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Global analysis of immune evasion strategies in *Staphylococcus aureus* clinical isolates

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1 Transparency statements

This study was sponsored by GSK, which was involved in all stages of the study conduct and analysis. Tarcisio Brignoli is a PhD student of the University of Bologna and supervised by GSK.

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A manuscript containing the same results shown in this thesis is currently in preparation.

2 Abstract

Staphylococcus aureus is a major human pathogen, responsible for a wide range of diseases. Its remarkable ability to develop resistance to antibiotics made *S. aureus* a worldwide issue in clinical medicine. One of the causes of its success as a pathogen is the peculiar array of immune evasion factors that enable the bacterium to avoid host defenses. Among these factors, the staphylococcal protein A (SpA), is thought to have a crucial role in staphylococcal immune evasion thanks to its IgG-binding activities. Indeed, SpA is able to bind the Fc region of IgG, hence preventing the recognition of the Fc by the host immune system and allowing escape from antibody-mediated neutrophil phagocytosis. Moreover SpA can also bind the V_H3 domain of B cell receptors acting as a superantigen and thus leading to an impairment of the B cell response. With the intent of determining the prevalence of SpA expression in staphylococcus isolates, we screened a large panel of strains for SpA expression. Interestingly, in about 7% of the isolates, SpA was not detectable by Western blot despite the presence of the gene. Of note, the strains lacking SpA expression (SpA^- strains) are mainly associated with the USA100/CC5 lineages, which are responsible for Hospital Acquired (HA) infections. The analysis of the genomes of the SpA^- strains revealed that the loss of SpA expression may have more than one genetic basis, since only a subset of SpA^- isolates carried a conserved mutation in the *spA* 5'UTR sequence. The analysis of transcript levels of more than 90 virulence factors showed a unique feature in SpA^- strains, in that a higher capsule biosynthesis operon (*cap* operon) mRNA was identified along with lower *spA* transcripts. The negative correlation between *spA* and *cap* operon transcripts is shared by strains coming from distant geographic origins, thus indicating this as a common adaptation in SpA^- strains. The analysis of the regulatory network controlling *spA* and *cap* operon transcription highlights how a number of factors contribute to the balance of these two virulence factors and their mutually exclusive expression. The difference in *cap* operon RNA levels was reflected in the amount of capsule produced, which is significantly higher in the SpA^- strains. We then investigated the ability of anti-capsule antibodies to induce phagocytic uptake by neutrophils, which resulted in specific internalization only in the SpA negative background.

Capsule and SpA are both important immune evasion factors that prevent opsonophagocytosis. Given their inversely regulated expression, we hypothesize that while both implicated in protection from opsonophagocytosis, the relevance of balancing the roles

of SpA and capsule in immune evasion is of utmost importance in *S. aureus* isolates. Moreover, the expression profile of capsule and SpA among different *S. aureus* isolates makes them good therapies target if used in combination.

3 Introduction

3.1 Disease and pathology

Staphylococcus aureus (Fig.1) is a Gram-positive bacterium that colonizes the human nares and skin [1, 2]. Despite the fact that about 30% of the population carries *S. aureus* asymptomatically [2], it is a frequent cause of opportunistic infections that lead to a huge variety of diseases, ranging from skin and soft tissue infection to infective endocarditis and bacteremia [3]. Of note, *S. aureus* is one of the leading causes of both hospital and community acquired infections [4-6]. The mortality rate of staphylococcal bacteremia in the US is approximately 2 to 10 deaths annually per 100,000 population and is higher than mortality rates of AIDS, tuberculosis and viral hepatitis, and comparable to that of breast and prostate cancer [7]. The elevated incidence of staphylococcal infections in hospital settings (above all, staphylococcal bacteremia) has been attributed to several factors, for example the breaching of the skin or the mucosa upon surgical intervention allows the pathogen to overcome the external body barrier, while its ability to form biofilm *S. aureus* particularly adept at infecting foreign bodies within the human host, such as vascular and urinary catheters, prosthetic cardiac valves or prosthetic joint devices [3]. The remarkable versatility of *S. aureus* renders it is also one of the major causes of community (CA) acquired infections, where it is able to infect otherwise healthy individuals not associated with any predisposing risk factor. CA infections are usually associated with strains with distinct genetic background from health care associated (HA) strains, and are characterized by enhanced virulence [8-10]. Besides the multiple pathology types and the different settings in which they are involved, staphylococcal infections are an increasing concern in human health because of antibiotic resistance. During the past decades indeed we witnessed the emergence of *S. aureus* strains resistant to vast classes of antibiotics, rendering staphylococcal infection increasingly harder to treat[11].

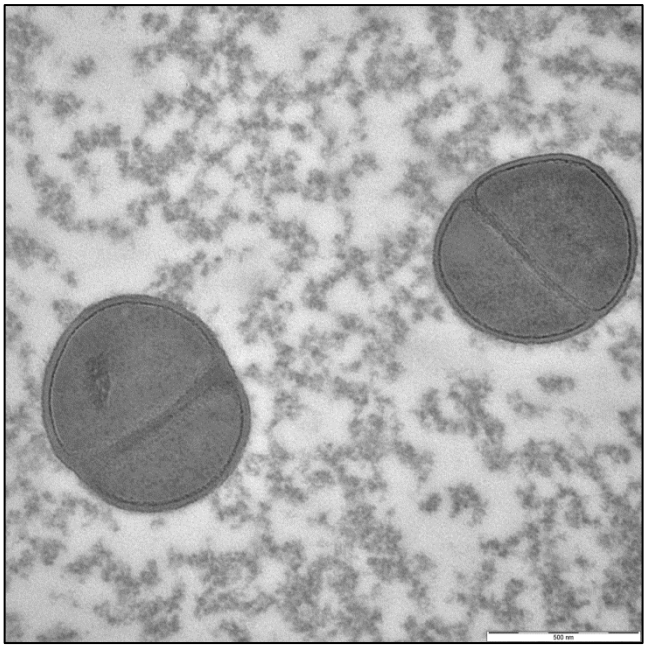

Figure 1. Electron microscopy image of *Staphylococcus aureus*

3.2 Antibiotic resistance

Since the introduction of penicillin in the 1940s, *S. aureus* has shown a remarkable ability to gain new antibiotic resistance. The emergence of new antibiotic resistant strains can be seen as series of waves (Fig.2), as result of the introduction of newly discovered antimicrobial compounds [11]. The first wave started in the mid-1940s, with the emergence of penicillin resistant *S. aureus* strains in USA hospitals. From the hospitals those strains rapidly spread causing community acquired infections, and by the end of 1950s they were pandemic. The mechanism of resistance was based on a plasmid encoded penicillase that lead to the inactivation of the antibiotic. The introduction of methicillin lead to the disappearance of the infections due to the most relevant penicillin resistant clone, but already in 1961 the first methicillin resistant *S. aureus* (MRSA) strains were identified. These first MRSA strains were limited to European hospitals with sporadic reports in USA, without spreading to the community or the rest of the world. The mechanism of this resistance was not associated with drug inactivation, but it conferred protection to a broad set of antibiotics: the entire class of β-lactam including penicillins, cephalosporins, and carbapenems. Later, the mechanism was associated with a specific gene *mecA* which encodes for the low affinity penicillin binding protein PBP2a. By the 1980s the infections determined by those archaic MRSA strains waned in Europe and from the late 1970s, outbreaks of MRSA strains were reported in the US and spread into the hospitals, a pandemic that continues to present times. Given the rising emergence of methicillin resistant clones, vancomycin was increasingly used as reliably antimicrobial in *S. aureus* infections. This selective pressure led to the emergence of two distinct mechanisms of resistance. In 1996 the first Vancomycin Intermediate *Staphylococcus aureus* strain (VISA) was isolated in Japan [12]. This clone was characterized by a vancomycin minimum inhibitory concentration of 4-8 μg ml^{-1} determined by an increased thickness of the cell wall able to block antibiotic molecules. The VISA strains are not associated to the acquisition of specific antibiotic resistance genes, but the phenotype is reached through a step wise process in which several mutations are accumulated in the genome, particularly in cell wall metabolism regulatory genes [13]. In 2002, shortly after the first VISA report, also vancomycin resistant strains (VRSA) emerged [14]. VRSA strains are associated to the acquisition of *vanA* gene, which confers resistance up to 32 μg ml^{-1} of

vancomycin. So far VISA strains are mostly associated with health care settings, while VRSA strains are extremely rare.

Almost in the same period of the emergence of VISA strains, increasing numbers of MRSA infections were reported to be community acquired (CA-MRSA) and not associated to nosocomial settings. Those strains show distinctive characteristics when compared to HA-MRSA, like a different pulsed field gel electrophoresis pattern and the susceptibility to most antibiotics other than β-lactams, indicating that their evolution occurred in a separate way. Moreover, while the HA-MRSA strains were unable to disseminate in the community setting, CA-MRSA strains disseminated also in the health care setting. The reasons for this difference reside in peculiar virulence ability and diverse pathogenesis, like the greater ability to spread by skin to skin contact [11, 15, 16]. The characterization by multilocus sequence type (MLST) and Pulsed field gel electrophoresis (PFGE) indicates that nowdays the most common lineage of HA MRSA in United states is Clonal complex 5 (ST5) and USA100 PFGE type, while the predominant CA MRSA clone belongs to Clonal complex 8 (ST8) and USA300 PFGE type.

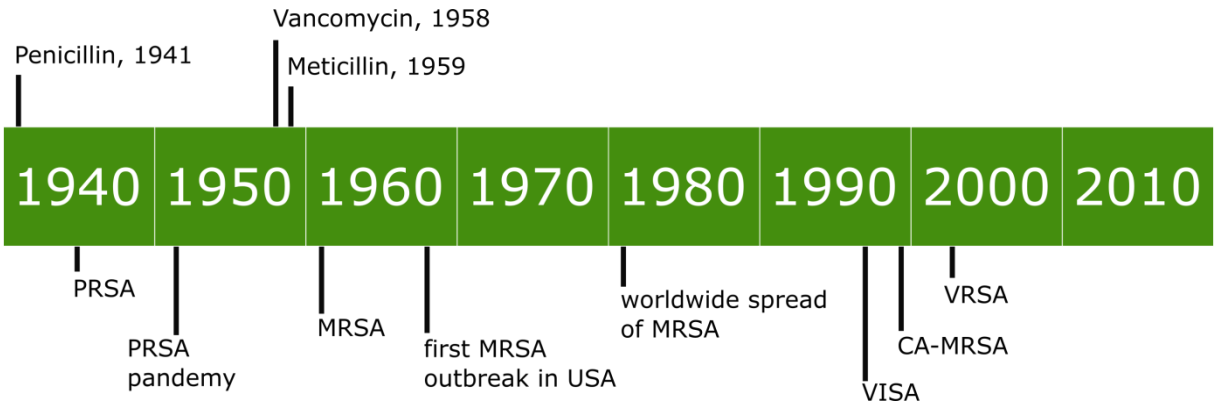

Figure 2 Emergence of antibiotic resistance in time. The panel shows the timeline of the introduction of new antibiotics in medical use, and the following emergence of antibiotic resistant strains. Figure adapted from *Chambers et al.2009* and *McGuinness et al. 2017* [11, 17]

3.3 Staphylococcus aureus virulence factors

The ability to act both as a commensal or a pathogen and the ability to cause different types of infection exemplifies the versatile nature of *Staphylococcus aureus*. One of the reasons for its success in its multi-faced physiology reside in its unique array of virulence and immune evasion factors that allows the bacterium to invade and escape host defenses (Fig.3). These include factors that help the bacterium in the different environments encountered during commensal colonization and the different stages of infection, like adhesion factors, factors able to block complement cascade, impair neutrophils chemotaxis, inhibit opsonophagocytosis and kill immune host cells [18-22]. In the complex scenario of a staphylococcal infection, it is not uncommon that the same factor is responsible for more than one function, or that the same functions are carried out by several proteins. Although the principal role of the various virulence factors may not be the same, it is interesting to note that *S. aureus* employs multiple strategies to accomplish one single task. Considering this redundancy it is likely that none of these factors is strictly necessary for virulence. In fact, infection-related clinical isolates may naturally be deficient in a range of these factors [23, 24]. Furthermore, animal model studies comparing virulence of isogenic single mutants show attenuated but not completely abolished ability to infect [25-27].

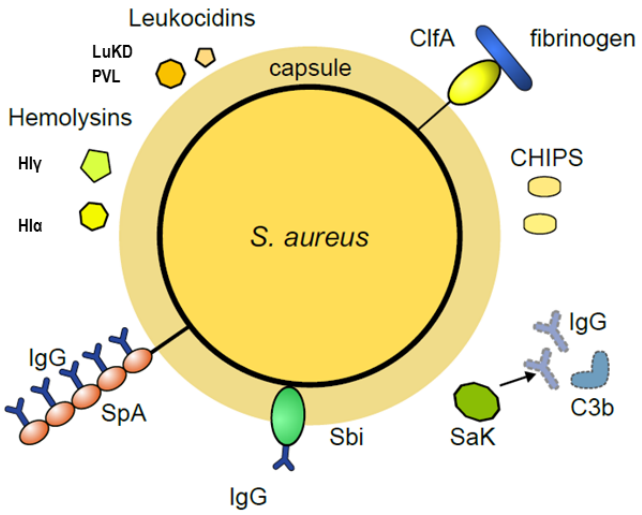

Figure 3 Schematic representation of major staphylococcal virulence factors. Staphylococcal virulence determinants include secreted and surface attached factors, involved in adhesion, immune evasion and tissue damage. These factors often exert several functions, which are frequently redundant.

3.3.1 Cell Wall Anchored proteins

S. aureus can express up to 24 surface proteins covalently bound to the cell wall, known as Cell Wall Anchored (CWA) proteins. Those proteins have secretory signals in the N terminus, which directs the precursor to the secretory system in the cell membrane, and a characteristic sorting signal containing the LPXTG motif in the C terminus, required for the anchorage to the cell wall. The CWA family can be divided in several major groups of proteins, based on structural and functional similarities [28]. The microbial surface component recognizing adhesive matrix molecules (MSCRAMM) is the most represented group of CWA.

Clumping factor A (ClfA) is the archetype of the MSCRAMM family, and is the major staphylococcal fibrinogen-binding protein. The ClfA N-terminus is able to bind the γ-chain located in the C-terminus of fibrinogen, which is formed of two specular tripeptide chains connected at the N-terminus [29-31]. The same fibrinogen molecule can be bound by two ClfA molecules coming from different bacterial cells, which determines cells clumping *in vitro* [31]. *In vivo* bacterial cells are coated with fibrinogen molecules, which impair opsonin deposition and phagocytosis [32]. Furthermore, ClfA can bind to complement factor I, which results in the cleavage of C3b to inactive iC3b [33, 34], impairing complement cascade and complement-mediated phagocytosis[35].

Another fibrinogen binding protein that belongs to the MSCRAMM family is Clumping factor B (ClfB) [36]. Differently to ClfA, it binds to α- and β- chains of fibrinogen [37], as well as cytokeratin 10 [38]. ClfB was shown to promote adherence to human keratinocyte [39], and it has a major role *S. aureus* nasal carriage [40, 41]. The different role of ClfA and ClfB is underlined by their reported different expression profile in *in vitro* growth: where *clfB* is mainly transcribed in the exponential phase of growth, *clfA* is upregulated during stationary phase [42].

A class of CWA proteins is characterized by the presence of the Near iron transporter (NEAT) motif, which can bind hemoglobin or haem [28]. The Iron surface determinants (Isd) A,B,H proteins can harbor from one to three NEAT motifs, and are involved in iron acquisition within the host, were it is limited [43]. Haem is bound on the cellular surface and then transported in to the cytoplasm, where iron is extracted. The CWA Isd proteins have several other functions: IsdA binds several host protein including cytokeratin 10, promoting nasal

colonization [44], IsdB was shown to contribute to adherence and internalization by non-phagocytic human cells [45], while IsdH enhances the inactivation of C3b [46].

3.3.1.1 Staphylococcal protein A

Staphylococcal protein A (SpA) is one of the major virulence factors of *Staphylococcus aureus*. SpA is a 45-60 k Da protein that can be cell wall associated or secreted [47, 48]. It is composed by three regions: the IgG binding domain in the N terminus, the X variable region and the peptidoglycan tail in the C terminus (Fig.4) [48-50]. The IgG binding region is composed normally by five homologous domains (called EABCD), each one folds into triple α helical bundles each responsible for two main binding activities, such as the Fcγ portion of human IgM, IgD and class I, II and IV IgGs[51-53], the V_H3 portion of human B cell receptor [52, 54, 55], tumor necrosis factor 1[56], epithelial growth factor receptor [57] and Von Willebrand Factor [58, 59]. The X region divides the IgG binding domains from the cell surface and it is composed by an Xr variable region and the constant region Xc. The Xr region is formed by highly variable numbers of octapeptide repeats, and its sequencing can be used as typing method for staphylococcal strains [60]. The constant Xc region contains the LPXGT motif sorting signal, that is needed for cell-wall anchoring [61]. When the protein is released from cell surface it presents an attached peptidoglycan tail of variable length, which has been shown to be relevant in the immunological functions of the protein [62].

