKVMPGDYQYVVTYRDEHGKEHQKQYTISVNDKKP
MITQGRFDTINGVDHFTPDKTKALDSSGIVREEVFYLAKKNGRKFDVTEGKDGITVSDNKVYIPKNPDGSYTISKRDGV
TLSDYYYLVEDRAGNVSFATLRDLKAVGKDKAVVNFGLDLPVPEDKQIVNFTYLVRDADGKPIENLEYYNNSGNSLILP
YGKYTVELLTYDTNAAKLESDKIVSFTLSADNNFQQVTFKITMLATSQITAHFDHLLPEGSRVSLKTAQDQLIPLEQSL
YVPKAYGKTVQEGTYEVVVSLPKGYRIEGNTKVNTLPNEVHELSLRLVKVGDASDP(S)TGDHKVMSKNNSQALTASAT
PTKSTTSATAKALPSTLEHHHHHH*
```

The protein has the HIS tag at both N & C termini. The theoretical molecular weight of SPy0416B is 103.11 kDa.

Spy0416B$_{Fn2Fn3}$

Protein sequence

```
MAFENLAVAEESIYRLKSQGKTGFYFDESGPKDDIYVGKHFTGLVTLGSETNVSTKTISDNGLHTLGTFKNADGKFILE
KNAQGNPVLAISPNGDNNQDFAAFKGVFLRKYQGLKASVYHASDKEHKNPLWVSPESFKGDKNFNSDIRFAKSTTLLGT
AFSGKSLTGAELPDGHYHYVVSYYPDVVGAKRQEMTFDMILDRQKPVLSQATFDPETNRFKPEPLKDRGLAGVRKDSVF
YLERKDNKPYTVTINDSYKYVSVEDNKTFVERQADGSFILPLDKAKLGDFYYMVEDFAGNVAIAKLGDHLPQTLEHHHH
HH*
```

The protein has the HIS tag at its N-terminus. The theoretical molecular weight of Spy0416B$_{Fn2Fn3}$ is 34.8 kDa

Spy0416B$_{Fn2'Fn3'}$

Protein sequence

```
MALGKTPIKLKLTDGNYQTKETLKDNLEMTQSDTGLVTNQAQLAVVHRNQPQSQLTKMNQDFFISPNEDGNKDFVAFKG
LKNNVYNDLTVNVYAKDDHQKQTPIWSSQAGASVSAIESTAWYGITARGSKVMPGDYQYVVTYRDEHGKEHQKQYTISV
NDKKPMITQGRFDTINGVDHFTPDKTKALDSSGIVREEVFYLAKKNGRKFDVTEGKDGITVSDNKVYIPKNPDGSYTIS
KRDGVTLSDYYYLVEDRAGNVSFATLRDLEHHHHHH*
```

The protein has the HIS Tag at its N-terminus. The theoretical molecular weight of Spy0416B$_{Fn2'Fn3'}$ is 28.9 kDa

Spy0416B$_{Fn2'Fn3'Y}$

Protein sequence

```
MALGKTPIKLKLTDGNYQTKETLKDNLEMTQSDTGLVTNQAQLAVVHRNQPQSQLTKMNQDFFISPNEDGNKDFVAFKG
LKNNVYNDLTVNVYAKDDHQKQTPIWSSQAGASVSAIESTAWYGITARGSKVMPGDYQYVVTYRDEHGKEHQKQYTISV
NDKKPMITQGRFDTINGVDHFTPDKTKALDSSGIVREEVFYLAKKNGRKFDVTEGKDGITVSDNKVYIPKNPDGSYTIS
KRDGVTLSDYYYLVEDRAGNVSFATLRDLKAVGKDKAVVNFGLDLPVPEDKQIVNFTYLVRDADGKPIENLEYYNNSGN
SLILPYGKYTVELLTYDTNAAKLESDKIVSFTLSADNNFQQVTFKITMLATSQITAHFDHLLPEGSRVSLKTAQDQLIP
LEQSLYVPKAYGKTVQEGTYEVVVSLPKGYRIEGNTKVNTLPNEVHELSLRLVKVGDASDSTGDHKVMSKNNSQALTAS
ALEHHHHHH*
```

The protein has the HIS tag at its N-terminus. The theoretical molecular weight of Spy0416B$_{Fn2'Fn3'Y}$ is 52 kDa.

Spy0416B$_{Y}$

Protein sequence

```
MALKAVGKDKAVVNFGLDLPVPEDKQIVNFTYLVRDADGKPIENLEYYNNSGNSLILPYGKYTVELLTYDT
NAAKLESDKIVSFTLSADNNFQQVTFKITMLATSQITAHFDHLLPEGSRVSLKTAQDQLIPLEQSLYVPKA
YGKTVQEGTYEVVVSLPKGYRIEGNTKVNTLPNEVHELSLRLVKVGDASDSTGDHKVMSKNNSQALTASAL
EHHHHHH*
```

The protein has the HIS tag at its N-terminus. The theoretical molecular weight of Spy0416B$_{Y}$ is 32.1 kDa.

Spy0416B$_{\Delta Fn1}$

Protein sequence

```
HHHHHHSMAFENLAVAEESIYRLKSQGKTGFYFDESGPKDDIYVGKHFTGLVTLGSETNVSTKTISDNGLHTLGTFKNA
DGKFILEKNAQGNPVLAISPNGDNNQDFAAFKGVFLRKYQGLKASVYHASDKEHKNPLWVSPESFKGDKNFNSDIRFAK
STTLLGTAFSGKSLTGAELPDGHYHYVVSYYPDVVGAKRQEMTFDMILDRQKPVLSQATFDPETNRFKPEPLKDRGLAG
VRKDSVFYLERKDNKPYTVTINDSYKYVSVEDNKTFVERQADGSFILPLDKAKLGDFYYMVEDFAGNVAIAKLGDHLPQ
TLGKTPIKLKLTDGNYQTKETLKDNLEMTQSDTGLVTNQAQLAVVHRNQPQSQLTKMNQDFFISPNEDGNKDFVAFKGL
KNNVYNDLTVNVYAKDDHQKQTPIWSSQAGASVSAIESTAWYGITARGSKVMPGDYQYVVTYRDEHGKEHQKQYTISVN
DKKPMITQGRFDTINGVDHFTPDKTKALDSSGIVREEVFYLAKKNGRKFDVTEGKDGITVSDNKVYIPKNPDGSYTISK
RDGVTLSDYYYLVEDRAGNVSFATLRDLKAVGKDKAVVNFGLDLPVPEDKQIVNFTYLVRDADGKPIENLEYYNNSGNS
LILPYGKYTVELLTYDTNAAKLESDKIVSFTLSADNNFQQVTFKITMLATSQITAHFDHLLPEGSRVSLKTAQDQLIPL
EQSLYVPKAYGKTVQEGTYEVVVSLPKGYRIEGNTKVNTLPNEVHELSLRLVKVGDASDSTGDHKVMSKNNSQALTASA
LE
```

The protein has the HIS tag at its N-terminus. The theoretical molecular weight of Spy0416B$_{\Delta Fn1}$ is 85.9 kDa.

Spy0416A$_{\Delta Fn2}$

Protein sequence