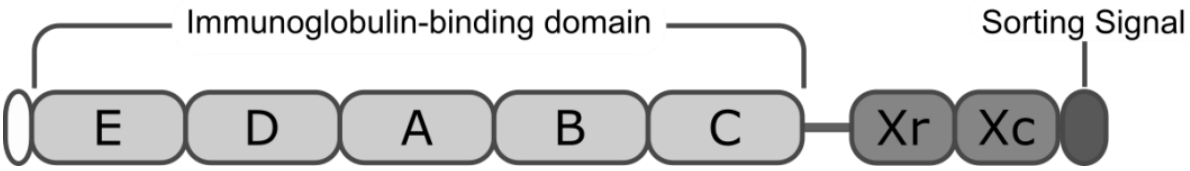

Figure 4 Schematic representation of staphylococcal protein A. In white, at the N-terminus the signal sequence, followed by five IgG binding domains, then the repetitive Xr region and the X constant region. At the C-terminus the sorting signal.

SpA plays a central role in the multilayered phenomenon which is staphylococcal immune evasion, exerting several functions. The Fc binding activity allows SpA to sequester the antibodies and to display them with the wrong orientation, determining a reduced antibody dependent phagocytosis [63, 64]. The combination of Fc binding activity and the Fab binding of V_H3, is thought to determine immune complexes that affect Neutrophil recruitment and complement activation [65].

Through V_H3 Fab binding SpA can act also as a B cell superantigen[66], activating B cells in a non-specific manner [67]. The results of this B cell activation are not clear yet. Initial studies suggested a role in B cell apoptosis [68], while further studies showed that SpA leads to B cell expansion and antibody expression [62].

The inhibition of opsonophagocytosis and the superantigen activity of SpA are thought to be the main reasons for the poor efficacy of adaptive immune response against *Staphylococcus aureus* infections [64].

3.3.2 Other surface exposed factors

3.3.2.1 Second IgG binding protein

A second immunoglobulin-binding protein (Sbi) is a cell envelope-associated factor that can be secreted [69]. Its N-terminus portion contains two IgG binding domains that share similarity to SpA IgG binding domains, followed by two domains that bind the C3 complement component. The C-terminus part is composed of a proline-rich domain and a tyrosine threonine-rich domain that is involved in the attachment of the protein to the cell envelope. The C3 binding domain is also able to bind the factor H complement component, forming a tripartite complex with factor H and C3, leading to the consumption of C3 [70].The two binding domains confer to the protein multiple immune evasion properties, which have different relevance if the protein is secreted or attached to the cell membrane. In fact the IgG binding activity protects the bacteria when the protein is exposed on the cell surface, while the C3 binding domain preserves the pathogen when the protein is released [69]. Considering these peculiar properties, it is clear how Sbi is a unique immune evasion factor that specifically targets both innate and acquired immunity.

3.3.2.2 Capsule

Most staphylococcal clinical isolates express a thin layer of capsule, often referred to as microcapsule, composed of different serotypes. The serotype 5 and the serotype 8 account for more than the 70% of clinical isolates[71]. The *cap* operon is composed of 16 genes (*capA-capP*) which encode for the enzymes needed for capsule synthesis [72]. A cluster of four genes (*capH-capK*) gives serotype 5 or 8 specificity, while the rest of the operon shares more than the 97% of aminoacidic identity among the two serotypes [72]. The contribution of capsular polysaccharide in *S. aureus* virulence has been debated [73], and its relevance seems to depend on the specific strain and growth conditions [27]. In fact several external stimuli influence capsule expression, which for instance changes drastically if the strain is grown on plates or in liquid [27]. More recent works showed that the expression of either capsule 5 or 8 serotype enhances bacterial protection from opsonophagocytosis *in vitro* and increased virulence *in vivo* [27], even though with different contribution by the two serotypes [74].

3.3.3 Secreted factors

Among the virulence and immune evasion factors of *S. aureus* there is a great number of secreted molecules that exerts several functions. Staphylokinase (SAK) is a plasminogen activator that binds to host plasminogen, and mediates the cleavage of surface bound C3b and antibodies [75]. Chemotaxis inhibitory protein of *S. aureus* (CHIPS) is able to inhibit C5a- and fMLP-induced response in neutrophils and macrophages, impairing their recruitment to the infection site [76]. CHIPS have several homologs in *S. aureus*, called FPR-like 1 inhibitory proteins (FLIPr and FLIPr-like), which act inhibiting the first chemoattractants from migrating to the site of infection [77].

Killing of immune cells is a key feature in staphylococcal pathogenesis, especially relevant in abscess formation. This is achieved through the secretion of a large number of toxins, able to damage host cell membrane and eventually lysis. There are three classes of molecules able to damage host cell membrane: the pore-forming toxins, β-hemolysin and phenol soluble modulins (PSM). The pore-forming toxins are the largest family of such toxins including the α-hemolysin, the bi-component leukocidins γ-hemolysin, the Panton Valentine leucocidin (PVL), LukED and LukGH/AB. The α-hemolysin is secreted as a monomer, which assembles into a homo-heptamer on target cell surface that determines pore formation and cell lysis.

In the other pore-forming toxins the formation of the pore is mediated by two different subunits named F (fast) and S (slow) based on their electrophoresis mobility. All the bi-component leukocidins share sequence homology, and their mechanism of action is thought to be similar. The two components assemble sequentially on the cell surface creating a hetero-octamer, in which the S and F subunits are alternatively disposed.

In contrast to pore forming toxins, the β-hemolysin is a neutral sphingomyelinase hydrolysing sphingomyelin, which is a plasma membrane lipid and is thought to destabilize membrane structure.

The PSM are small amphipathic peptides, divided in two sub classes based on their length: PSMα that includes γ-hemolysin, PSMα1-4 and PSM mec that have a length of 20-26 aminoacids, while the PSMβ1 PSMβ2 are 44 aminoacids long.

3.4 Regulation of *S. aureus* gene expression

The huge array of virulence factors and immune evasion systems harbored by *S. aureus* is not always expressed in an indiscriminate way. In fact, *S. aureus* colonization and infection are complex processes that need the activation of specific functions in a coordinate manner in response to determined task. The same happens in *in vitro* growth curves were each virulence factor has a peculiar expression profile, which reflects a specific temporal expression during the different phases of infection [78]. As a general rule, factors involved in colonization (Cell Wall Associated proteins with adhesive and tissue binding functions) are preferentially expressed during the exponential phase of an *in vitro* growth curve, while proteins involved in dissemination and spreading of the infection (exoproteins, proteases, toxins, haemolysins) are more likely to be expressed in the stationary phase [78]. This expression profile is the result of a highly complex and interconnected regulation that enables the pathogen to respond to external stimuli and environmental changes [79, 80]. In *Staphylococcus aureus* two major families of global regulators have been identified: (1) the two component signal transduction systems (TCS) and (2) the SarA homologs, a global regulator of virulence factors. The TCS family normally consists of a membrane bound sensor histidine kinase (HK) and a cytosolic response regulator that induces transcriptional responses [79]. The phosphorylation of the response regulator mediated by the histidine kinases determines conformational changes that modifies the affinity of the DNA binding domain for its target sequence [81]. This simple and general scheme of the TCS can vary in different systems, including other accessory proteins or cytosolic sensor histidine kinases. *S. aureus* genome encodes for 16 TCS, involved in sensing a variety of external stimuli and affecting diverse cellular processes, ranging from quorum sensing and virulence regulation (*agr*, SaeRS), response to antimicrobials and cell wall damage (VraSR, GraXSR, BraRS), cell wall metabolism (WalRK), autolysis (ArlRS, LytSR) and cellular metabolism (SrrAB, NreCBA, AirRS, HssSR, KdpDE, PhoRP). SarA homologs are composed of single proteins with multiple specific targets across the genome. The *S. aureus* genome shows the presence of eleven SarA homologs (SarA, SarR, SarS, SarT, SarU, Rot, SarX, MgrA, SarZ, SarV, SarY) [82]. Those regulators control highly interconnected regulons, in which the expression of a target gene is the result of several diverse regulations. Moreover, SarA homologs and TCS can influence each other expression, adding a further level of regulation.

S. aureus circulating strains can harbor different alleles of these regulators, as well as mutations within the regulatory network, resulting in diverse expression patterns. This can influence several aspects of staphylococcal physiology, ranging from antibiotic resistance (VISA phenotype), to persistence and adaptation in human host like the Small Colony Variant phenotype (SCV) reference.

These observations point out further the importance that gene regulation has in staphylococcal pathogenesis, and its role during infections.

3.4.1 Accessory gene regulator

The accessory gene regulator system (*agr*) is the major quorum sensing system of *S. aureus* as well as a major global regulator controlling the expression of a wide number of virulence factors. The *agr* system controls the expression of more than 100 genes, determining the transition from a colonizing to an invasive phenotype in a cell density dependent manner. As general rule, it promotes transcription of secreted virulence factors (i.e. lipases, protease, PSM, haemolysins, leukocidins) and inhibits the expression of cell surface proteins involved in adhesion and aggregation (SpA, FnbA, FnbB). The *agr* locus encodes for two divergent transcripts, the RNAII and RNAIII, controlled by the promoters P2 and P3, respectively. The RNAII contains the coding sequences of the quorum sensing system *agrBDCA*. *agrB* encodes for a transmembrane peptidase that is involved in processing and secreting the AgrD propeptide into the active pheromone called autoinducing peptide (AIP). AgrC is the sensor kinase that undergoes transient phosphorylation after binding AIP through the extracellular sensor domain. The activation of the sensor kinase *AgrC* occurs only when the accumulation of AIP exceeds a concentration threshold. AgrC then transfers the phosphate group to the response regulator AgrA triggering its activity. Phosphorylated AgrA binds to the P2 promoter inducing RNAII transcription and completing the autoinducing cycle of the quorum sensing system. AgrA binds also to the P3 promoter, activating the transcription of RNAIII, which is the main effector molecule of the quorum sensing system and functions as a non-coding RNA as well as a coding RNA encoding the *hld* gene. AgrA binds to P3 with lower affinity compared to P2, indicating that the *argBDCA* autoinduction occurs before RNAIII transcription activation. RNA III modulates the expression of most *agr* system target genes by interacting with their mRNA. Moreover, AgrA is able to activate transcription of α and

βphenol soluble modulins (PSM), binding directly to their promoters in a RNAIII independent manner. Four different types of AIP are known to date and strains can be grouped according to the AIP produced. Each AIP type is able to activate *agr* system only in strains belonging to the same group (Fig.5). Conversely, AIP molecules can inhibit response of *agr* belonging to different groups. The *agr* system is further tuned by direct or indirect interconnection with several other transcription factors. The most relevant regulators of *agr* are SarA homologs. In fact, SarA induces RNAII transcription binding directly to the P2 promoter, while SarR downregulates transcription from P2.

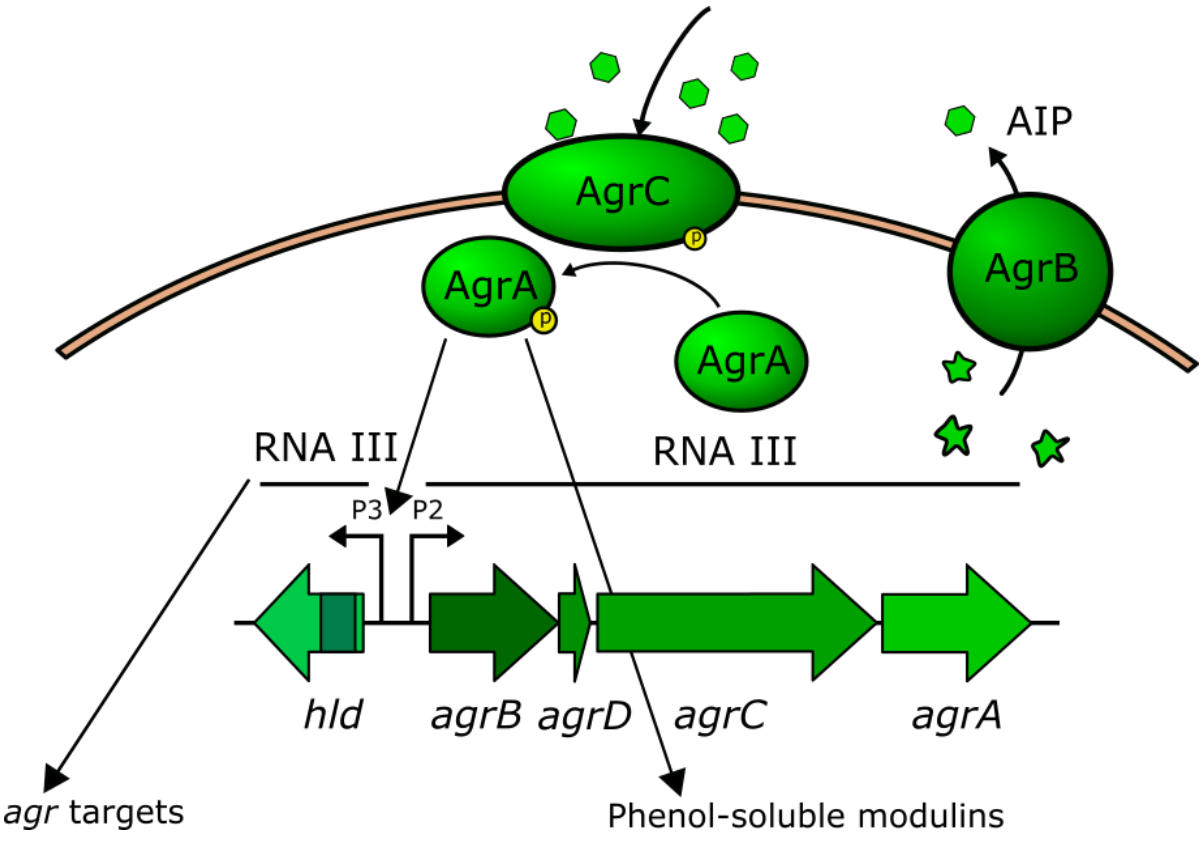

Figure 5. Schematic representation of the *agr* system. The system is encoded by two divergent transcripts, the RNAII that comprise *agrBDCA* genes and RNAIII transcript. *agrD* encodes for the propeptide, which is secreted and matured in AIP by AgrB. The AgrC senses the AIP, and activates AgrA. AgrA induces transcription from P2 and P3 promoters, and activates transcription of α and β PSMs. RNAIII is the effector molecule that controls the expression of *agr* system targets

3.4.2 Vancomycin resistance associated sensor-regulator

The vancomycin resistance associated sensor-regulator (*vraSR*) TCS was firstly described as upregulated in Vancomycin Intermediate *Staphylococcus aureus* strains (VISA) [83]. VraS is the sensor HK that is composed by an N-terminal transmembrane domain and a C-terminal HK domain, while VraR is the response regulator of the system. The *vraRS* system is encoded downstream of a transcript containing other two genes of unclear function *vraU* and *vraT* (previously named *yvqF*). VraS responds to cell-wall affecting antimicrobials like glycopeptides, β-lactams, bacitracins by autophosphorylation and subsequent phosphorylation of VraR. VraR induces *vra* operon (*vraUTRS*) transcription and presumably controls the transcription of *vra* stimulon. It has been proposed that VraT is the actual sensor that interacts with VraS, influencing its autophosphorylation [84], while VraU role remains unknown to date. Mutations in this TCS can modulate (either increasing or diminishing) vancomycin resistance, as well as other antimicrobials. The broad spectrum of the stimulating agents suggests that the *vraSR(T)* senses cell wall damage and its activation determines the expression of cell wall biosynthesis enzymes that increase cell wall thickness.

3.5 *S. aureus* vaccine development

Considering the great burden of staphylococcal infections, and the remarkable ability to overcome antibiotic treatment by this pathogen, a vaccine against *S. aureus* would have an extremely beneficial impact on public health. Several attempts have been made for the development of a *S. aureus* vaccine, with different antigen composition [85-87], but only two have progressed to phase III clinical trials [88]. Merck's V710, based on surface protein IsdB showed safety concerns in phase III trial [89], despite being protective in murine model and inducing of high antibody titers in rhesus macaque. NABI's StaphVAX, composed by the capsular polysaccharide 5 and 8 coupled with a carrier protein, failed to meet the primary end point in two phase III clinical trials [90, 91].

Several reasons behind this failure have been proposed [88, 92], but it is worth considering that so far both strategies are based on single antigen vaccines approaches. Given the redundancy and complexity of *S. aureus* pathogenesis, a multicomponent strategy may be more successful and other vaccines formulations in earlier stages of development include multivalent approaches. Despite the failures obtained so far, *S. aureus* vaccine research programs are still active [86-88].

In recent years, SpA has been proposed as a promising vaccine candidate, showing efficacy in both passive and active immunization in animal models [64, 93-95]. A mutation within the IgG binding domains lead to the creation of a stable form of the protein void of ability to bind antibodies, named SpA_{KKAA} [96]. Vaccination with this mutated protein in mouse elicited higher SpA specific antibodies and an increase of the IgG titers against other staphylococcal antigens after challenge with MRSA epidemic strain [96]. Moreover, vaccination with the SpA_{KKAA} showed protection after challenge with different staphylococcal strains [96]. Passive immunization of mice with monoclonal antibodies was able to protect against MRSA and MSSA strains [93]. A later study demonstrated that immunization of mice with non-toxigenic SpA reduces nasal carriage rates in susceptible murine strains [97]. Similar results were obtained in guinea pig model, were the V_H3 fraction in B cell population resemble human physiology whereas in mice is lower [94].

4 Aims of the study

Given the central role of SpA in staphylococcal pathogenesis and the raising interest in SpA as potential vaccine antigen, we aimed at understanding the prevalence of SpA expression in staphylococcal isolates. To do so, we set out to screen a large collection of strains for the expression of this virulence factor. We identified a subset of strains carrying the gene but void of expression to detectable levels of SpA (SpA^- strains). We investigated if the absence of SpA could be associated with genetic polymorphisms common to the SpA^- subset of strains and whether other changes in the virulence factor expression profile are associated with the SpA^- phenotype. The hypotheses are: 1) the lack of a major staphylococcal immune evasion system such as SpA may be complemented with upregulation of another factor with a redundant function, and 2) a regulatory effect altering SpA expression may also drive other changes in the regulatory network of additional virulence factor gene expression. We verified these hypotheses by investigating the expression dynamics of a large number of virulence factors during *in vitro* growth curves using a high throughput qRT PCR approach.

On identifying other genes that exhibit significant alteration in their transcription profiles we characterized the phenotypic differences other than SpA expression that are exhibited in the SpA^- and SpA^+ groups of test strains.

To understand the regulatory circuitry that may be involved in the phenotypic observations, we mapped the regulatory networks that exert control on the identified genes with altered expression.

The study of the expression interplays among the most significant immune evasion systems will give a deeper insight on staphylococcal pathogenesis and support to the design of new therapies against this important human pathogen.

5 Results

5.1 Identification of a small subset of strains not expressing SpA

Our first aim was to evaluate the presence of the *spA* gene and its expression in our collection of *S. aureus* strains. The collection was composed of 133 strains with different origins and different typing systems. In particular 72 isolates were collected in Vanderbilt University Medical Center (US) between 2005 and 2015 and were characterized according to the Pulse Field Gel Electrophoration typing (PFGE typing)[98], while the remaining 61 strains, including well characterized lab strains such as Newman, were collected from different locations and typed by Multilocus Sequence Typing (MLST) (Fig.6).

The Vanderbilt subset was composed of 10 different PFGE types: USA100 (33 isolates), USA300 (16 isolates) USA400 (14 isolates), USA700 (8 isolates), USA200 (4 isolates), USA400 (3 isolates), USA1100 (3 isolates), USA 500 (2 isolates), USA1000, USA800, USA 900 (1 isolate each), plus 2 isolates with uncertain typing.

The remaining collection included strains belonging to 15 different clonal complexes (CCs) and 28 unique genotypes (STs) (Fig.6). The largest portion of the subset typed by MLST was composed of CC30 (9 isolates, STs 30-34-36), CC8 (10isolates, STs 8-239-250-254) and CC5 (12 isolates, STs 5-228), while the rest of the CCs were represented by less than 4 isolates.

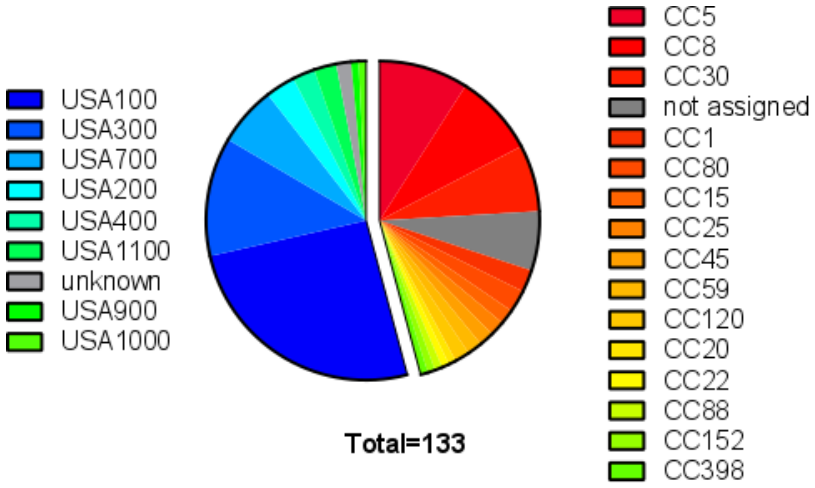

Figure 6. Lineages in the *S. aureus* collection. The collection is divided in two main subsets according to the typing method used. The strains from the Vanderbilt subset were characterized by PFG electrophoration, the other by MLST. The majority of the strains belongs to USA100/CC5 and USA300/CC8 lineages.

The collection was tested by PCR for the presence of the *spA* gene, and by Western blot for the expression of the SpA protein. Although all the strains carried the *spA* locus (data not shown), nine of them resulted negative by Western blot analysis (Fig.7). The SpA negative (SpA^-) strains included the Mu50 strain [12], four USA100 strains (MB01, MM1, MM2, S27), two CC5 isolates from Italy (ITSA18, ITSA19) and two laboratory strains belonging to CC25, Lowenstein [99] and Reynolds [100]. To further investigate the reasons of the loss of SpA expression in the above-mentioned isolates, an equal number of strains were chosen as representatives of the SpA^+ group. In particular, N315 strain [101], four USA100 and four CC5 strains were selected.

N315 and Mu50 are well characterized strains, isolated in Japan in 1982 and 1998 respectively [12]. These two strains were previously sequenced and compared, showing 96% of sequence identity [101] and both belong to USA100 lineage [98, 102]. Given their well-established characterization, Mu50 and N315 were chosen as representatives for the SpA^- and SpA^+ group respectively.

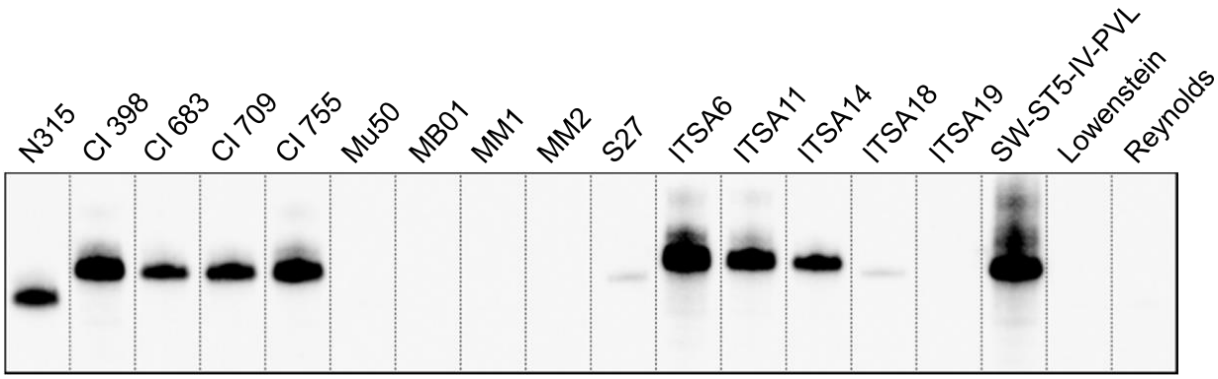

Figure 7. SpA expression in a representative panel of strains. Western blot analysis on the SpA^- strains and the representative panel of SpA^+ strains. Samples were taken from exponential phase of *in vitro* growth curve, SpA was detected by direct binding with rabbit secondary antibody.

5.2 Comparative genomic analysis shows deletion in *spA* 5'UTR affects SpA expression in a subset of SPA$^-$ strains

After the identification of the SpA$^-$ phenotype, we investigated the possible reasons for the loss of SpA expression. In particular, we investigated if at the level of genome sequence we could identify the genetic basis for the SpA$^-$ phenotype that could be shared by the different SpA$^-$ strains. For this purpose, we analyzed the genomes of the ten USA100 strains (five SpA$^+$ and five SpA$^-$), including N315 and Mu50. Comparative genomics analysis was performed using either N315 or Mu50 as reference, to find possible insertions or deletions in the genomes of SpA$^-$ strains.

The analysis showed that all the strains shared high sequence identity if compared to N315 (Fig.8A). As expected, Mu50 resulted with higher similarity to the N315 genome, while S27 is the isolate that carried the highest number of mutations. Most of the identified mutations are harbored in all the USA isolates, supporting that they are not responsible for the phenotype.

Similar results were obtained using Mu50 as reference (Fig.8B). This analysis revealed a higher number of gaps in the other genome sequences, indicating several regions that are present only in the Mu50 strain. Moreover, the distribution of these mutations is more heterogeneous, as some of them are not carried by all the strains. Anyway, also in this case the same mutations are harbored in strains coming from both groups. As already reported, N315 showed a deletion in the coding sequence of *arlR/S*, a two component system known to be also a repressor of *spA* [103]. It was not possible to identify a single mutation carried by all the SpA$^-$ strains and not present in any of the SpA$^+$. However, three of the SpA$^-$ strains (MB01, MM1, MM2) showed a deletion of eight nucleotides, six nucleotides upstream the *spA* start codon (Fig.9).

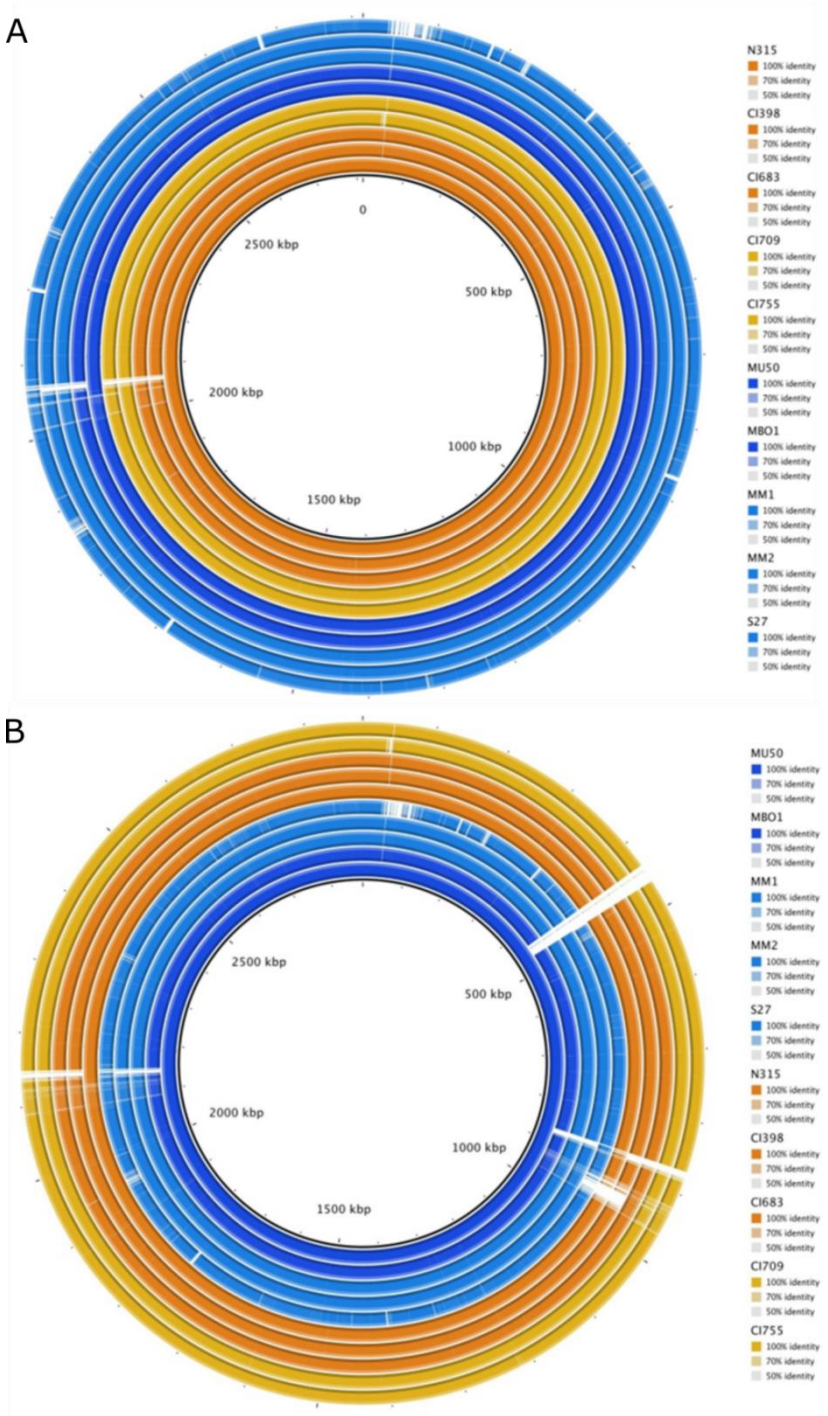

Figure 8. Comparative genomic analysis using N315 or Mu50 as reference. The figure shows the genetic identity between the USA100 strains and the reference strains N315 and Mu50. The SpA^+ strains are depicted in orange, while the SpA^- strains in blue. The color scale is different for each strain. White gaps show regions present in the reference strain but absent in the analyzed genomes. The inner circle represents the reference strains. (A) Genomic comparison using N315 as reference. (B) Genomic comparison using Mu50 as reference.

The sequence corresponding to the deletion (CAGGGGGT) and its position make it likely to contain a Ribosome Binding Site (RBS) sequence for the *spA* gene.

Further analysis on the *spA* locus showed additional differences among the isolates (Fig.9). The *spA* gene in the Mu50 and N315 strain carried a deletion of 174 nucleotides that causes the expression of a shorter SpA protein that lacks the last IgG binding domain, as already shown in the Western blot (Fig.7). Moreover, S27 isolate carries several SNPs across the sequence, including the promoter, as well as a shorter X variable region. Most of these mutations are silent, and none of these is responsible for an early stop codon.