```
MADELSTMSEPTITNHAQQQAQHLTNTELSSAESKSQDTSQITLKTNREKEQSQDLVSEPTTTELADTDAASMANTGSD
ATQKSASLPPVNTDVHDWVKTKGAWDKGYKGQGKVVAVIDTGIDPAHQSMRISDVSTAKVKSKEDMLARQKAAGINYGS
WINDKVVFAHNYVENSDNIKENQFEDFDEDWENFEFDAEAEPKAIKKHKIYRPQSTQAPKETVIKTEETDGSHDIDWTQ
TDDDTKYESHGMHVTGIVAGNSKEAAATGERFLGIAPEAQVMFMRVFANDIMGSAESLFIKAIEDAVALGADVINLSLG
TANGAQLSGSKPLMEAIEKAKKAGVSVVVAAGNERVYGSDHDDPLATNPDYGLVGSPSTGRTPTSVAAINSKWVIQRLM
TVKELENRADLNHGKAIYSESVDFKDIKDSLGYDKSHQFAYVKESTDAGYNAQDVKGKIALIERDPNKTYDEMIALAKK
HGALGVLIFNNKPGQSNRSMRLTANGMGIPSAFISHEFGKAMSQLNGNGTGSLEFDSVVSKAPSQKGNEMNHFSNWGLT
SDGYLKPDITAPGGDIYSTYNDNHYGSQTGTSMASPQIAGASLLVKQYLEKTQPNLPKEKIADIVKNLLMSNAQIHVNP
ETKTTTSPRQQGAGLLNIDGAVTSGLYVTGKDNYGSISLGNITDTMTFDVTVHNLSNKDKTLRYDTELLTDHVDPQKGR
FTLTSHSLKTYQGGEVTVPANGKVTVRVTMDVSQFTKELTKQMPNGYYLEGFVRFRDSQDDQLNRVNIPFVGFKGQLEH
HHHHH*
```

The protein has the HIS tag at its N-terminus. The theoretical molecular weight of Spy0416A$_{\Delta Fn2}$ is 87.3 kDa.

spy0895 Knock Out Construct

Nucleotide sequence

ATATAGGTACCTGCCTTCGATCTCTATCTATGCTCGTGAATTAATCGTTGATTACGGTGTCAAAACATTGATCCGTGTT
GGTACTGCGGGTGCGATTGACCCAGAAGTTCATGTGCGTGAATTGGTGCTTGCCCAAGCAGCAGCAACCAACTCAAATA
TTATCCGTAATGACTTCCCAGAATTTGATTTCCCACAAATTGCTGATTTTGGTTTACTTGACAAGGCTTACCATATCGC
CAGAGAAATGGGAGTAACAACTCACGTTGGGAATGTGCTTTCTTCTGATGTCTTTTACACTAACATGCCTGAGCGTAAT
ATGGCTCTTGGTAAATTGGGTGTTAAAGCCATCGAAATGGAAGCAGCAGCTCTTTACTATTTAGCTGCTCAGCACCATG
TCAAAGCCCTTGGAATCATGACGATCTCAGATAACTTGAATGATCCAACCGAAGATACTACAGCTGAAGAGCGCCAAAC
AACTTTCACTGATATGATGAAGGTCGGCTTGGAAACCTTAATTGCCAATGACTAATAATCAAACACTAGACATCCTTTT
GGATGTCTATGCTTATAATCACGCCTTTAGAATTGCTAAAGCCTTGCCAAATATCCCTAAAACTGCCCTCTATTTACTA
GAGATGTTAACTTTGATGACATGAATGCCATTATTAAAAAGGAGTTGAGCCTTTGGAGACAATCTATTGTCTGATATTT
ATCAAAAAACACGATAACTTGACCGATACAGGTCTTACTACCGTGTTTTTTAGTATGACGATTGAAAAAGACATTAATC
AAAGGTTACTTCTTCGAGAAGGTATTCGATAAAACGTTCCCCCATTTTGGACAAGTTGGCTTTTTCATGCTTGATAAAA
ACAATGTCAATTTCGTCAGGGACATCTAGCGGGATAGCCACAATCTGGTCACCATTTAGATTGCTATTTAGAATACCAC
TGGCAACGGTGTAGCCATCTAAACCAATCATTAAGTTAAAGAGGGTGGCACGGTCACTGACCACAATTGATTTGTTGTG
GGGCATCTGGGACATCATCTCTTCTGAAAAATAAAAGGAGTTGTGAATCCCTTGGTCATAGCTCAGGTAAGGAAAATCT
CTCAAGTCGTCCATGCTTAATAGGCTTTTAGTTGCTAGAGGATTTGATTTGCTGACAAAAATATGAGGCTGTGCCTTAA
ATAATGGAGAGGCGGTCAAATGATTGTCATCAAATAATTTGGTCAAGACATCTCGGTTATAGTCGTTGATAAAGAGAGG
ATCCATATA

The underlined nucleotides show restriction enzyme recognition sites Kpn*I* and Bam*HI,* respectively. Sequence highlighted in grey represents the sequence of the N-terminal region of *spy0895*. The C-terminal *spy0895* region is the non-highlighted sequence.

spy1536 Knock Out Construct

Nucleotide sequence

ATATAGGTACCCGCTGTCAAGTTAGATATGTTTTATGTAAAAAATGATTGAACCCAAAAGACTGGTCAAGGATCTGTCT
ATCTGGTTCAACCGTTTTTTATGAGTTAATAGCGTGAATATAATTGAATATAATACATTCATAGTTATTTTATTTGCGA
AGATAAACCAGTGCCTCTTGAACAGTCGTAACAGGAACAATCTTCATTTTGGTCTTTAGTCGTTTGGCTGCCCGTTTGG
CTTCTTCGTAATTACTTATAGCATTTGGATTAACTTTTTTAATTTCCTTATCAACAGGATTATTCGGAACAAAAAATAT
ATCTGCACCAGCTTCAGCTGCTGCAACTACTTTAAGACCTGCACCACCAATATCTCCTACTTCGCCATCCTTGCCAATA
GTTCCTGTACCTGCAATTGTACGGCCCTTGCGTAAATCTTCTTTAGTTATTTGATCATATATATCAAGAGTAAACATTA
GACCAGCACTAGGTCCTCCTACTCCTTTAGTACTAAAGATCACTGTGTCTTCTGAATTGACACTTGTATGATCAGTCAA
GGCAATGCCAATCCCAGTAAGCGCCTCCAGGCATTTCAATATAATAAGGTAGCGGAAAAAATAACGCTAGCAACAAAGA
GATTAAAGCTAGCAGACCCACTAACCACCATTTGATTTTTTTAAGTCTTTTCATTCATTTTCTCCACTTGAGCAATAAC
CGACTGAGGAACCAAACCTTCTAAGGAAGACTGAAAATGAATTAGCTCTCTCACACGACTAGAGCTAAGGGCCTGCCAT
TTATTTCTTGATATCAAATAGACGGTTTCAAGGATCCATATA

The underlined nucleotides show restriction enzyme recognition sites Kpn*I* and Bam*HI,* respectively. Sequence highlighted in grey represents the sequence of the N-terminal region of *spy1536.* The C-terminal *spy1536* region is the non-highlighted sequence.

Sequence alignments of *spy0895* gene deletion in strain ΔSpy0895

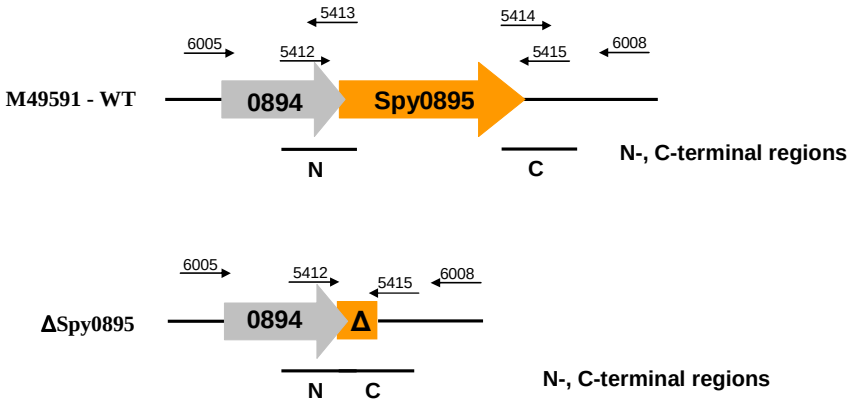

Figure: Region view of spy0895 operon in wildtype and spy0895 gene deletion mutant with a schematic representation of primer localization used for sequencing

Sequence alignment *spy0895* gene deletion mutant (deletion highlighted in yellow)