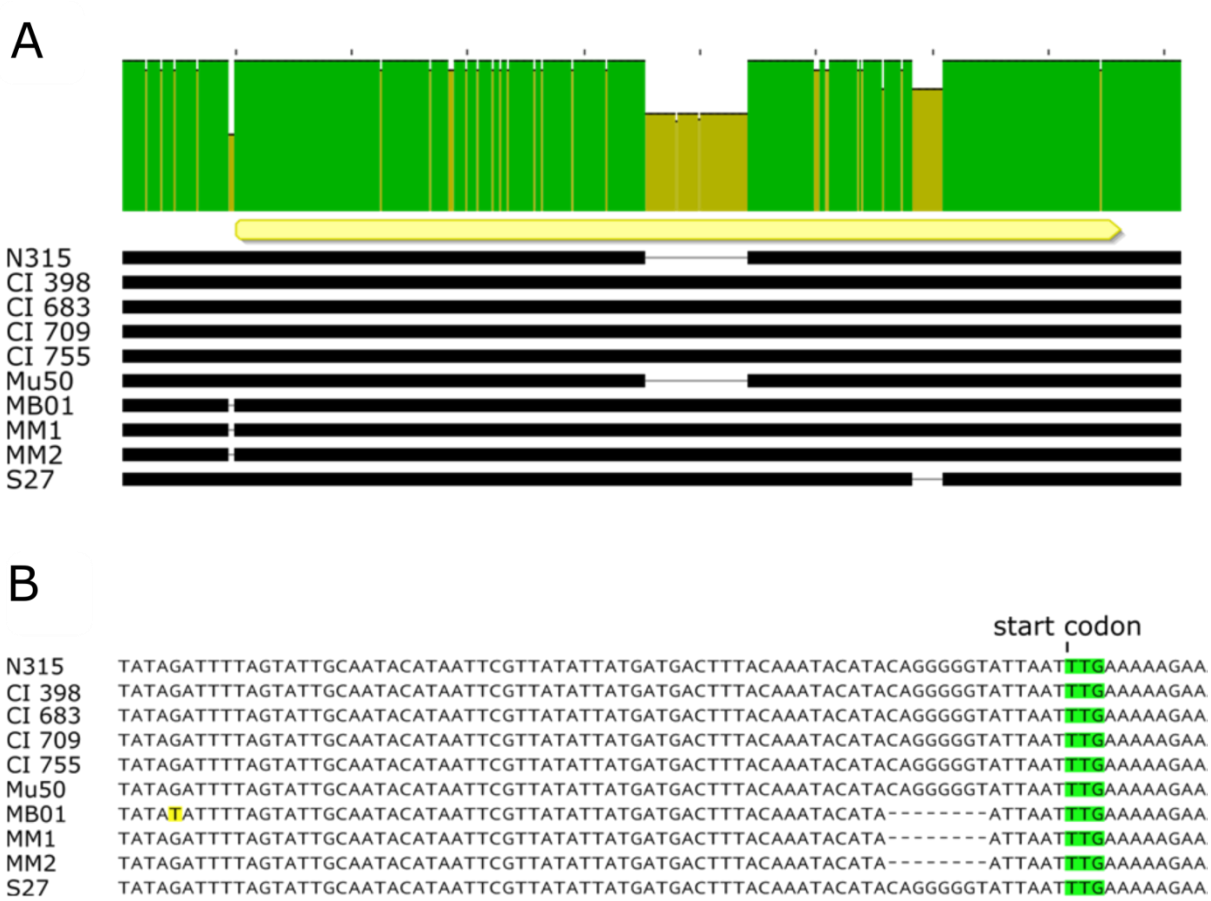

Figure 9. Alignment of *spA* locus in the USA100 subset of strains . (A) Alignment of the *spA* locus of the ten strains analyzed. The upper bar highlights the sequence identity of the *spA* locus. The yellow arrow shows the position of *spA* coding sequence, and its orientation. The black bars represent the sequence in each strain and gaps correspond to deletions. (B) Magnification of the region upstream the coding sequence of *spA*. The start codon is highlighted in greenand the mismatch present in the MB01 sequence is highlighted in yellow. The dashed gaps underline the deletion present in the 5'UTR region of *spA* gene in MB01, MM1 and MM2 strains.

In order to investigate whether the deletion found in three of the SpA$^-$ strain could be responsible for their SpA$^-$ phenotype, we generated two translational fusions of the *spA* promoter and the 5'UTR region to a mCherry reporter with or without the deletion (Fig.10). While expression of the reporter was detected when the N315 *spA* gene upstream sequence was fused to mCherry, fusion of the sequence containing the deletion resulted in no fluorescence signal. This suggests that the mutation containing the RBS region may be responsible for abolishing SpA expression in the three identified strains. This genomic analysis showed that the genetic reasons for SpA$^-$ phenotype are probably different among the different strains, however for the MB01, MM1 and MM2 strains the loss of SpA expression is caused by a mutation in *spA* untranslated region.

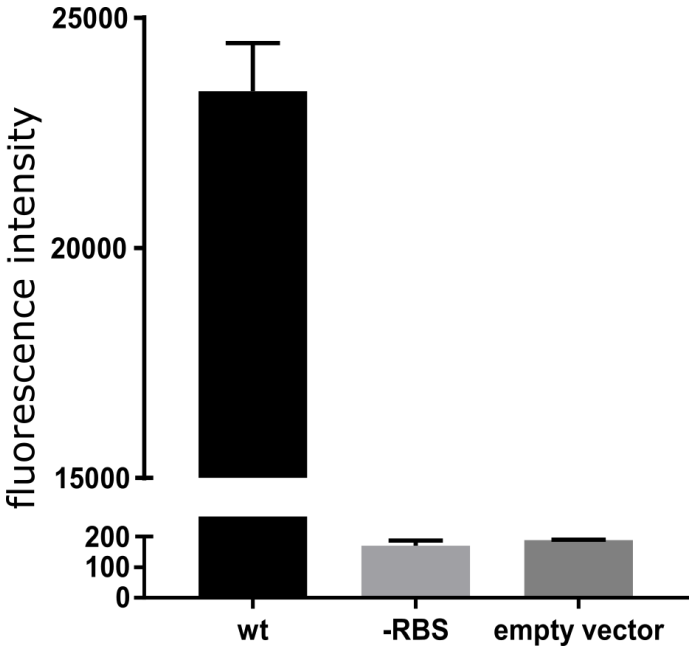

Figure 10. Effect of the 5' UTR mutation on reporter production. The graph shows the fluorescence signal obtained from overnight growth of *E.coli* transformed with pOS1 plasmid containing either the wt *spA* promoter, or carrying the RBS mutation fused to mCherry reporter gene. The empty vector control shows the signal from *E.coli* strain transformed with pOS1 backbone. The graph represents three independent experiments from different clones.

5.3 Comparative transcript profiling of 84 virulence related genes in the subpanel of USA 100 strains

Our genomic analysis showed that the genetic reasons for the SpA^- phenotype are probably different among the different strains. For this reason, the SpA^- strains were further investigated using a high-throughput qRT PCR approach to analyze the transcripts of virulence related genes. A panel of 84 TaqMan assays of selected virulence related genes, was used together with the HD Biomark microfluidics system were used to perform comparative transcription profiling. The panel of genes has been previously selected (Haag et al., manuscript in preparation) to cover diverse factors expressed in different stages of staphylococcal infection process, including adhesion, invasion and immune evasion. It is known that virulence genes exhibit *in vitro* growth phase regulation [78], therefore the transcriptional profiling was performed by measuring the transcript levels of the 84 virulence-related genes at five representative time points (early, mid and late exponential phase, early and late stationary phase) of the *in vitro* growth curve. The Heatmap in Fig. 11 shows the transcription kinetics of the selected genes in all the strains tested. Several assays were not able to detect any target transcript in S27 isolate, probably because of the high rate of mismatch between the probes and the targets sequences. All the genes were then clustered according to their transcription profiles through the growth and among the isolates. The majority of the genes expression is increased with respect to early log phase (0,5 OD) indicated by the yellow predominance in the heatmap. There are four main clusters representing different transcript kinetic profile. Cluster 1 comprises the genes whose transcription decreases during the growth like *clfB* and *fnbB* that are known to be upregulated in exponential phase (Fig.12) [42]. Cluster 2 includes the genes which are upregulated in mid-exponential phase and then downregulated in stationary phase. This second cluster contains the *isdA isdB, isdC* and *isdG* genes, which are involved in iron metabolism and are part of the iron-regulated surface determinant pathway (Isd). *sirA*, an iron-regulated lipoprotein involved in iron metabolism, is also included in this cluster [104]. The genes comprised in cluster 3 are highly upregulated as the growth progress (Fig12). As expected, this cluster includes genes involved in the quorum sensing system *agr*, such as *agrA* and *hld*. In particular, *agrA* encodes for the response regulator of the system, while *hld* coding sequence is located onto the RNAIII transcript, which is the RNA effector molecule of the system.

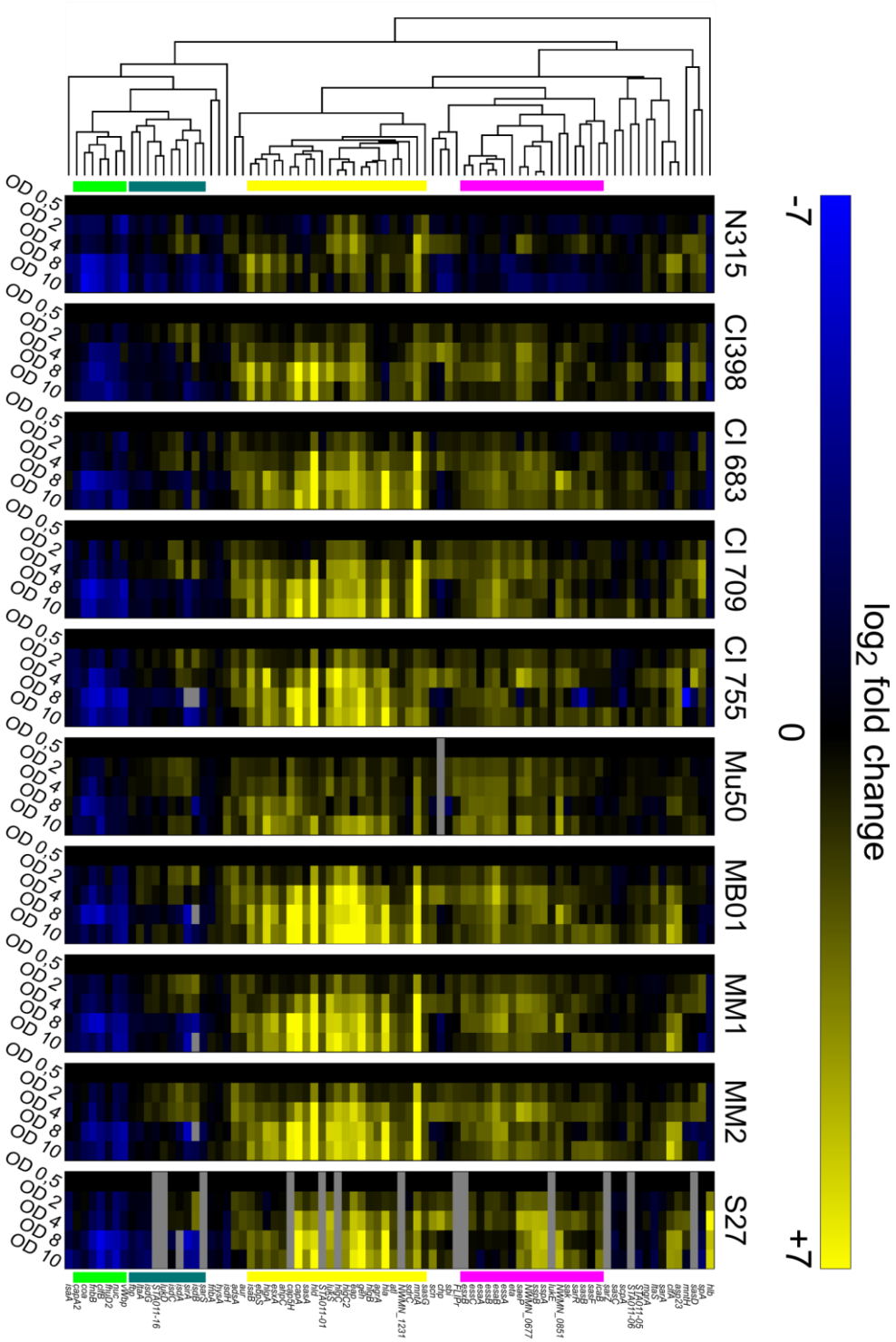

Figure 11. Transcript levels variation during *in vitro* growth curve. Each column represents one assay, while each row corresponds to a sample. Values are normalized to the first growth point (early stationary phase, OD0,5). The genes transcribed at lower levels compared to the first growth point are depicted in blue, and in yellow genes that are up-regulated. The bars on the two sides of the heatmap represent the four major clusters: cluster1 (green), cluster 2(dark green), cluster3(yellow) cluster 4(pink).

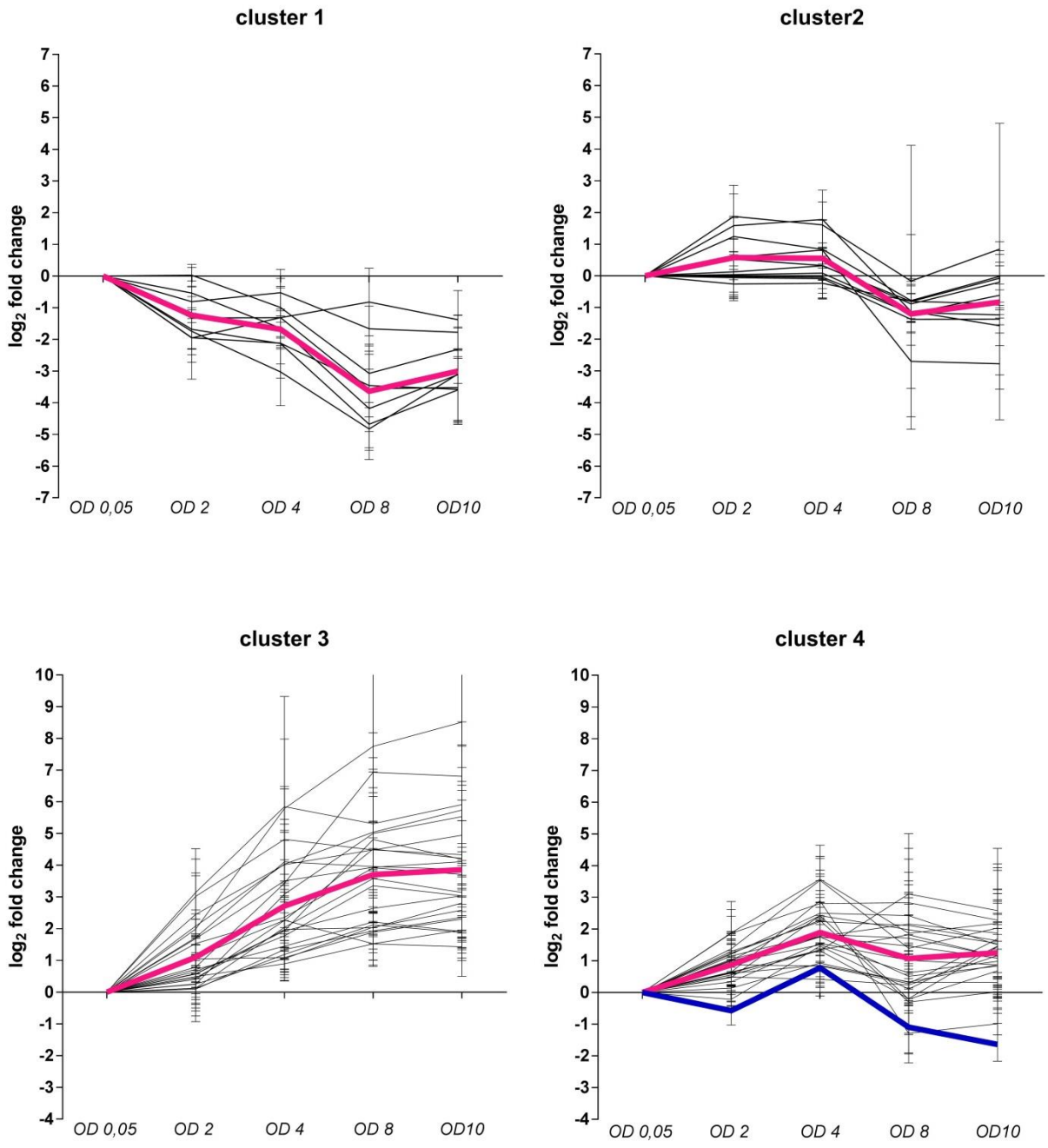

Figure 12. Kinetic profiles of the four major transcription clusters. Black lines represent the mean values of single genes and the error bars show the standard deviation among the isolates. The mean profile of the genes within the cluster is showed in pink. In cluster 4, the blue line represents the mean of the cluster in N315 strain.

Several genes that are known to be targets of the *agr* system are grouped in this cluster, like the capsule genes *capA* and *cap5H*, the alpha haemolysin *hla*, the gamma haemolysin (component B-C) *hlgB* and *hlgC* [105].

The genes in cluster 4 are moderately up-regulated during the growth, with the exception of the N315 where they appear down-regulated (Fig.11, Fig.12). This is particularly evident comparing the mean kinetic profile of N315 with the other strain (Fig.12, cluster 4).

The *spA* gene is not present in any of these major clusters, but it clusters together with *sasD*, their transcription increases in mid log phase and goes down during the stationary phase. However, their kinetic varies considerably among the isolates, although the transcription profile of these two genes is similar within the same strain. For example, both *spA* and *sasD* show flat curves in Mu50 and MB01 strains (Fig.11).