```
                    1673                                                                                                1772
    Spy0895 K.O.  (1673) GTGGAAAGCTCGGAAACTAGATAAACTAACTTTTGAGCTCACACATGGCGTTAGTTCCCTAAATTTCAGTCGCTTTCTGTCCGCCCTCTGACCTTCTACA
   0895K.O.-5412    (1) ----------------------------------------------------------------------------------------------------
0895K.O.-5415rev    (1) ----------------------------------------------------------------------------------------------------
0895K.O.-6008rev    (1) ----------------------------------------------------------------------------------------------------
   0895K.O.-6005    (1) ----------------------------------------------------------------------------------------------------
        WT-0895    (73) GTGGAAAGCTCGGAAACTAGATAAACTAACTTTTGAGCTCACACATGGCGTTAGTTCCCTAAATTTCAGTCGCTTTCTGTCCGCCCTCTGACCTTCTACA
        WT-5412    (1) ----------------------------------------------------------------------------------------------------
        WT-5414    (1) ----------------------------------------------------------------------------------------------------
        WT-6005    (1) ----------------------------------------------------------------------------------------------------
     WT-5413rev    (1) ----------------------------------------------------------------------------------------------------
     WT-5415rev    (1) ----------------------------------------------------------------------------------------------------
     WT-6008rev    (1) ----------------------------------------------------------------------------------------------------
       Consensus  (1673)
                    1773                                                                                                1872
    Spy0895 K.O.  (1773) AAATAAAAGGAAAGGTAGGGCTAACTGTATTCATTGAATTGAATACGGGCGGAGAACTGTTTTTCTTCCTTATAAAAAATAAAAAAATAAGAAAGGTGAG
   0895K.O.-5412    (1) ----------------------------------------------------------------------------------------------------
0895K.O.-5415rev    (1) ----------------------------------------------------------------------------------------------------
0895K.O.-6008rev    (1) ----------------------------------------------------------------------------------------------------
   0895K.O.-6005    (1) ----------------------------------------------------------------------------------------------------CT
        WT-0895   (173) AAATAAAAGGAAAGGTAGGGCTAACTGTATTCATTGAATTGAATACGGGCGGAGAACTGTTTTTCTTCCTTATAAAAAATAAAAAAATAAGAAAGGTGAG
        WT-5412    (1) ----------------------------------------------------------------------------------------------------
        WT-5414    (1) ----------------------------------------------------------------------------------------------------
        WT-6005    (1) ----------------------------------------------------------------------------------------------------
     WT-5413rev    (1) ----------------------------------------------------------------------------------------------------
     WT-5415rev    (1) ----------------------------------------------------------------------------------------------------
     WT-6008rev    (1) ----------------------------------------------------------------------------------------------------
       Consensus  (1773)
                    1873                                                                                                1972
    Spy0895 K.O.  (1873) GGCTGAGTTCATTAATTTGAATTCGGGCTAAGGACTTGGTCAAAAAGATAAGTTGTCTAGAAAATCAGGATTTTCTGCCAACTTCCTATTTTGCCATCGT
   0895K.O.-5412    (1) ----------------------------------------------------------------------------------------------------
0895K.O.-5415rev    (1) ----------------------------------------------------------------------------------------------------
0895K.O.-6008rev    (1) ----------------------------------------------------------------------------------------------------
   0895K.O.-6005    (3) AACTGTATTCATTGAATTGAATACGGGCGGAGAACTGTGTAAAAAAGATAAACTGTCTAGCATCTG-GGATGCGTCGTCAGTTTCCTATTTTTACTTTGT
        WT-0895   (273) GGCTGAGTTCATTAATTTGAATTCGGGCTAAGGACTTGGTCAAAAAGATAAGTTGTCTAGAAAATCAGGATTTTCTGCCAACTTCCTATTTTGCCATCGT
        WT-5412    (1) ----------------------------------------------------------------------------------------------------
        WT-5414    (1) ----------------------------------------------------------------------------------------------------
        WT-6005    (1) AACTGTATTCATTGAATTGAATACGGGCGGAGAACTGTGTAAAAAAGATAAACTGTCTAGCATCTG-GGATGCGTCGTCAGTTTCCTATTTTTACTTTGT
     WT-5413rev    (1) ----------------------------------------AAAAAGATAAACTGTCTAGCATCTG-GGATGCGTCGTCAGTTTCCTATTTTTACTTTGT
     WT-5415rev    (1) ----------------------------------------------------------------------------------------------------
     WT-6008rev    (1) ----------------------------------------------------------------------------------------------------
       Consensus  (1873)
                    1973                                                                                                2072
    Spy0895 K.O.  (1973) TCTTTTAACGCCCTCAGTATCATAATTTATGTCTATCCATATTTCTGCCAAAAAAGGCGATATTGCTGATAAAATTCTTCTACCTGGAGATCCTTTGCGC
   0895K.O.-5412    (1) ----------------------------------------------------------------------------------------------------
0895K.O.-5415rev    (1) ----------------------------------------------------------------------------------------------------
0895K.O.-6008rev    (1) ----------------------------------------------------------------------------------------------------
   0895K.O.-6005  (102) TCTTTGT-CGCCTTTAATATCTTAATA-ATGTCTATCCATATTTCTGCCAAAAAAGGTGATATTGCTGATAAAATTCTTCTACCTGGAGACCCTTTGCGC
        WT-0895  (373) TCTTTTAACGCCCTCAGTATCATAATTTATGTCTATCCATATTTCTGCCAAAAAAGGCGATATTGCTGATAAAATTCTTCTACCTGGAGATCCTTTGCGC
        WT-5412    (1) ----------------------------------------------------------------------------------------------------
        WT-5414    (1) ----------------------------------------------------------------------------------------------------
        WT-6005  (100) TCTTTGT-CGCCTTTAATATCTTAATA-ATGTCTATCCATATTTCTGCCAAAAAAGGTGATATTGCTGATAAAATTCTTCTACCTGGAGACCCTTTGCGC
     WT-5413rev   (59) TCTTTGT-CGCCTTTAATATCTTAATA-ATGTCTATCCATATTTCTGCCAAAAAAGGTGATATTGCTGATAAAATTCTTCTACCTGGAGACCCTTTGCGC
     WT-5415rev    (1) ----------------------------------------------------------------------------------------------------
     WT-6008rev    (1) ----------------------------------------------------------------------------------------------------
       Consensus  (1973)
                    2073                                                                                                2172
    Spy0895 K.O.  (2073) GCGAAATTTATCGCTGAAAATTTCCTAGAAGATGCAGTGTGTTTTAACGAAGTGCGTAACATGTTTGGCTACACAGGAACTTACAAAGGGCACCGCGTTT
   0895K.O.-5412    (1) ----------------------------------------------------------------------------------------------------
0895K.O.-5415rev    (1) ----------------------------------------------------------------------------------------------------
0895K.O.-6008rev    (1) ----------------------------------------------------------------------------------------------------
   0895K.O.-6005  (200) GCGAAATTTATCGCTGAAAATTTCCTAGAAGATGCAGTGTGTTTTAACGAAGTGCGTAACATGTTTGGCTACACAGGAACTTACAAAGGGCACCGCGTTT
        WT-0895  (473) GCGAAATTTATCGCTGAAAATTTCCTAGAAGATGCAGTGTGTTTTAACGAAGTGCGTAACATGTTTGGCTACACAGGAACTTACAAAGGGCACCGCGTTT
        WT-5412    (1) ----------------------------------------------------------------------------------------------------
        WT-5414    (1) ----------------------------------------------------------------------------------------------------
        WT-6005  (198) GCGAAATTTATCGCTGAAAATTTCCTAGAAGATGCAGTGTGTTTTAACGAAGTGCGTAACATGTTTGGCTACACAGGAACTTACAAAGGGCACCGCGTTT
     WT-5413rev  (157) GCGAAATTTATCGCTGAAAATTTCCTAGAAGATGCAGTGTGTTTTAACGAAGTGCGTAACATGTTTGGCTACACAGGAACTTACAAAGGGCACCGCGTTT
     WT-5415rev    (1) ----------------------------------------------------------------------------------------------------
     WT-6008rev    (1) ----------------------------------------------------------------------------------------------------
       Consensus  (2073)
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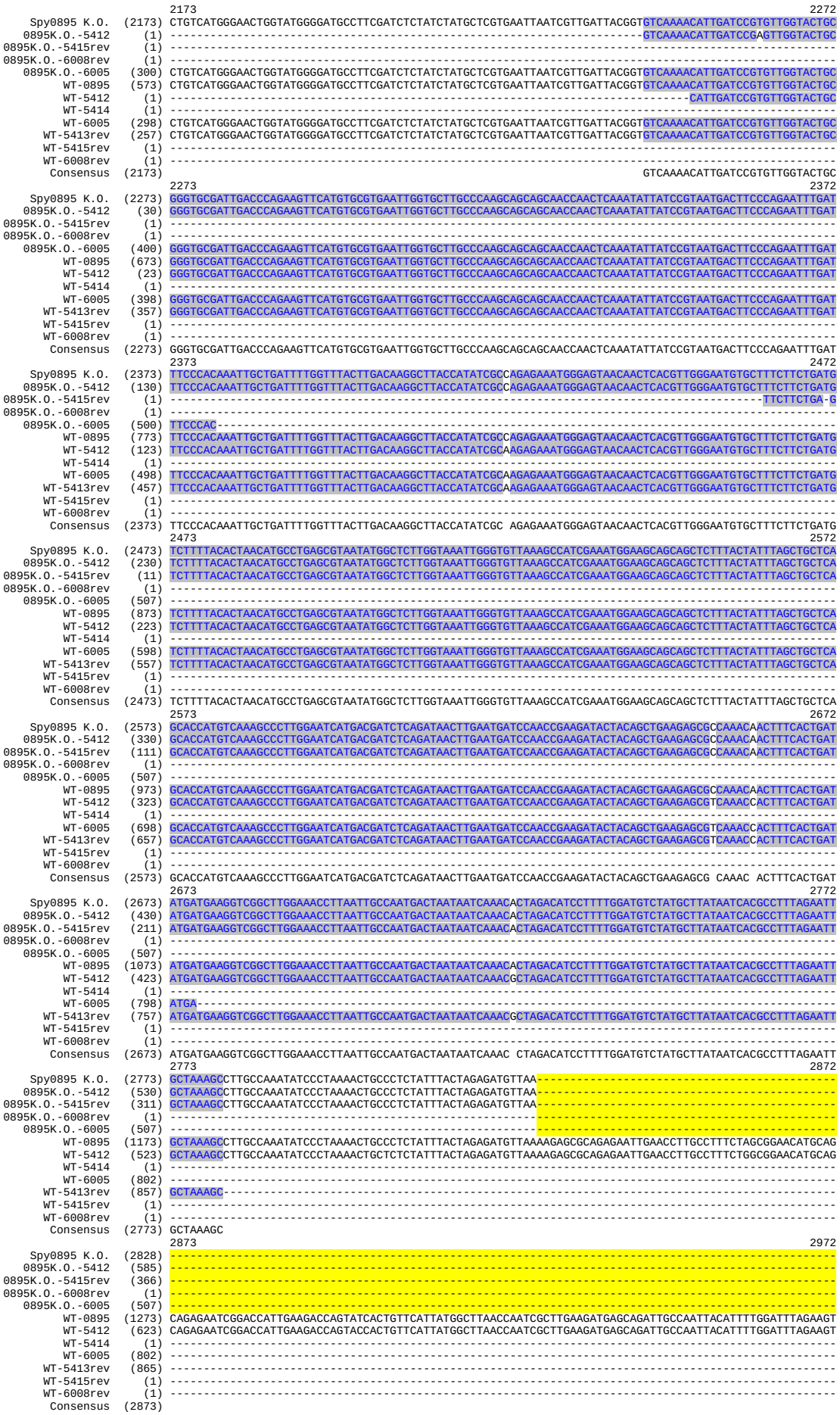

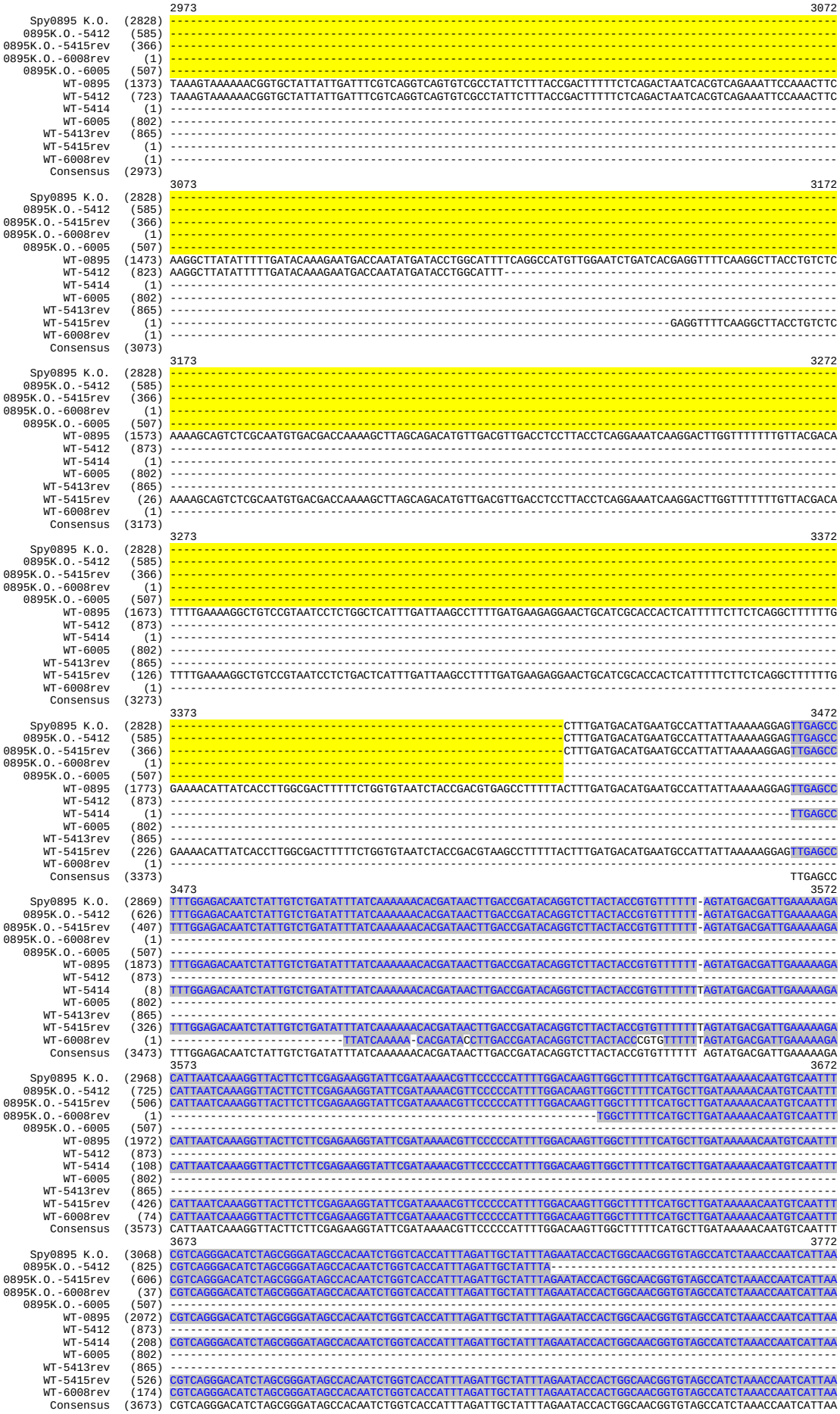

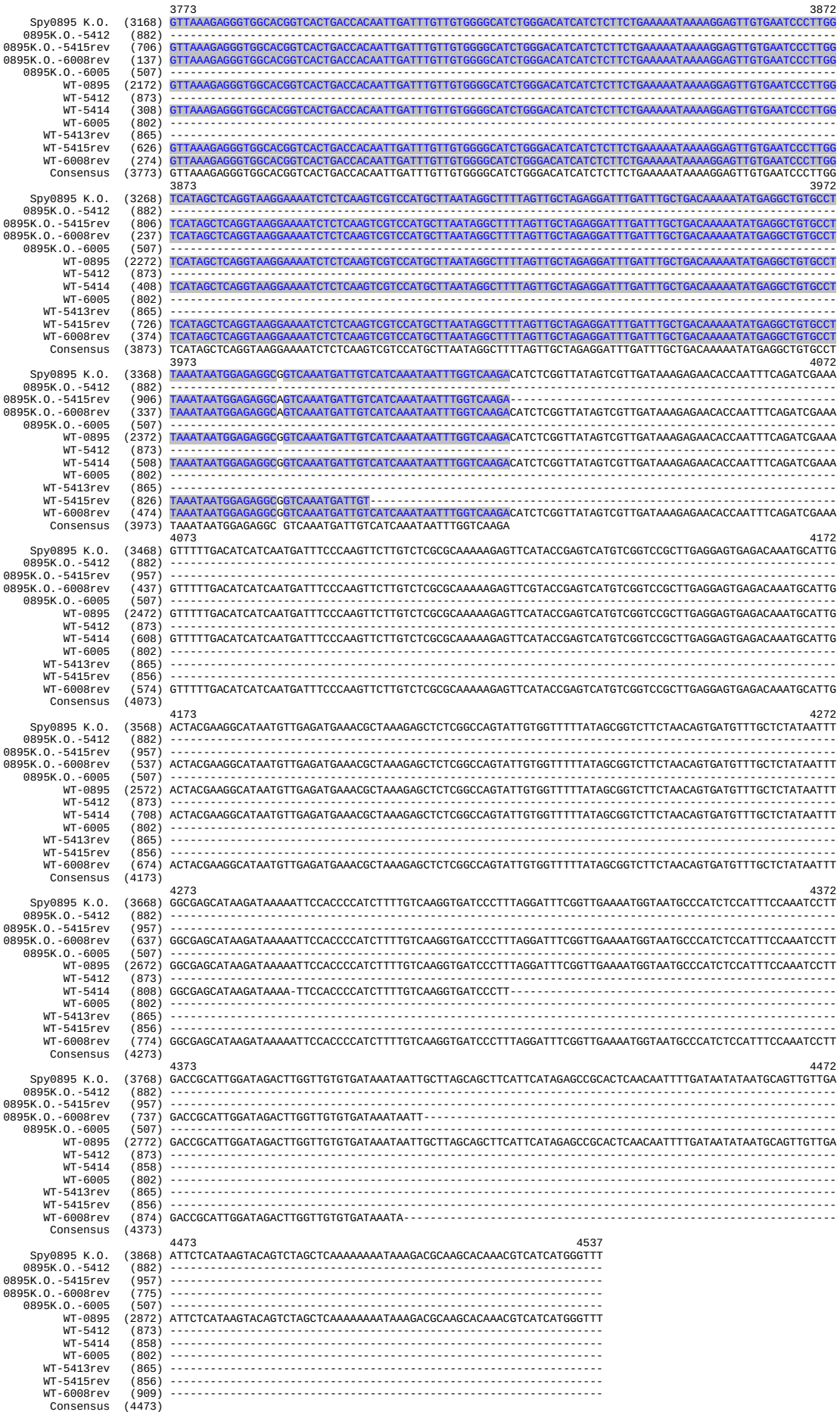
3773 3872
Spy0895 K.O. (3168) GTTAAAGAGGGTGGCACGGTCACTGACCACAATTGATTTGTTGTGGGGCATCTGGGACATCATCTCTTCTGAAAAATAAAAGGAGTTGTGAATCCCTTGG
0895K.O.-5412 (882) ----------
0895K.O.-5415rev (706) GTTAAAGAGGGTGGCACGGTCACTGACCACAATTGATTTGTTGTGGGGCATCTGGGACATCATCTCTTCTGAAAAATAAAAGGAGTTGTGAATCCCTTGG
0895K.O.-6008rev (137) GTTAAAGAGGGTGGCACGGTCACTGACCACAATTGATTTGTTGTGGGGCATCTGGGACATCATCTCTTCTGAAAAATAAAAGGAGTTGTGAATCCCTTGG
0895K.O.-6005 (507) ----------
WT-0895 (2172) GTTAAAGAGGGTGGCACGGTCACTGACCACAATTGATTTGTTGTGGGGCATCTGGGACATCATCTCTTCTGAAAAATAAAAGGAGTTGTGAATCCCTTGG
WT-5412 (873) ----------
WT-5414 (308) GTTAAAGAGGGTGGCACGGTCACTGACCACAATTGATTTGTTGTGGGGCATCTGGGACATCATCTCTTCTGAAAAATAAAAGGAGTTGTGAATCCCTTGG
WT-6005 (802) ----------
WT-5413rev (865) ----------
WT-5415rev (626) GTTAAAGAGGGTGGCACGGTCACTGACCACAATTGATTTGTTGTGGGGCATCTGGGACATCATCTCTTCTGAAAAATAAAAGGAGTTGTGAATCCCTTGG
WT-6008rev (274) GTTAAAGAGGGTGGCACGGTCACTGACCACAATTGATTTGTTGTGGGGCATCTGGGACATCATCTCTTCTGAAAAATAAAAGGAGTTGTGAATCCCTTGG
Consensus (3773) GTTAAAGAGGGTGGCACGGTCACTGACCACAATTGATTTGTTGTGGGGCATCTGGGACATCATCTCTTCTGAAAAATAAAAGGAGTTGTGAATCCCTTGG
3873 3972
Spy0895 K.O. (3268) TCATAGCTCAGGTAAGGAAAATCTCTCAAGTCGTCCATGCTTAATAGGCTTTTAGTTGCTAGAGGATTTGATTTGCTGACAAAAATATGAGGCTGTGCCT
0895K.O.-5412 (882) ----------
0895K.O.-5415rev (806) TCATAGCTCAGGTAAGGAAAATCTCTCAAGTCGTCCATGCTTAATAGGCTTTTAGTTGCTAGAGGATTTGATTTGCTGACAAAAATATGAGGCTGTGCCT
0895K.O.-6008rev (237) TCATAGCTCAGGTAAGGAAAATCTCTCAAGTCGTCCATGCTTAATAGGCTTTTAGTTGCTAGAGGATTTGATTTGCTGACAAAAATATGAGGCTGTGCCT
0895K.O.-6005 (507) ----------
WT-0895 (2272) TCATAGCTCAGGTAAGGAAAATCTCTCAAGTCGTCCATGCTTAATAGGCTTTTAGTTGCTAGAGGATTTGATTTGCTGACAAAAATATGAGGCTGTGCCT
WT-5412 (873) ----------
WT-5414 (408) TCATAGCTCAGGTAAGGAAAATCTCTCAAGTCGTCCATGCTTAATAGGCTTTTAGTTGCTAGAGGATTTGATTTGCTGACAAAAATATGAGGCTGTGCCT
WT-6005 (802) ----------
WT-5413rev (865) ----------
WT-5415rev (726) TCATAGCTCAGGTAAGGAAAATCTCTCAAGTCGTCCATGCTTAATAGGCTTTTAGTTGCTAGAGGATTTGATTTGCTGACAAAAATATGAGGCTGTGCCT
WT-6008rev (374) TCATAGCTCAGGTAAGGAAAATCTCTCAAGTCGTCCATGCTTAATAGGCTTTTAGTTGCTAGAGGATTTGATTTGCTGACAAAAATATGAGGCTGTGCCT
Consensus (3873) TCATAGCTCAGGTAAGGAAAATCTCTCAAGTCGTCCATGCTTAATAGGCTTTTAGTTGCTAGAGGATTTGATTTGCTGACAAAAATATGAGGCTGTGCCT
3973 4072
Spy0895 K.O. (3368) TAAATAATGGAGAGGCGGTCAAATGATTGTCATCAAATAATTTGGTCAAGACATCTCGGTTATAGTCGTTGATAAAGAGAACACCAATTTCAGATCGAAA
0895K.O.-5412 (882) ----------
0895K.O.-5415rev (906) TAAATAATGGAGAGGCAGTCAAATGATTGTCATCAAATAATTTGGTCAAGA----------
0895K.O.-6008rev (337) TAAATAATGGAGAGGCAGTCAAATGATTGTCATCAAATAATTTGGTCAAGACATCTCGGTTATAGTCGTTGATAAAGAGAACACCAATTTCAGATCGAAA
0895K.O.-6005 (507) ----------
WT-0895 (2372) TAAATAATGGAGAGGCGGTCAAATGATTGTCATCAAATAATTTGGTCAAGACATCTCGGTTATAGTCGTTGATAAAGAGAACACCAATTTCAGATCGAAA
WT-5412 (873) ----------
WT-5414 (508) TAAATAATGGAGAGGCGGTCAAATGATTGTCATCAAATAATTTGGTCAAGACATCTCGGTTATAGTCGTTGATAAAGAGAACACCAATTTCAGATCGAAA
WT-6005 (802) ----------
WT-5413rev (865) ----------
WT-5415rev (826) TAAATAATGGAGAGGCGGTCAAATGATTGT----------
WT-6008rev (474) TAAATAATGGAGAGGCGGTCAAATGATTGTCATCAAATAATTTGGTCAAGACATCTCGGTTATAGTCGTTGATAAAGAGAACACCAATTTCAGATCGAAA
Consensus (3973) TAAATAATGGAGAGGC GTCAAATGATTGTCATCAAATAATTTGGTCAAGA
4073 4172
Spy0895 K.O. (3468) GTTTTTGACATCATCAATGATTTCCCAAGTTCTTGTCTCGCGCAAAAAGAGTTCATACCGAGTCATGTCGGTCCGCTTGAGGAGTGAGACAAATGCATTG