Focusing on *spA* transcription, it is clear how the variability of *spA* transcript in SpA^- strains follows the upregulation of *spA* itself, which has the highest expression in the exponential phase. In Fig. 13 the *spA* transcription kinetic is shown in detail for each strain, considering the different relative steady state levels, and highlighting three different behaviors. Two of the strains with the RBS mutation (MM1, MM2) maintained the kinetic of expression of the SpA^+ strains, even though at lower steady state in comparison with SpA^+ strains. Strains MB01 (that harbor the RBS mutation) and S27 maintained only partially this profile as reflected by the flatter curve. MU50 shows a completely flat trend, with similar transcript levels across the growth. This means that some of the SpA^- strains lost *spA* regulation during growth other than expressing lower steady state *spA* transcript.

The difference in transcription kinetics among the isolates was further investigated by clustering the data both by assays and by samples. Vertical colored bars in Fig.14 show that N315, Mu50 and S27 samples are grouped by strain, while the samples from the other isolates cluster according to the growth phases. This indicates that the overall differences within the growth phases of N315, Mu50 and S27 are lower than the one displayed with the other strains, whose similarity is higher within the same growth phase rather than within the same strain. In particular, mid and late exponential phases are well distinguished, while early and late stationary phases cluster together. This analysis showed that the profiles of N315, Mu50 and S27 strains are independent from the other isolates.

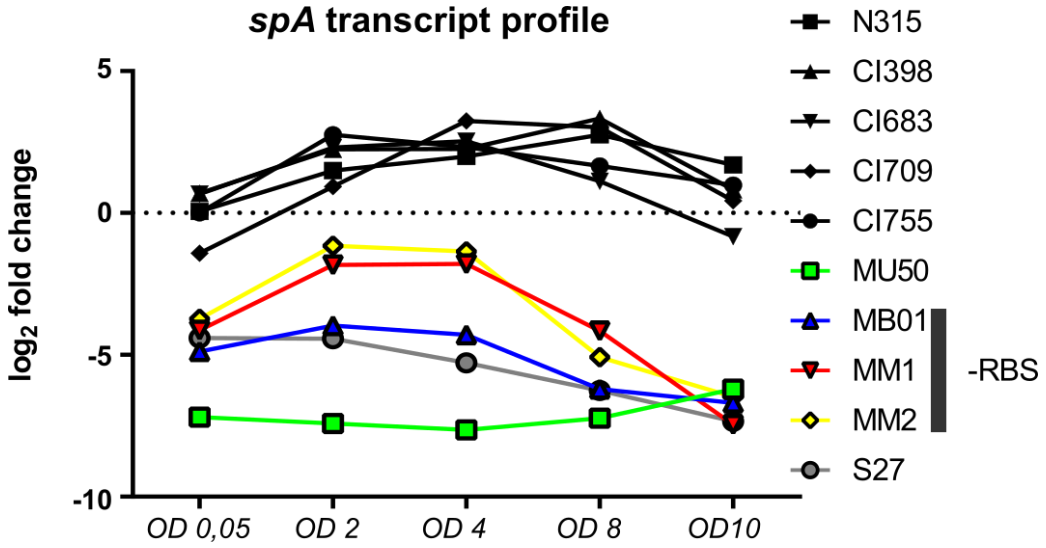

Figure 13. *spA* transcript profiles in all the SpA+ and SpA- strains. The black lines indicate the transcript profile of the SpA^+ strains, while the coloured ones represent the *spA* RNA levels in the SpA^- strains. All the values are normalized to the mean of the SpA^+ strains in the early exponential phase. The three strains that carry the RBS mutation are highlighted in the figure legend.

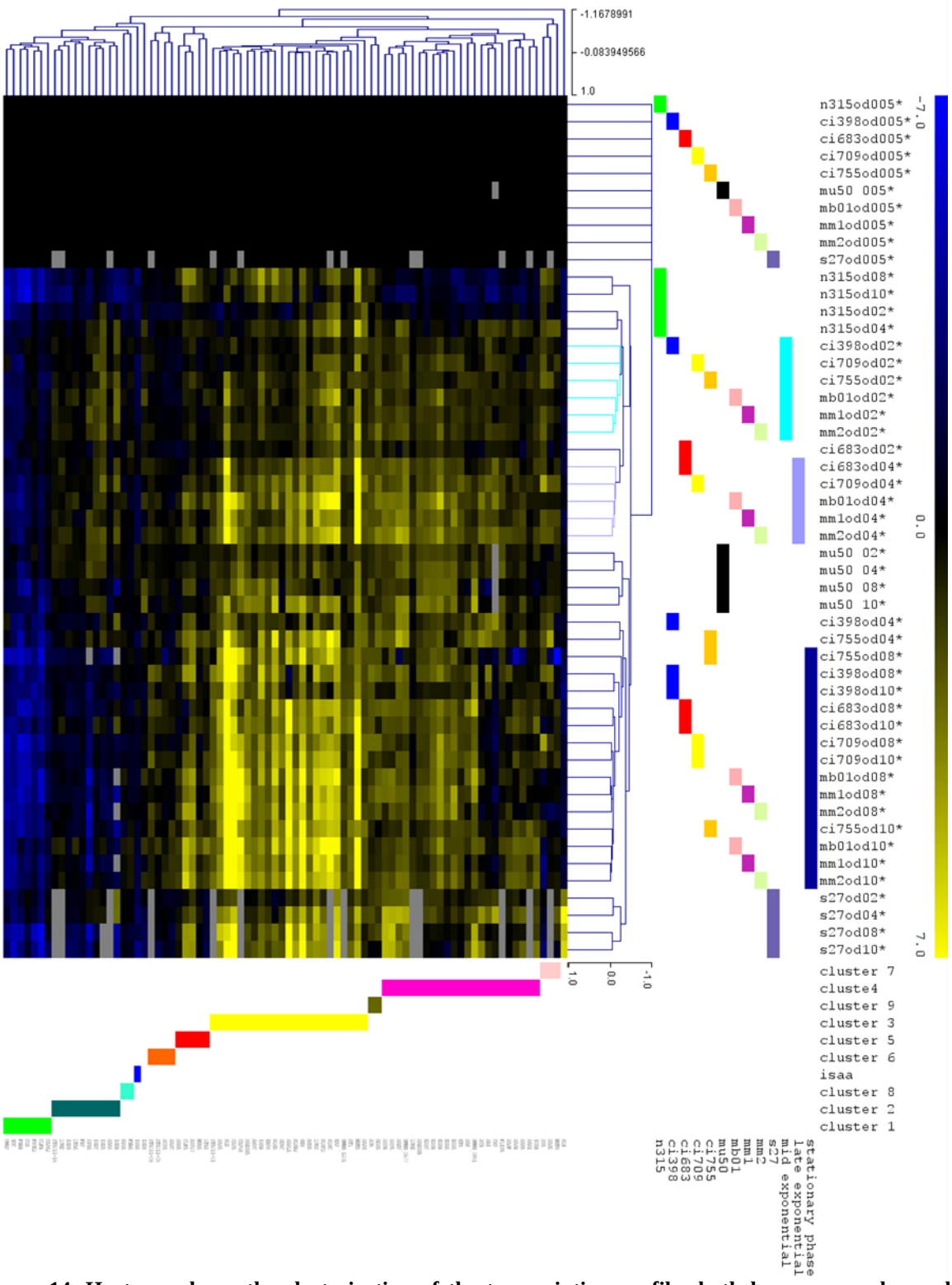

Figure 14. Heatmap shows the clusterization of the transcription profiles both by assays and samples. Colored bars indicate the different clusters and the ten strains. Horizontal colored bars indicate assays clusters, as already shown in Fig.5. Vertical coloured bars represent the ten strains tested and samples clusters.

5.4 Capsule biosynthesis operon transcription is higher in SpA$^-$ strains

To highlight the transcriptional differences between SpA$^+$ and SpA$^-$ strains, we calculated the difference in gene expression between the mean RNA levels of SpA$^+$ and SpA$^-$ strains. The genes were ranked based on their up-regulation in one or the other group and plotted in to a heatmap (Fig.15). At the two extremities of the heatmap are shown the genes that were diversely expressed in each point of the growth, *i.e.* genes that are always upregulated in either group. The center of the heatmap shows that some genes had time point specific differences.

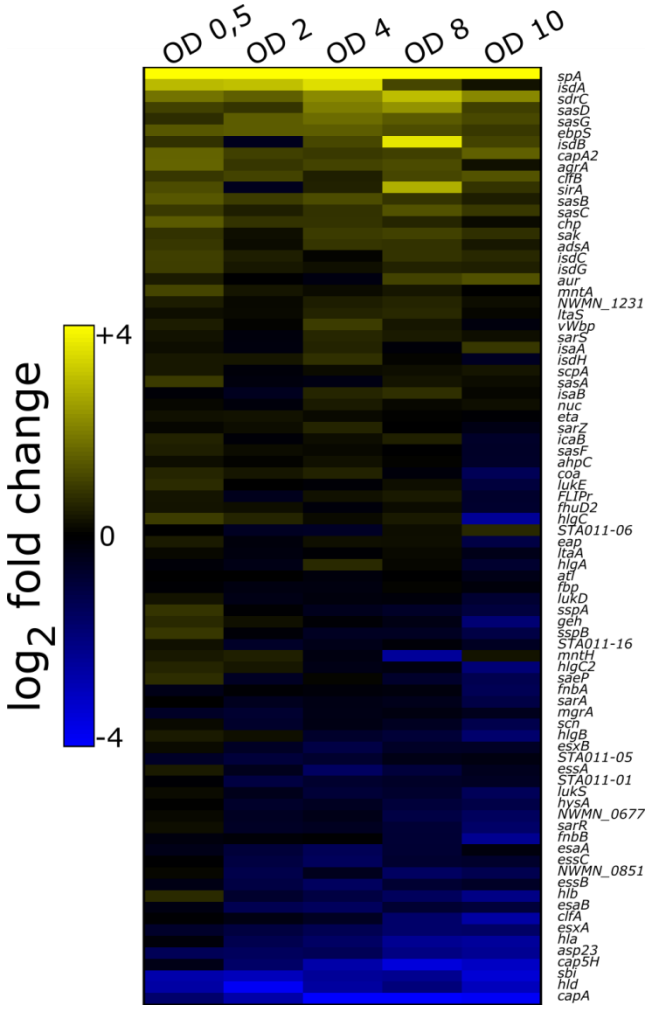

Figure 15. Heatmap showing the differential transcription between SpA$^+$ and SpA$^-$ strains. The heatmap shows the difference in transcription for all the genes tested between SpA$^+$ and SpA$^-$ strains at each time point. The genes that are more transcribed in the SpA$^+$ strains are depicted in yellow, while the ones that are more transcribed in the SpA$^-$ strains are depicted in blue.

The significance of the difference in transcription for each gene was assessed through a two-way ANOVA, as shown by the volcano plot in Fig.16. This analysis highlighted five genes that show significant difference in transcript levels, i.e. genes with a fold change higher than two and p-value lower than 0.05 among the two groups of strains. As expected the *spA* gene exhibits the highest upregulation in the SpA^+ strains, while other two genes, *sdrC* and *sasD*, were upregulated in SpA^+ strains with lower significance. Interestingly, two genes were upregulated in the SpA^- strains, the capsule biosynthesis related genes *capA* and *cap5H*. These two genes belong to the capsule biosynthesis operon (Fig.17) and are the first gene of the operon (*capA*) and the first gene of the capsular polysaccharide type 5 specific region (*cap5H*). Of note, *cap5H* transcript was not detected in S27 isolate, indicating that it probably belongs to a different serotype.

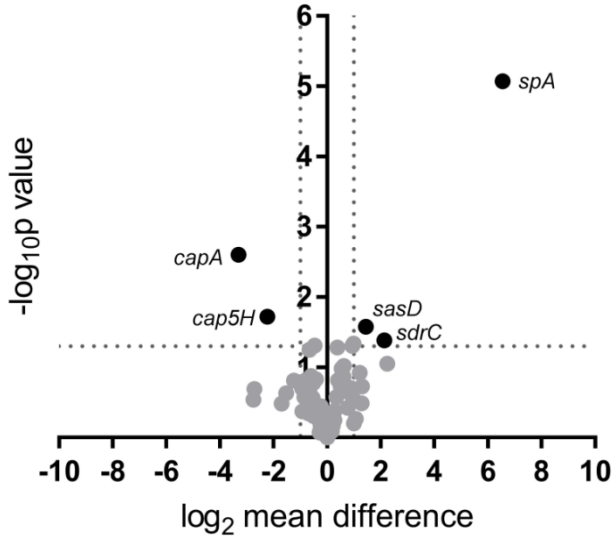

Figure 16. Volcano plot showing genes with significant difference in transcription. Genes are displayed according to the mean difference in transcript levels between SpA^+ and SpA^- strain along the entire growth, and the p value measuring the statistical significance associated. On the right, with positive mean difference, the genes that are more transcribed in the SpA^+ strains, on the left, with negative mean difference, the genes that are more transcribed in the SpA^- strains.

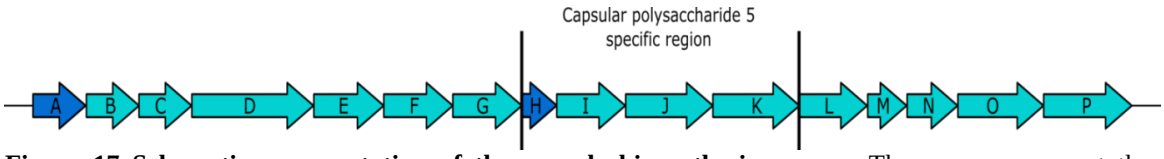

Figure 17 Schematic representation of the capsule biosynthesis operon. The arrows represent the open reading frames. In blue are highlighted the two genes present in the transcriptional screening, *capA* and *cap5H* are highlighted in blue. The operon is composed by 16 genes, four of which are specific for the capsular polysaccharide type 5.

The transcriptional changes of *spA*, *capA* and *cap5H* genes during the growth are shown in the boxplots in Fig.18, highlighting how the difference in RNA levels between the two groups of strains is not constant, but changes during the growth.

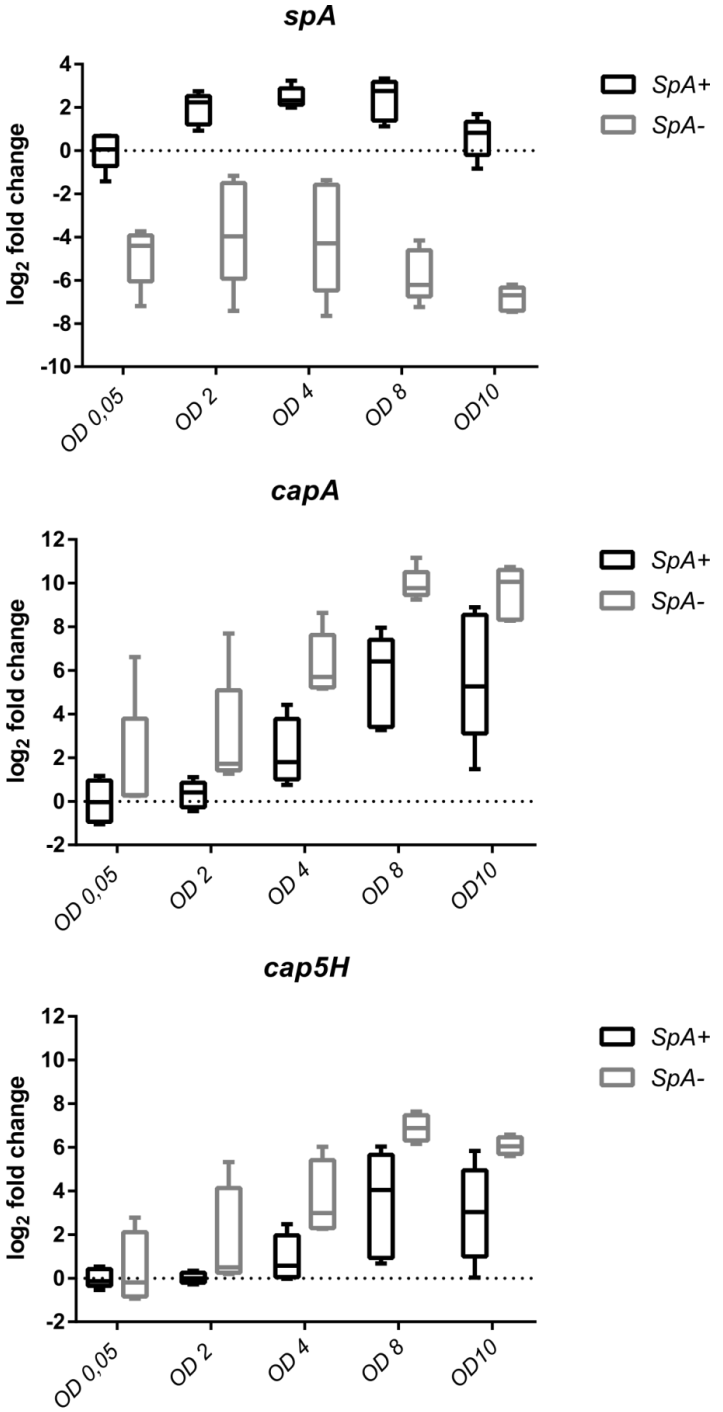

Figure 18. Transcription trends for *spA*, *capA* and *cap5H*. The boxplots show the transcription of the three genes among the SpA^+ and SpA^- strains; bars show the median and whiskers the maximum and minimum values. The values are normalized by the mean of the SpA^+ strains at OD 0,5. The dotted line represents the SpA^+ mean level in early exponential phase.