0895K.O.-5412 (882) ----------
0895K.O.-5415rev (957) ----------
0895K.O.-6008rev (437) GTTTTTGACATCATCAATGATTTCCCAAGTTCTTGTCTCGCGCAAAAAGAGTTCGTACCGAGTCATGTCGGTCCGCTTGAGGAGTGAGACAAATGCATTG
0895K.O.-6005 (507) ----------
WT-0895 (2472) GTTTTTGACATCATCAATGATTTCCCAAGTTCTTGTCTCGCGCAAAAAGAGTTCATACCGAGTCATGTCGGTCCGCTTGAGGAGTGAGACAAATGCATTG
WT-5412 (873) ----------
WT-5414 (608) GTTTTTGACATCATCAATGATTTCCCAAGTTCTTGTCTCGCGCAAAAAGAGTTCATACCGAGTCATGTCGGTCCGCTTGAGGAGTGAGACAAATGCATTG
WT-6005 (802) ----------
WT-5413rev (865) ----------
WT-5415rev (856) ----------
WT-6008rev (574) GTTTTTGACATCATCAATGATTTCCCAAGTTCTTGTCTCGCGCAAAAAGAGTTCATACCGAGTCATGTCGGTCCGCTTGAGGAGTGAGACAAATGCATTG
Consensus (4073)
4173 4272
Spy0895 K.O. (3568) ACTACGAAGGCATAATGTTGAGATGAAACGCTAAAGAGCTCTCGGCCAGTATTGTGGTTTTTATAGCGGTCTTCTAACAGTGATGTTTGCTCTATAATTT
0895K.O.-5412 (882) ----------
0895K.O.-5415rev (957) ----------
0895K.O.-6008rev (537) ACTACGAAGGCATAATGTTGAGATGAAACGCTAAAGAGCTCTCGGCCAGTATTGTGGTTTTTATAGCGGTCTTCTAACAGTGATGTTTGCTCTATAATTT
0895K.O.-6005 (507) ----------
WT-0895 (2572) ACTACGAAGGCATAATGTTGAGATGAAACGCTAAAGAGCTCTCGGCCAGTATTGTGGTTTTTATAGCGGTCTTCTAACAGTGATGTTTGCTCTATAATTT
WT-5412 (873) ----------
WT-5414 (708) ACTACGAAGGCATAATGTTGAGATGAAACGCTAAAGAGCTCTCGGCCAGTATTGTGGTTTTTATAGCGGTCTTCTAACAGTGATGTTTGCTCTATAATTT
WT-6005 (802) ----------
WT-5413rev (865) ----------
WT-5415rev (856) ----------
WT-6008rev (674) ACTACGAAGGCATAATGTTGAGATGAAACGCTAAAGAGCTCTCGGCCAGTATTGTGGTTTTTATAGCGGTCTTCTAACAGTGATGTTTGCTCTATAATTT
Consensus (4173)
4273 4372
Spy0895 K.O. (3668) GGCGAGCATAAGATAAAAATTCCACCCCATCTTTTGTCAAGGTGATCCCTTTAGGATTTCGGTTGAAAATGGTAATGCCCATCTCCATTTCCAAATCCTT
0895K.O.-5412 (882) ----------
0895K.O.-5415rev (957) ----------
0895K.O.-6008rev (637) GGCGAGCATAAGATAAAAATTCCACCCCATCTTTTGTCAAGGTGATCCCTTTAGGATTTCGGTTGAAAATGGTAATGCCCATCTCCATTTCCAAATCCTT
0895K.O.-6005 (507) ----------
WT-0895 (2672) GGCGAGCATAAGATAAAAATTCCACCCCATCTTTTGTCAAGGTGATCCCTTTAGGATTTCGGTTGAAAATGGTAATGCCCATCTCCATTTCCAAATCCTT
WT-5412 (873) ----------
WT-5414 (808) GGCGAGCATAAGATAAAA-TTCCACCCCATCTTTTGTCAAGGTGATCCCTT----------
WT-6005 (802) ----------
WT-5413rev (865) ----------
WT-5415rev (856) ----------
WT-6008rev (774) GGCGAGCATAAGATAAAAATTCCACCCCATCTTTTGTCAAGGTGATCCCTTTAGGATTTCGGTTGAAAATGGTAATGCCCATCTCCATTTCCAAATCCTT
Consensus (4273)
4373 4472
Spy0895 K.O. (3768) GACCGCATTGGATAGACTTGGTTGTGTGATAAATAATTGCTTAGCAGCTTCATTCATAGAGCCGCACTCAACAATTTTGATAATATAATGCAGTTGTTGA
0895K.O.-5412 (882) ----------
0895K.O.-5415rev (957) ----------
0895K.O.-6008rev (737) GACCGCATTGGATAGACTTGGTTGTGTGATAAATAATT----------
0895K.O.-6005 (507) ----------
WT-0895 (2772) GACCGCATTGGATAGACTTGGTTGTGTGATAAATAATTGCTTAGCAGCTTCATTCATAGAGCCGCACTCAACAATTTTGATAATATAATGCAGTTGTTGA
WT-5412 (873) ----------
WT-5414 (858) ----------
WT-6005 (802) ----------
WT-5413rev (865) ----------
WT-5415rev (856) ----------
WT-6008rev (874) GACCGCATTGGATAGACTTGGTTGTGTGATAAATA----------
Consensus (4373)
4473 4537
Spy0895 K.O. (3868) ATTCTCATAAGTACAGTCTAGCTCAAAAAAAATAAAGACGCAAGCACAAACGTCATCATGGGTTT
0895K.O.-5412 (882) ----------
0895K.O.-5415rev (957) ----------
0895K.O.-6008rev (775) ----------
0895K.O.-6005 (507) ----------
WT-0895 (2872) ATTCTCATAAGTACAGTCTAGCTCAAAAAAAATAAAGACGCAAGCACAAACGTCATCATGGGTTT
WT-5412 (873) ----------
WT-5414 (858) ----------
WT-6005 (802) ----------
WT-5413rev (865) ----------
WT-5415rev (856) ----------
WT-6008rev (909) ----------
Consensus (4473)

Sequence alignments of *spy1536* gene deletion in strain ΔSpy1536

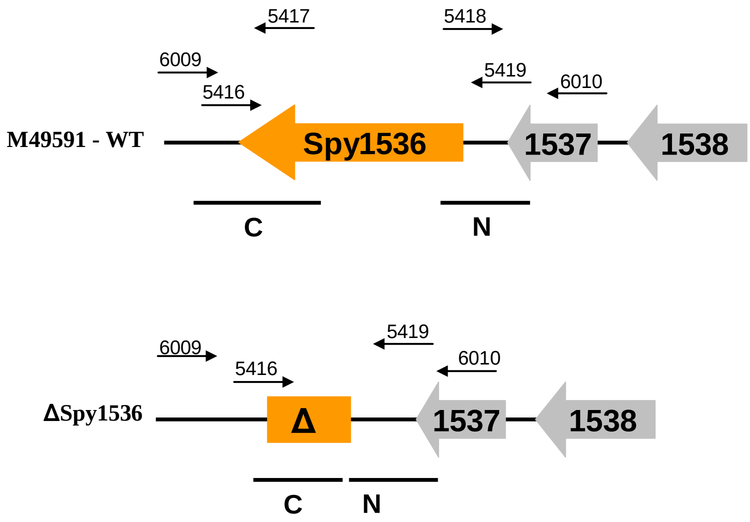

Figure: Region view of *spy1536* operon in wildtype and *spy1536* gene deletion mutant with a schematic representation of primer localization used for sequencing

Sequence alignment *spy1536* gene deletion mutant (deletion highlighted in yellow)