The analysis performed allowed the identification of genes whose transcription is different among the two groups of strains throughout the growth, however, less evident transcriptional differences could occur in single phase of the growth. To deeper understand the differences in gene regulation between SpA^+ and SpA^- strains, we investigated whether some of the genes had a significant difference in transcription at single growth points. In this analysis, all the genes were evaluated in each growth point separately, and the results are reported in the volcano plot in Fig.19. The *spA* gene resulted different in all the growth points, while *capA* and *cap5H* showed a significant difference in three and two growth phases respectively (late log phase, early and late stationary phase). All the remaining genes detected by this analysis had significantly different RNA levels only in one time point of the growth. The genes that were transcribed with significant difference throughout the growth, i.e. *spA*, *capA*, *cap5H* and *sdrC*, showed a similar profile, with the highest upregulation in the early stationary phase (Fig.20). The common kinetics in differential regulation makes it reasonable to address this expression profile to the same factor or combination of factors. Despite *sasD* was identified as one of the genes with a significant difference throughout the growth, no singular growth point was highlighted by this analysis (in early stationary phase the mean difference was 5, the p-value 0.0581). Interestingly, all the remaining genes were differentially transcribed in late stationary phase (Fig.19, Fig.20B). In particular, *sarA* that is a well-known regulator [105-107], was detected among them. To summarize, the comparative analysis of transcription kinetic highlighted two different groups of genes with similar profiles. The genes that showed different transcript levels in the late stationary phase have different transcript levels among SpA^+ and SpA^- strains only in this growth phase. Conversely, the genes that have a significant difference in RNA levels throughout the growth show the highest difference in transcription during early stationary phase.

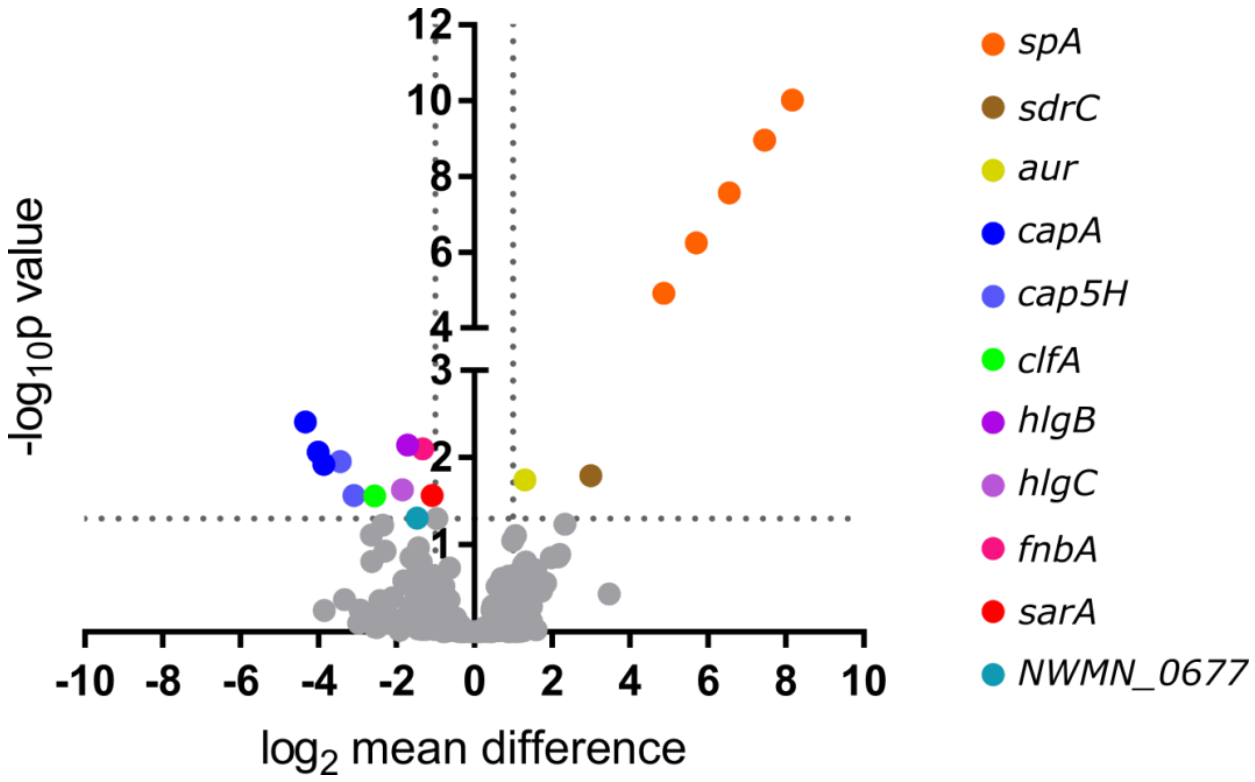

Figure 19. Volcano plot showing the genes with significant difference in specific growth phases. Each dot represents one gene in one growth point. The genes and the corresponding OD are ordered by significance: *spA* (OD 8; OD10; OD 4; OD 2; OD 0,5), *capA* (OD 8, OD4, OD 10), *cap5H* (OD8, OD10) *sdrC* (OD 8), *aur*, *clfA*, *hlgB*, *hlgC*, *fnbA*, *sarA*, *NWMN_0677* (OD 10).

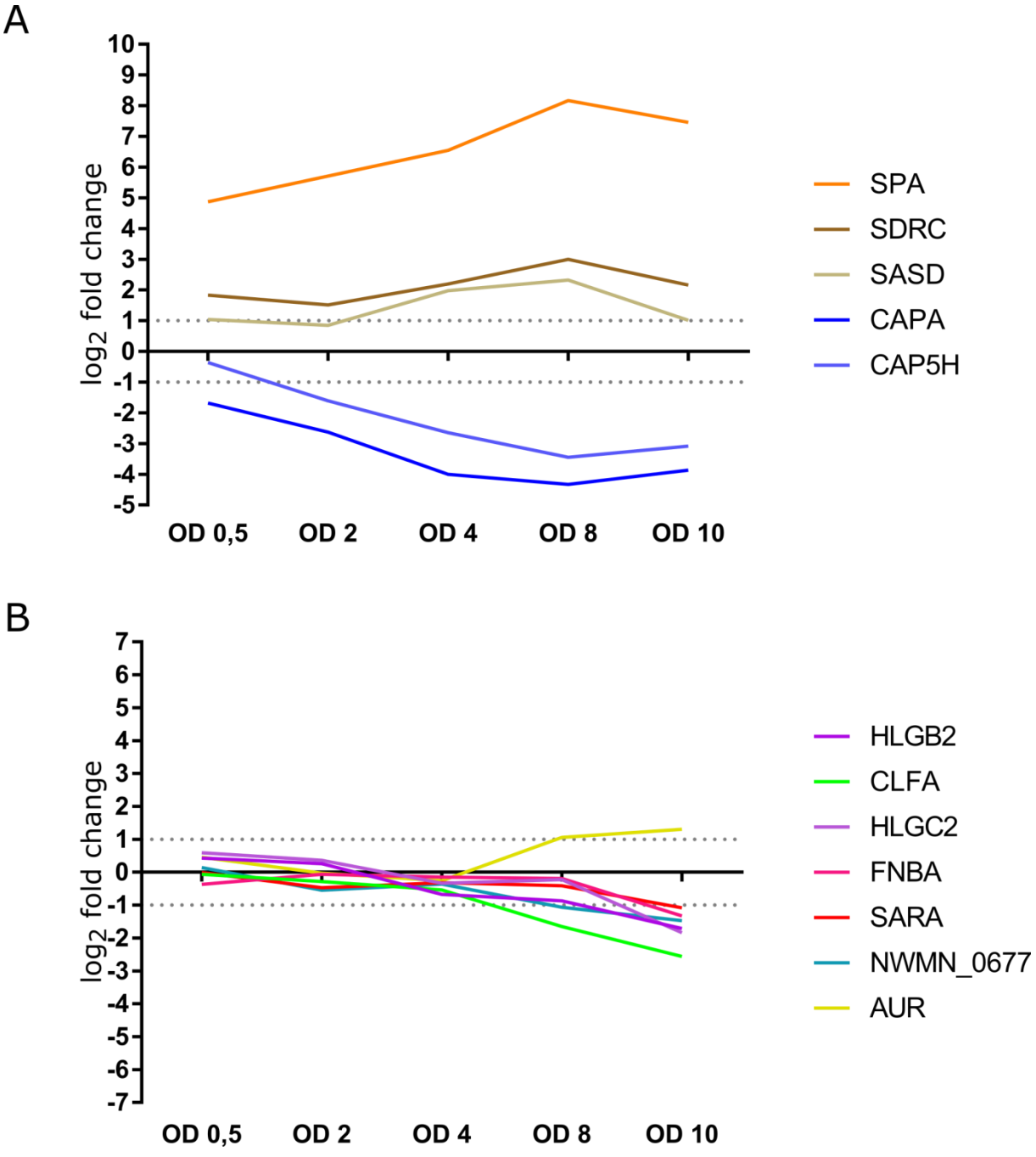

Figure 20. Differential transcription kinetic of significant genes. The graphs represent the log2 of the ratio between SpA^+ and SpA^- mean RNA levels, dotted lines highlights the fold two difference. (A) Differential transcription kinetic of genes with significant difference throughout the growth. (B) Differential transcription kinetic of genes with significant difference in one single growth phase.

5.5 Absence of SpA does not influence directly *cap* operon transcription

The anti-correlation between the *spA* and capsule transcripts, and their common localization on the cell wall, suggested that the two factors could compete for the same localization on bacterial surface. It is possible that the two factors are linked to a feedback loop regulation, in which the presence of one of the two directly inhibits the expression of the other. To investigate this hypothesis, we measured the RNA levels of capsule biosynthetic genes in a Δ*spA* background. Figure 21 shows *capA* and *cap5H* RNA levels in strain Newman wt and Δ*spA*. The transcript levels are similar in the two strains, probably slightly lower in the *spA* mutant. This suggests that the expression of SpA does not influence the transcription of the capsule biosynthetic genes, so their expression is probably balanced by an upstream regulatory network. The increased transcription of the capsule biosynthetic genes and the down regulation of *spA* could be determined by the impairment of this regulation.

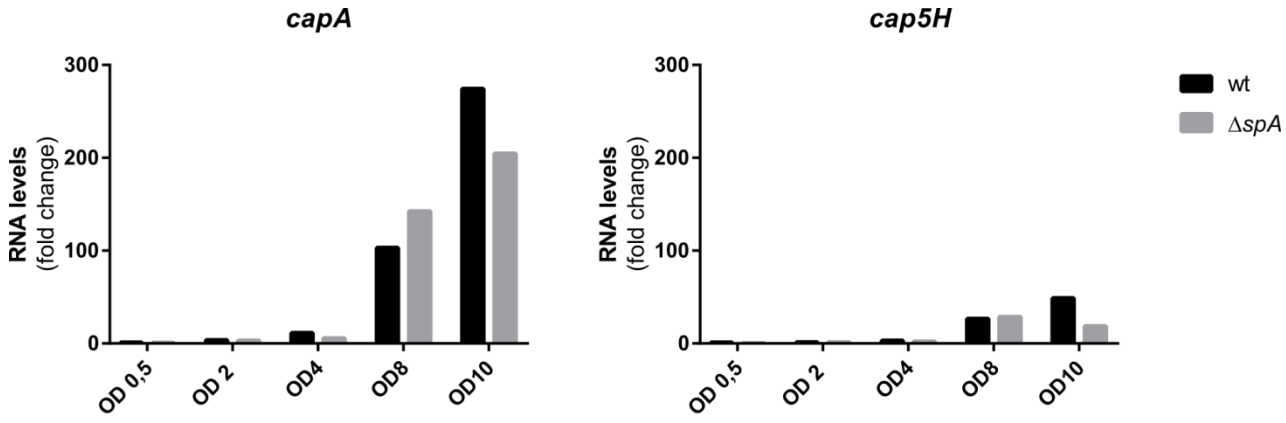

Figure 21. *capA* and *cap5H* RNA levels in Δ*spA* background. The graphs show *capA* and *cap5H* RNA levels in five growth points of an *in vitro* growth curve. Newman wild type and Δ*spA* were used. The experiment was performed in single replicate.

5.6 Regulatory network analysis of genes with distinct transcription profiles in SpA^+ and SpA^- strains

Since *spA* and capsule expression do not interfere directly on each other transcription, we focused on the dissection of the regulatory network upstream the differentially transcribed genes, to identify factors that could be responsible for the phenotype. The genes whose deletion affects the transcription of the genes selected for transcript analysis were extracted from the **SATMD** *Staphylococcus aureus* Transcriptome Meta-Database [108]. The relevant factors for the transcription of the genes having different RNA levels in SpA^+ and SpA^- strains throughout the growth are shown in Fig.22. Three different types of genes can be identified in the regulatory network. Seven genes (Fig.22, group marked 1) are known to have similar regulatory activity on both group of genes upregulated by SpA^+ or SpA^- strains, indicating that they are unlikely involved in the SpA^- phenotype. Twenty genes were shown to influence the transcription of only one of the two groups of genes differently expressed, implying that one single gene of this group cannot be responsible for SpA^- phenotype. Ten of the regulators (Fig.22 group marked 2) are reported to have an opposite regulatory activity (induction or inhibition) on the genes identified as having distinct profiles in SpA+ and SpA- group of strains. This suggests that a shift in the regulatory output of one of these could result in the transcriptional difference observed in the SpA- strains. Interestingly, a relevant number of regulators whose activity is consistent with SpA^- phenotype is involved in cell envelope stress signal sensing, cell wall synthesis regulation and antimicrobial resistance (v*raTSR, walR, , stk1, stp1, rpoB, clpP, nsaRS, tcaR, ecsA*) [84, 109-113].