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                         269                                                                                                368
   Spy1536 K.O.    (269) CTTGTTTAATGGTTACCATAGCCTTACCTCCTTGTAAAATAAGTTTATGGGGATTTTTCAATCTCAAGTTTTTAATTTAGTTGTTATAACGTTAACTTGT
   1536K.O.-5416     (1) ----------------------------------------------------------------------------------------------------
   1536K.O.-6009     (1) ----TTTAATGGTTACCATAGCCTTACCTCCTTGTAAAATAAGTTTATGGGGATTTTTCAATCTCAAGTTTTTAATTTAGTTGTTATAACGTTAACTTGT
1536K.O.-5419rev     (1) ----------------------------------------------------------------------------------------------------
1536K.O.-6010rev     (1) ----------------------------------------------------------------------------------------------------
         WT-1536   (169) CTTGTTTAATGGTTACCATAGCCTTACCTCCTTGTAAAATAAGTTTATGGGGATTTTTCAATCTCAAGTTTTTAATTTAGTTGTTATAACGTTAACTTGT
         WT-5416     (1) ----------------------------------------------------------------------------------------------------
      WT-5417rev     (1) --------------------------------TGTAAAATAAGTTTATGGGGATTTTTCAATCTCAAGTTTTTAATTTAGTTGTTATAACGTTAGCTTGT
         WT-5418     (1) ----------------------------------------------------------------------------------------------------
      WT-5419rev     (1) ----------------------------------------------------------------------------------------------------
         WT-6009     (1) ---GTTTAATGGTTACCATAGCCTTACCTCCTTGTAAAATAAGTTTATGGGGATTTTTCAATCTCAAGTTTTTAATTTAGTTGTTATAACGTTAGCTTGT
      WT-6010rev     (1) ----------------------------------------------------------------------------------------------------
       Consensus   (269)
                         369                                                                                                468
   Spy1536 K.O.    (369) CTATTGCAAGGCTTTCCAGAATAATAGAGTCATGGAATGGACTCATGATTATCTGCGCAAGTTCTTGGATGAGCTAGTGAAAAAAATCAATTTTCACTAG
   1536K.O.-5416     (1) ----------------------------------------------------------------------------------------------------
   1536K.O.-6009    (97) CTATTGCAAGGCTTTCCAGAATAATAGAGTCATGGAATGGACTCATGATTATCTGCGCAAGTTCTTGGATGAGCTAGTGAAAAAAATCAATTTTCACTAG
1536K.O.-5419rev     (1) -------------------------------------------------------------TTCTTGGATGAGCTAGTGAAAAAAATCAATTTTCACTAG
1536K.O.-6010rev     (1) ----------------------------------------------------------------------------------------------------
         WT-1536   (269) CTATTGCAAGGCTTTCCAGAATAATAGAGTCATGGAATGGACTCATGATTATCTGCGCAAGTTCTTGGATGAGCTAGTGAAAAAAATCAATTTTCACTAG
         WT-5416     (1) ----------------------------------------------------------------------------------------------------
      WT-5417rev    (69) CTATTGCAAGGCTTTCCAGAATAATAGAGTCATGGAATGGACTCATGATTATCTGCGCAAGTTCTTGGATGAGCTAGTGAAAAAAATCAATTTTCACTAG
         WT-5418     (1) ----------------------------------------------------------------------------------------------------
      WT-5419rev     (1) ----------------------------------------------------------------------------------------------------
         WT-6009    (98) CTATTGCAAGGCTTTCCAGAATAATAGAGTCATGGAATGGACTCATGATTATCTGCGCAAGTTCTTGGATGAGCTAGTGAAAAAAATCAATTTTCACTAG
      WT-6010rev     (1) ----------------------------------------------------------------------------------------------------
       Consensus   (369)                                                              TTCTTGGATGAGCTAGTGAAAAAAATCAATTTTCACTAG
                         469                                                                                                568
   Spy1536 K.O.    (469) GTGTTTTTAGTATGCTCTATTTCCAGATAATCGACTAAAGTTTACTCAATTCTCTATTGCCTTTTTACC-ACTAGACTTTTATAACATCTTTTTTACGCT
   1536K.O.-5416     (1) ----------------------------------------------------------------------------------------------------
   1536K.O.-6009   (197) GTGTTTTTAGTATGCTCTATTTCCAGATAATCGACTAAAGTTTACTCAATTCTCTATTGCCTTTTTACC-ACTAGACTTTTATAACATCTTTTTTACGCT
1536K.O.-5419rev    (40) GTGTTTTTAGTATGCTCTATTTCCAGATAATCGACTAAAGTTTACTCAATTCTCTATTGCCTTTTTACCCACTAGACTTTTATAACATCTTTTTTACGCT
1536K.O.-6010rev     (1) ----------------------------------------------------------------------------------------------------
         WT-1536   (369) GTGTTTTTAGTATGCTCTATTTCCAGATAATCGACTAAAGTTTACTCAATTCTCTATTGCCTTTTTACC-ACTAGACTTTTATAACATCTTTTTTACGCT
         WT-5416     (1) ----------------------------------------------------------------------------------------------------
      WT-5417rev   (169) GTGTTTTTAGTATGCTCTATTTCCAGATAATCGACTAAAGTTTACTCAATTCTCTATTGCCTTTTTACC-ACTAGACTTTTATAAAATCTTTTTTACGCT
         WT-5418     (1) ----------------------------------------------------------------------------------------------------
      WT-5419rev     (1) ----------------------------------------------------------------------------------------------------
         WT-6009   (198) GTGTTTTTAGTATGCTCTATTTCCAGATAATCGACTAAAGTTTACTCAATTCTCTATTGCCTTTTTACC-ACTAGACTTTTATAAAATCTTTTTTACGCT
      WT-6010rev     (1) ----------------------------------------------------------------------------------------------------
       Consensus   (469) GTGTTTTTAGTATGCTCTATTTCCAGATAATCGACTAAAGTTTACTCAATTCTCTATTGCCTTTTTACC ACTAGACTTTTATAA ATCTTTTTTACGCT
                         569                                                                                                668
   Spy1536 K.O.    (568) GTCAAGTTAGATATGTTTTATGTAAAAAATGATTGAACCCAAAAGACTGGTCAAGGATCTGTCTATCTGGTTCAACCGTTTTTTATGAGTTAATAGCGTG
   1536K.O.-5416     (1) -------------------------------------------AGACTGGTCAAGGATCTGTCTATCTGGTTCAACCGTTTTTTATGAGTTAATAGCGTG
   1536K.O.-6009   (296) GTCAAGTTAGATATGTTTTATGTAAAAAATGATTGAACCCAAAAGACTGGTCAAGGATCTGTCTATCTGGTTCAACCGTTTTTTATGAGTTAATAGCGTG
1536K.O.-5419rev   (140) GTCAAGTTAGATATGTTTTATGTAAAAAATGATTGAACCCAAAAGACTGGTCAAGGATCTGTCTATCTGGTTCAACCGTTTTTTATGAGTTAATAGCGTG
1536K.O.-6010rev     (1) ----------------------------------------------------------------------------------------------------
         WT-1536   (468) GTCAAGTTAGATATGTTTTATGTAAAAAATGATTGAACCCAAAAGACTGGTCAAGGATCTGTCTATCTGGTTCAACCGTTTTTTATGAGTTAATAGCGTG
         WT-5416     (1) --------------------------------------------GACTGGTCAAGGATCTGTCTATCTGGTTCAACCGTTTTTTATGAGTTAATAGCGTG
      WT-5417rev   (268) GTCAAGTTAGATATGTTTTATGTAAAAAATGATTGAACCCAAAAGACTGGTCAAGGATCTGTCTATCTGGTTCAACCGTTTTTTATGAGTTAATAGCGTG
         WT-5418     (1) ----------------------------------------------------------------------------------------------------
      WT-5419rev     (1) ----------------------------------------------------------------------------------------------------
         WT-6009   (297) GTCAAGTTAGATATGTTTTATGTAAAAAATGATTGAACCCAAAAGACTGGTCAAGGATCTGTCTATCTGGTTCAACCGTTTTTTATGAGTTAATAGCGTG
      WT-6010rev     (1) ----------------------------------------------------------------------------------------------------
       Consensus   (569) GTCAAGTTAGATATGTTTTATGTAAAAAATGATTGAACCCAAAAGACTGGTCAAGGATCTGTCTATCTGGTTCAACCGTTTTTTATGAGTTAATAGCGTG
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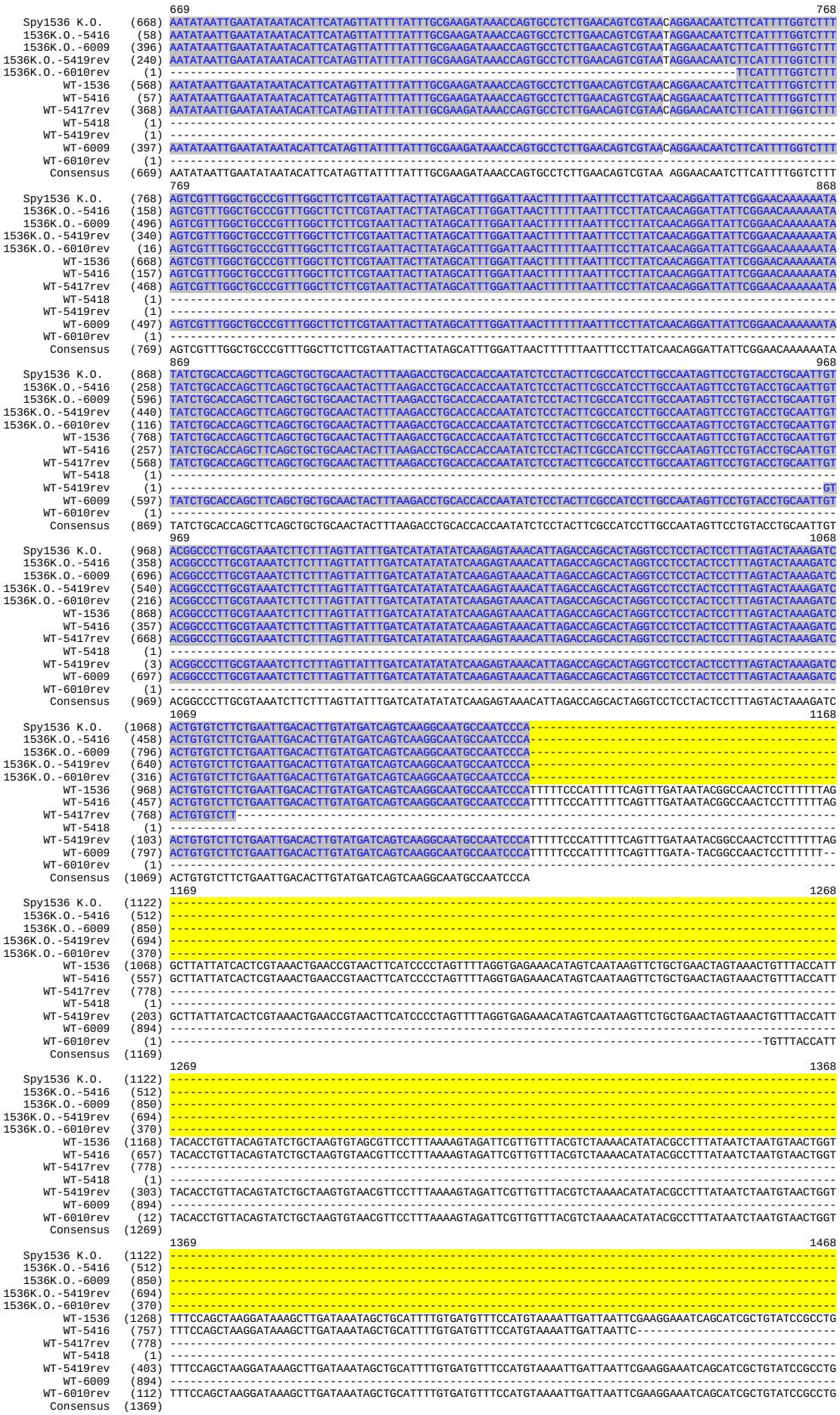

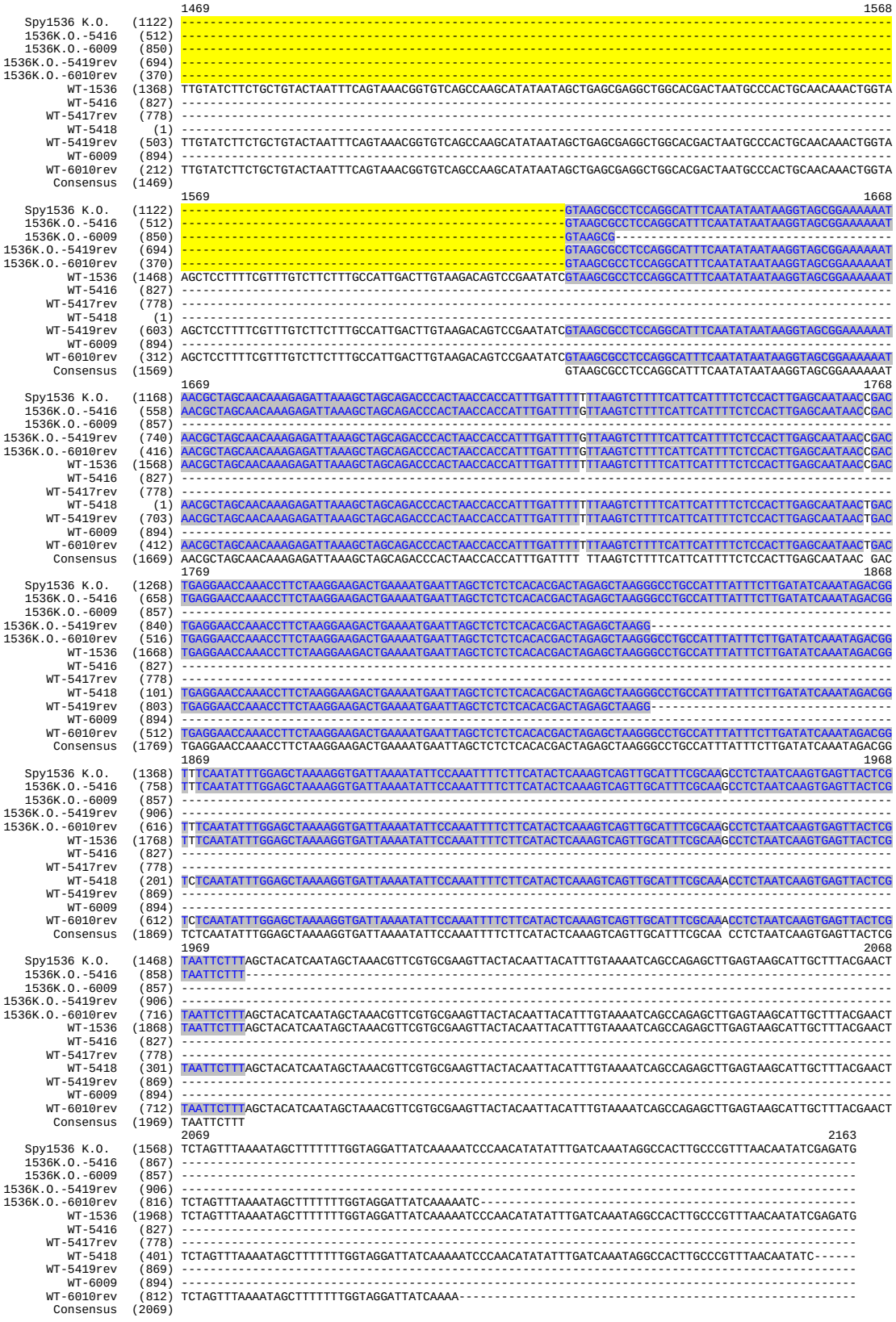

11 CURRICULUM VITAE

Personal information

Name	Andrea Fritzer
Place of birth	9020 Klagenfurt, Austria
Date of birth	20th January 1983
Nationality	Austrian

Education and training

1989-1993	Elementary school, St. Martin, 9560 Feldkirchen, Austria
1993-2001	Secondary school, Bundesrealgymnasium, 9560 Feldkirchen, Austria
2001-2003	Study Biology at the University of Vienna
2003-2006	Studies of Genetic and Microbiology at the University of Vienna
11.01.05-26.06.05	Study at University of Lund
2005-2006	Diploma thesis "Characterization of protective antigens from the human pathogen *Streptococcus pyogenes*" at Intercell AG, Campus Vienna Biocenter 3, 1030 Vienna, Austria with Dr. Andreas Meinke and supervisor Prof. Dr. Alexander von Gabain
16.01.2006	Diploma examination (for degree)
2006-2009	Ph.D.-thesis "Characterization of protective antigens of *Streptococcus pyogenes* and their contribution to virulence" at Intercell AG, Campus Vienna Biocenter 3, 1030 Vienna, Austria

	with Dr. Andreas Meinke and supervisor Prof. Dr. Alexander von Gabain
2008	Part of the Organization Committee of VBC Student Symposium II Campus Vienna Biocenter, Vienna, Austria
08.02.09-28.02-09	Microarray analysis at University of Rostock Medical Faculty Inst. of Med. Microbiology, Virology and Hygiene Dept. of Med. Microbiology and Hospital Hygiene in Dr. Bernd Kreikemeyer's group, Schillingallee 70, 18055 Rostock, Germany

12 THESIS-RELATED PUBLICATIONS

12.1 Publications

Fritzer A, Noiges B, Schweiger D, Rek A, Kungl A.J, von Gabain A, Nagy E and Meinke A.L. **Chemokine degradation by the Group A streptococcal serine proteinase ScpC can be reconstituted in vitro and requires two separate domains.** 2009. Biochemical Journal 422(3):533-542

Fritzer A, Senn B.M, Bui Minh D, Hanner M, Gelbmann D, Noiges B, Henics T, Schulze K, Guzman C, Goodacre J, von Gabain A, Nagy E and Meinke A.L. **Novel protective candidates selected by the ANTIGENome technology to prevent group A streptococcal infections.** Manuscript in preparation

12.2 Poster and Oral Presentations at Scientific Conferences

A. Fritzer, B. Noiges, A. Rek, A. Kungl, A. von Gabain, E. Nagy and Meinke A.L.
Two separate domains are required for Il-8 degradation by a serine proteinase from group A streptococcus. Poster presentation at *Semmering Conference 2007, Baden, Austria*

A. Fritzer, B. Noiges, A. Rek, A. Kungl, A. von Gabain, E. Nagy and Meinke A.L.
Two separate domains are required for Il-8 degradation by a serine proteinase from group A streptococcus. Oral presentation at *VBC Student Symposium 2007, Vienna, Austria*

A. Fritzer, A. von Gabain, E. Nagy and Meinke A.L.
Characterization of protective antigens from *Streptococcus pyogenes* and their contribution to virulence. Poster presentation at *XVII Lancefield International Symposium on Streptococci and Streptococcal Diseases, Porto Heli, Greece*.

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