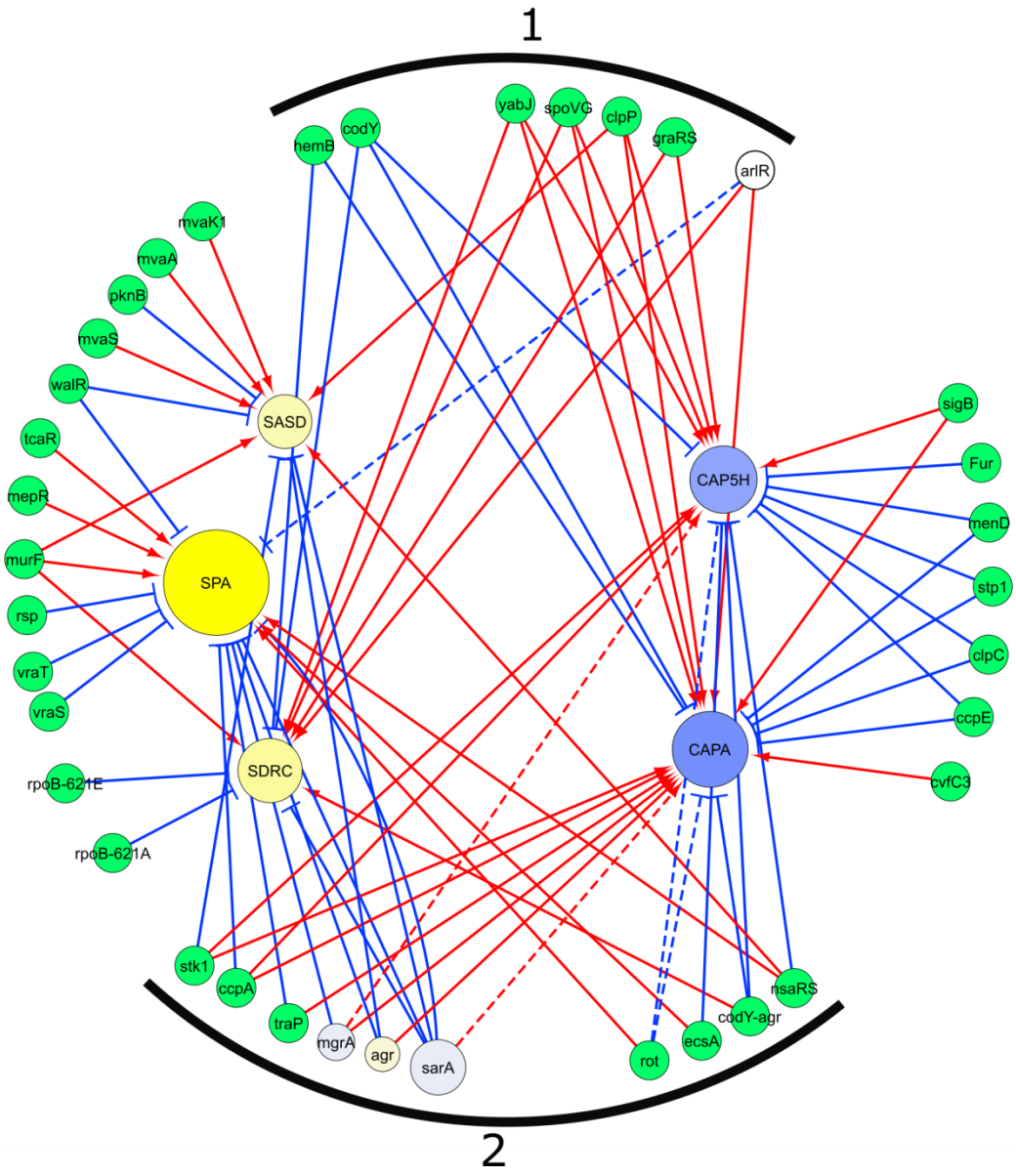

Figure 22. The regulatory network of the genes differentially transcribed between SpA$^+$ and SpA$^-$. The network was extracted from SATMD *Staphylococcus aureus* Transcriptome Meta-Database (core free). The yellow circles represent genes upregulated in SpA$^+$ strains, the blue circles represents the genes upregulated in the SpA$^-$ strains. The dimension of the circle indicates the significance of the upregulation. Green circles represent genes, or combination of genes, whose deletions influence the transcription of the five genes of interest. The three regulators present in the assays are depicted with the same graphic representation of the differential transcribed genes. Red arrows represent a positive regulation, blue arrows negative regulation. Dotted lines represent interaction not found in the database, but in the literature. 1) Genes that influence transcription of both groups of genes, but which is not consistent with transcript analysis. 2) Genes that exert a regulation on both groups of genes and which is consistent with transcript data.

Knowing that *sarA* gene is differently transcribed during the late stationary phase, we verified whether its regulon fits with our transcript data. The network represented in Fig.23 shows the known targets of SarA that were tested in the transcriptional screening, and their differential expression between SpA^+ and SpA^- strains. SarA represses all the genes that have significant upregulation in the SpA^+ strains, and induces those showing a significant upregulation in the SpA^- strains, except for *sarA* itself. This is consistent with *sarA* upregulation in the SpA^- strains. However, the majority of SarA targets (80%) do not show a significant difference in transcription in the SpA^+ or SpA^- strains (smaller circle in the network). This could be explained by the interference of others regulator that can impair SarA effect. Moreover, it is important to consider that the genes reported in the network are not necessarily direct targets of SarA, and overexpression of SarA alone could be not sufficient for their upregulation.

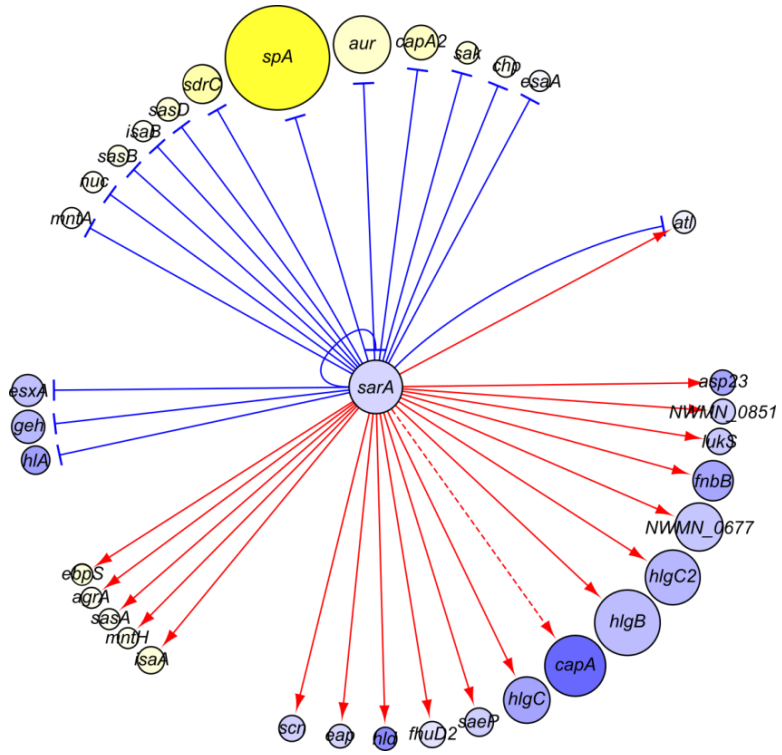

Figure 23. SarA regulon. The genes present in the screening and regulated by SarA are depicted in the figure according the group of strain in which they are up regulated (yellow for the genes upregulated in the SpA^+, blue for the genes upregulated in the SpA^- strains), and the p-value of the upregulation transcription at late stationary phase (dimension of the circle). Red arrows represent positive regulation, blue arrows negative regulation. Dotted lines represent interaction not found in the database, but in the literature.

5.7 Capsule gene transcription and capsule production is higher in SpA$^-$ isolates of geographically distinct origin

Having identified significant transcriptional differences in the USA100 subset of strains, we expanded the subset to include the remaining SpA$^-$ strains as well as a control SpA$^+$ group, verified the upregulation of *capA* and *cap5H* transcripts by qRT-PCR and quantified the relative amount of capsule produced by all strains. The *capA* and *cap5H* transcripts levels were quantified at a single time point (early stationary phase) by RT-PCR. As already observed in the USA100 subset of strains, the RNA levels of both genes were significantly higher in the SpA$^-$ strains (Fig.24).

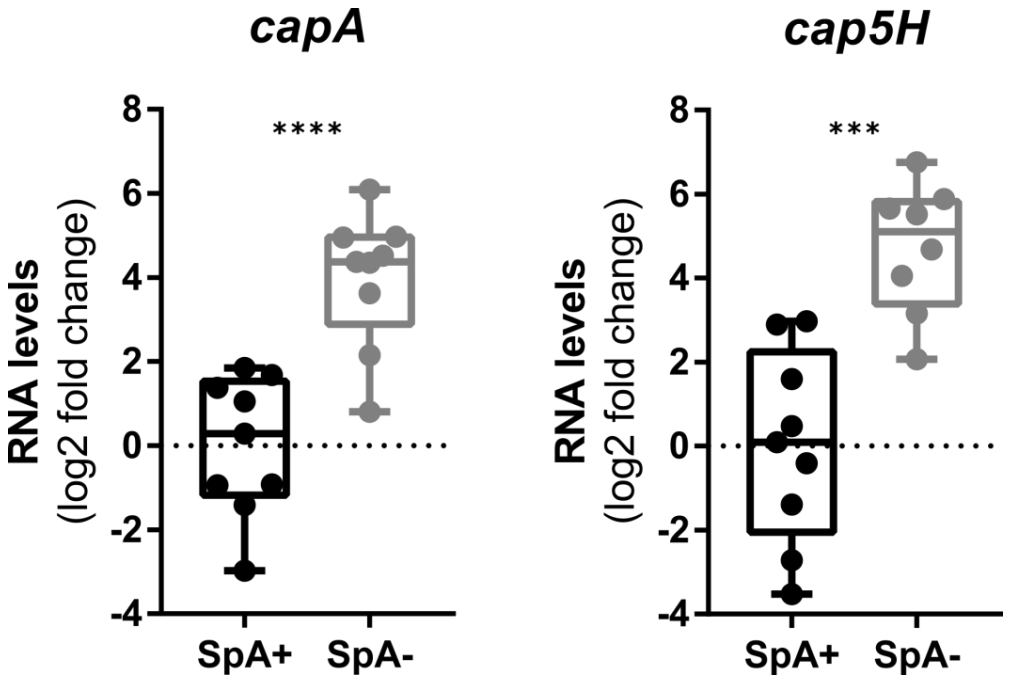

Figure 24. *capA* and *cap5H* transcript levels in SpA$^+$ and SpA$^-$ strains. Bars indicate the medians, whiskers the maximum and minimum values. All values are relative to the mean of SpA$^+$ strains levels. Statistical significance is calculated with t test.

To quantify the amount of capsule produced by the SpA^- strains, we performed a capsule immunoblot from single growth point (late stationary phase) (Fig.25).The analysis showed high variability in the amount of capsule among the different strains, although the highest quantity of capsular polysaccharide was detected in the SpA^- isolates. Moreover, four of the SpA^+ strains (N315, CI709, ITSA6, ITSA14) produced no detectable capsule at the time point tested, while all SpA^- strains expressed capsule at detectable levels.

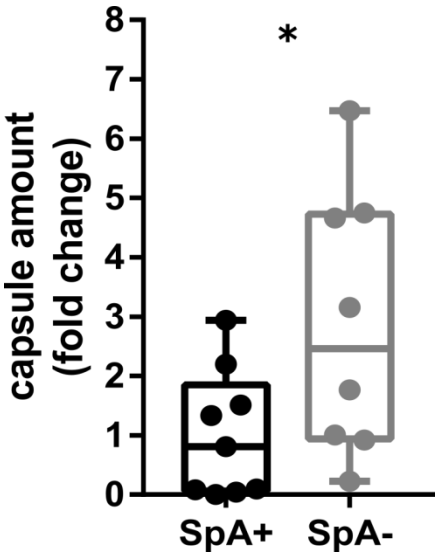

Figure 25. Capsule production among SpA^+ and SpA^- strains. Bars indicate the medians, whiskers the maximum and minimum values. All values are relative to the mean of SpA^+ strains levels. Statistical significance is calculated with t test.

5.8 SpA$^-$ phenotype does not influence biofilm production

Since the strains that do not express SpA produce a higher amount of capsule, we investigated whether the altered expression of these components could lead to altered biofilm formation. Previous studies showed that SpA has a role in biofilm formation in *Staphylococcus aureus* [114, 115]. Staphylococcal biofilms are usually encased in an extracellular matrix formed by Polysaccharide Intercellular Adhesin (PIA) or poly-N-acetylglucosamine (PNAG). Alternatively, biofilm formation can occur in a polysaccharide independent manner, in which SpA plays an essential role. This SpA-dependent biofilm was identified for the first time in an *arlRS* mutant, the two-component system that is naturally mutated in N315. It is reasonable to hypothesize that biofilm production in N315 strain could be SpA-dependent, as well as in other SpA$^+$ strains. Conversely, it is possible that the overexpression of the capsule in SpA$^-$ could play a role in biofilm formation. We addressed these hypotheses by measuring the biofilm formation in the USA100 subset of strain, using a N315 Δ*spA* to verify if SpA does have a role in biofilm formation. As shown in Fig.26, SpA deletion in N315 does not influence biofilm formation, which is comparable to the wt. Moreover, the totality of the other isolates shows lower levels of biofilm formation, irrespective of the presence or absence of SpA. This demonstrates that SpA is not necessary for N315 biofilm formation, and that the increased capsule amount in SpA$^-$ does not enhance it.

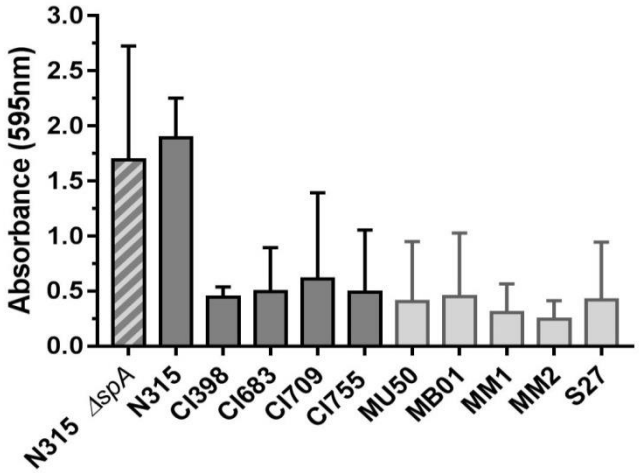

Figure 26. Biofilm formation in USA 100 isolates subset. The graph shows the absorbance relative to crystal violet staining, after 24h of incubation in 96 wells plates. The average of three independent experiments is represented for each strain. The error bars indicate the standard deviation.

5.9 SpA$^-$ strains are susceptible to phagocytosis mediated by capsule-specific antibodies

Knowing that SpA$^-$ and SpA$^+$ strains produce different amounts of capsule, we explored whether the quantity of capsule on the surface of the SpA$^-$ strains was enough to elicit the neutrophil uptake in the presence of capsule-specific antibodies. Mu50 and N315 were used as SpA$^-$ and SpA$^+$ reference strains respectively, and the phagocytosis assay was set up using capsule-specific rabbit antisera as described in materials and methods. Phagocytic uptake was visualized by confocal microscopy and quantified by Flow cytometry. Figure 27 shows a representative example of the interaction between Mu50 and neutrophils in different conditions. In the absence of complement and serum no bacteria were associated to neutrophils, and no phagocytosis was detected. The presence of both complement and specific serum resulted in the interaction of all the bacteria with the neutrophils, leading to an almost complete phagocytic uptake.

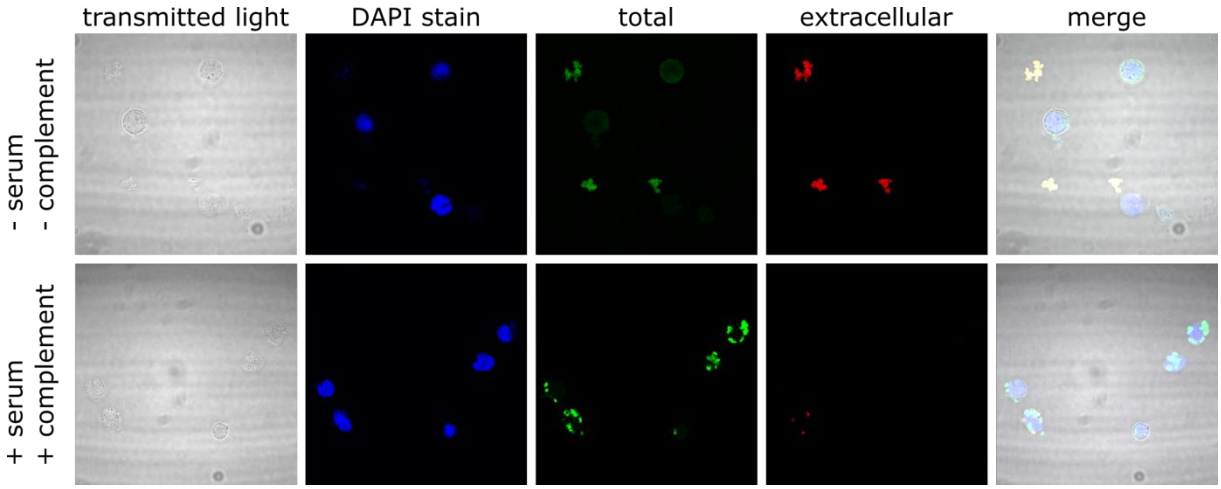

Figure 27. Effect of capsule specific serum and complement on Mu50 phagocytosis. Fixed bacterial cells were incubated with differentiated HL60 cells in the presence or absence of a source of complement and capsule specific antiserum. After 30' of phagocytosis the cells were fixed, and the samples stained with total anti *Staphylococcus aureus* antibodies. After cell permeabilization the samples were stained again, with Vancomycin BODYPIconjugate. Finally, DAPI stain was applied directly through the mounting medium. DAPI stain binds DNA and shows neutrophils nuclei and bacterial cells (with lower intensity). Total bacterial are shown by the staining after cell permeabilization, extracellular bacteria are shown by the staining before cell permeabilization.

The effect of different serum dilutions and the presence or absence of a complement source was tested on both Mu50 and N315 strains (Fig.28). The minimal level of uptake observed in absence of either complement or serum was used as baseline level for both strains. In the case of Mu50 strain, the addition of capsule-specific antibodies alone induced the internalization of the bacteria in a dose-dependent manner, while the phagocytic uptake of N315 strain remained similar in either presence or absence of serum. The presence of complement alone determined an increased uptake for both strains, but higher in Mu50: this is probably due to a reduced susceptibility to phagocytosis in the absence of complement of the encapsulated strain [116]. The addition of both specific serum and complement strongly increased the uptake of the Mu50 bacteria but not of those of the N315 strain. This inefficacy of capsule-specific antibodies is not due to an antiphagocytic effect of SpA because similar results were obtained in the N315 Δ*spA* control (data not shown). With these experiments we confirmed that SpA$^-$ strains express higher quantities of capsule, and that this capsule amount is enough to elicit phagocytosis in the presence of capsule-specific antibodies.

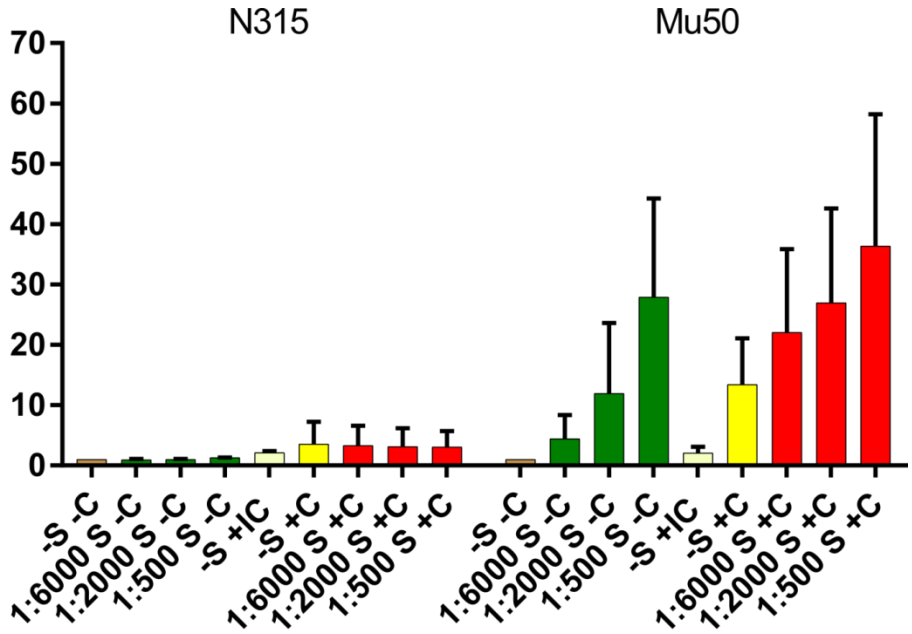

Figure 28. Effect of capsule specific antiserum on SpA$^+$ and SpA$^-$ phagocytosis. The graph shows the fluorescence associated to neutrophils after phagocytosis of fluorescent bacteria, under different conditions. The phagocytosis were performed in absence of both serum and complement (-S -C), in absence of complement and in presence of different serum dilution (S -C), in absence of serum and in presence of complement inactivated (IC), in absence of serum and in presence of complement (-S +C), or in presence of both complement and different dilutions of the serum (S+C). Each experiment was normalized by the corresponding –S –C sample.

6 Discussion

S. aureus is a major human pathogen, responsible for a wide range of diseases from both community and hospital acquired infections. The emergence of antibiotic resistant strains and the lack of alternative treatments to antimicrobials make it a recognized medical need. The vaccine approach has been considered promising, but no vaccine has yet been successfully developed to licensure. The reasons for this failure to date may be several and this should be taken into consideration in the perspective of the development of new therapies and interventions. *S. aureus* has evolved a plethora of immune evasion mechanisms, which impair host defenses and prevent bacterial clearance. Moreover, different strains can express different combinations of virulence factors, making it unlikely that strategies targeting single antigens result efficacious. Identifying a broadly expressed combination of antigens is therefore crucial for the development of new vaccine therapies. This study aims to address these concerns, investigating the expression of the recently proposed antigen SpA, and its interplays with other virulence determinants. SpA exerts several roles in staphylococcal infections. Its ability to bind IgGs through the Fc portion can prevent opsonophagocytosis by sequestering antibodies and by displaying them on the bacterial surface in an incorrect orientation [63, 64]. On the other hand, SpA can also bind the V_H3 domain of B cell receptors acting as a superantigen and thus leading to an impairment of the B cell response [66, 67]. Despite the crucial role of SpA in staphylococcal pathogenesis, the screening of a large library of strains, allowed us to identify a subset of strains not expressing SpA (SpA^-). These strains had heterogeneous origin and characteristics, and except for laboratory strains Reynolds and Lowenstein, all belong to the USA100/CC5 lineages which are associated to HA-MRSA infections. Genomic analysis of the SpA^- strains did not show mutations in *spA* or regulatory genes common to all SpA^- strains. However, in three of the analyzed strains we detected a mutation in the RBS of the *spA* gene that impairs the expression of SpA likely through affecting translational initiation of the protein. The fact that the two other SpA^- isolates carry an intact *spA* locus indicates that the genetic basis by which the protein is not expressed is diverse and may involve transcriptional regulation. SpA^- strains showed lower levels of *spA* transcript, indicating that the SpA^- phenotype is determined by changes in mRNA steady state regulation. Moreover, it seems that the regulatory layers by which the loss of SpA expression occurred are several. In fact the MM1 and MM2 strains, which carry the RBS mutation, have

similar *spA* transcription kinetic of SpA^+ strains but with lower steady state levels. Strains MB01, which carries the RBS mutation, and S27 exhibit a lower upregulation during growth, while *spA* transcription in Mu50 strain is largely void of *spA* transcription. This indicates that there are several independent factors that control both *spA* steady state level and its transcription kinetics, and that alterations in diverse regulators may contribute to the SpA^- phenotypes observed in the strains analyzed in this study. Many isolates of Staphylococcus have been described with polymorphisms within global regulators of virulence, for instance *agr* system, causing altered gene expression networks and resulting in heterogeneity in the circulating sub-populations.

Importantly the fact that the SpA^- phenotype has occurred repeatedly through diverse mechanisms in geographically distinct locations, suggests that the loss of SpA expression may represent the response to a selective pressure under specific conditions.

Given the major role of SpA in staphylococcal pathogenesis, we hypothesized that the loss of its expression may be associated to other changes in the virulence factors profile of the SpA^- strains. Through transcriptional profiling of a large number of virulence determinants, two other genes, *sdrC* and *sasD*, were identified as downregulated in the SpA^- strains. These two genes encode for cell wall anchored proteins, and thus belong to a similar regulatory network of *spA*. The major differentiating factor identified between SpA^+ and SpA^- strains resides in the upregulation of the capsule biosynthesis operon in SpA^- strains. It is important to notice that these differences are not the only transcriptional changes occurring in the SpA^- strains but represent a major relevant characteristic that is shared by all the SpA^- strains, suggesting that capsule upregulation is a common correlated adaptation. The analysis of the regulatory networks behind the expression of the genes identified in the transcriptional screening led to several observations. The regulators shared by *spA*, *sdrC* and *sasD* genes are known to affect the same regulatory activity on those three genes, confirming the fact that they are part of a similar regulatory network. Conversely, the analysis of the factors influencing *spA* and capsule transcription showed that several regulators determine opposite effects on those two virulence factors, indicating that multiple systems closely control inverse regulation to balance the expression of capsule and SpA. Alterations in any one of the factors involved in maintaining this tight balance would lead to the inverse shift that we observed between the SpA^- strains. This inverse regulation could be necessary due to their common localization on

the cellular surface and somewhat redundant nature of their functions, especially in relation to evading opsonophagocytosis. Risley et al. [117] showed how the presence of capsule on the bacterial surface masks another major surface protein Clumping factor A (ClfA), inhibiting its binding to fibrinogen probably through steric hindrance. Similarly to the effect on ClfA, it is possible that the capsule may interfere with SpA functions, and therefore the bacterium would express them in alternative way. Moreover, Nanra et al. [118] demonstrated that, in strains expressing both capsular polysaccharide and SpA, protein A does not elicit an anti-phagocytic effect towards anti-capsule specific antibodies. Nonetheless, expression kinetics in the *in vitro* growth curve shows a distinct temporal regulation, with *spA* transcribed mostly in exponential phase while the capsule biosynthetic genes were upregulated during stationary phase.

The genes identified in this study to be significantly altered in their transcription profiling between the Spa^- and Spa^+ strains (*spA*, *sdrC*, *sasD* and capsule) are all bound to and major components of the cell wall and it is reasonable that their expression must be finely-tuned in the context of cell wall synthesis. Interestingly, through the regulatory network analyses the regulators identified as 'fitting' the regulatory effects observed, namely v*raTSR, walR, stk1, stp1, rpoB, clpP* are involved in cell wall biosynthesis and often involved in the acquisition of low levels of vancomycin resistance in Vancomycin Intermediate Staphylococcus Aureus (VISA) isolates [110]. VISA strains acquire resistance through several cumulative mutations allowing the bacteria to reduce vancomycin susceptibility [13]. Those mutations can be different among the VISA isolates and are mostly associated to key regulatory genes that determine an increased thickness of the cell wall [13, 17, 110, 119]. This process is not only cumulative, but also reversible, indicating that there is a fitness cost that renders it advantageous only in determined condition [13, 17, 119]. Other features associated to the VISA phenotype have been reported, and in particular lower SpA expression and the higher capsule production [83, 119-122]. The production of capsular polysaccharide itself was shown to not alter the Vancomycin resistance [122], suggesting that its overexpression is the consequence of the rearrangements of the regulatory network modulating its transcription. The similar transcription profile shown by the strains analyzed in the present study led us to hypothesize that the SpA^- strains in our collection could have enhanced Vancomycin resistance. By performing a vancomycin resistance test we verified that the SpA^- analyzed in this study do not exhibit significant increase in Vancomycin resistance (data not shown),

although they share some phenotypic characteristic of the VISA isolates. Therefore, the overexpression of capsule in strains not expressing SpA is not a feature limited to the VISA phenotype.

It is possible that SpA^- phenotype, that involves two major virulence and immune evasion factors, has a strong influence on host pathogen interaction. Indeed, several studies, based on both clinical or laboratory evidences, reported that VISA strains are associated to reduced virulence [123-126]. Moreover, it seems that VISA strains are associated to lower risk of shock and reduced systemic inflammatory response [123], suggesting that those strains are less likely to cause acute clinical manifestation but more likely to be persistent [127, 128]. It was proposed that the phenotypic features of VISA strains may be the consequences not only of antimicrobial treatment, but also of changes in host pathogen interactions [119, 120]. In other words, the features exhibited by VISA phenotype suggest that the host environment has an impact in the adaptation of these isolates, prompting a combined resistance and persistent phenotype. Another staphylococcal phenotype associated to persistence is the Small Colony Variant (SCV) phenotype [127]. The SCV strains are associated with significant growth defects and other phenotypic changes, that lead to an increased persistence in host cell [129]. A recent comparative transcriptomic study, among other transcriptional changes, showed a strong increase in capsule gene transcription and a lower *spA* RNA in SCV clones compared to parental strain [130]. The presence of this common expression pattern in diverse clinically relevant phenotypes of *S. aureus* drives the hypothesis that the balance of SpA and capsule is a crucial feature in staphylococcal infection that contributes to determine virulence and host-pathogen interaction. Moreover, it is interesting to notice that despite the changes in expression of these two virulence factors, their genes are not affected by major mutations, suggesting that the bacterium may be able to switch from SpA^+ to SpA^- phenotype and *vice versa* to adapt to different conditions. It is worth noting that the SpA^- strains that we identified from 2 geographically distinct sets of clinical isolates are within the USA100/CC5 lineage associated with HA-MRSA strains, suggesting that the adaptation to a SpA^- phenotype may occur preferentially in hospital acquired infections.

In the context of vaccines research this peculiar balance is of major relevance when considering prophylactic strategies targeting one of these two major virulence factors, SpA or capsule. In particular, intervention strategies targeting solely SpA may not be successful

against all strains due to the identified population of SpA^- strains that are circulating and causing disease in geographically distinct locations. Here we show that a SpA^- strain is highly susceptible to the opsonic killing of anti-capsule antibodies, therefore suggesting that an intervention/vaccine strategy targeting both factors could be used for extending the coverage of a SpA–based vaccine to the broadest number of staphylococcal strains.

On the other hand, a vaccine strategy using capsular polysaccharide as unique antigen was already developed but failed in phase III clinical trial [90, 91]. Interestingly, the Lowenstein and Reynolds strains which exhibit SpA^- phenotype were used for assaying capsular functional antibodies in both vaccine development and in clinical readouts from vaccine intervention [131-135]. Indeed, these strains are optimal capsular test strains due to their SpA^- phenotype.

One of the reasons of the capsule-based vaccine failure could be the emergence of strains with no or low capsule, as we had shown that SpA^+ strains may be not susceptible to capsule specific antibodies due to the absence of the antigen. In particular the USA300 lineage, which in the past years became the predominant epidemic CA-MRSA strain in US, was shown to lack the capsular polysaccharide due to conserved mutations in the capsule biosynthetic operon [24]. The presence of a wide spread number of strains that differs in virulence, and the development of several phenotypes through the adaptation to the host suggest that a multicomponent strategy is fundamental in staphylococcal vaccine design. Using the high throughput qRT PCR approach we compared the transcription of a large number of selected virulence determinants in a panel of strain, obtaining a highly detailed and time resolved expression profile. Its analysis allowed us to identify peculiar interplays in the expression of virulence determinants, which would have been impossible to detect by genomic screening. From this study, we identify that SpA and capsule could be combined in a vaccine approach to effectively target a broader range of strains with phenotypic adaptations involving virulence determinants.

The implementation of this approach would provide a powerful tool for the evaluation of *S. aureus* virulence in a large number of strains and a useful support for the development of new therapies against this major human pathogen.

7 Materials and methods

7.1 Strains and growth conditions

S. aureus strains used in this study are listed in table 1. Staphylococcal strains were grown on plates at 37°C in trypticase soy agar (TSA) supplemented with 5% (v/v) of sheep blood. Liquid cultures were performed in tryptic soy brot (TSB, Dilfco Laboratories) at 37°C 250rpm, from a 0.05 optical density (OD_{600}) overnight preinoculum. Growth curves for each of the analyzed strains were performed in order to identify the OD corresponding to the diverse growth phases. The OD identified were OD_{600}=0.5 for early exponential phase, OD_{600}=2 for medium exponential phase, OD_{600}=4 for late exponential phase, OD_{600}=8 for early stationary phase, OD_{600}=10 for late stationary phase.

E. coli DH5α clones were grown at 37°C on LB plate or LB broth, supplemented with 100µg ml^{-1} of ampicillin if necessary.

7.2 Western blot analysis

S. aureus isolates were grown in liquid to OD_{600} = 0.6. Samples were pelletted and resuspended in Lysis buffer (Tris HCl 50mM, MgCl2 20mM, Raffinose penta-hydrate 30%) supplemented with complete protease inhibitor cocktail (Roche), then treated with lysostaphin 10' at 37°C. The suspensions were centrifuged at 6000g for 20'and the supernatant retained. Protein concentration was quantified through BCA. Equal protein amounts were loaded onto a 4-12% Bis-Tris precast gel (invitrogen) and then transferred onto a nitrocellulose membrane using iBlot Gel Transfer Device (ThermoFisher). For SpA detection the membrane was incubated with SpA-specific chicken antibodies, conjugated with biotin. The membrane was then incubated with HRP conjugated streptavidin and developed using Pierce ECL Western Blotting substrate (ThermoFisher). Alternatively SpA was detected as previously reported [48], probing the membrane with HRP-conjugated rabbit anti-mouse IgG (1:2,000 Dako).

7.3 Genomic DNA extraction, sequencing and assembly

Genomic DNA was extracted from overnight growth using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) and manufacturer instructions adapted for *S. aureus* DNA extraction. Overnight growth of *S.aureus* were pelleted and re-suspended in TSM buffer (50mM Tris-HCL pH 7.5, 10 mM MgCl2 and 0.5 M Sucrose), with the addition of 25µg of Lysostaphin.

The suspensions were incubated until lysis, before the addition of 2µl of proteinase K (GenElute Bacterial Genomic DNA Kit). The extraction continued following the kit instructions.

Sequencing was performed with HiSeq 2500 Sequencing System from Illumina and Paired-Ends.

Paired reads were assembled using CLC genomic work bench (Qiagen).

BRIGG analysis was performed for all the genomes, using either Mu50 or N315 as reference.

High resolution alignments of specific DNA regions were performed extracting the sequences using BLAST and then aligning them with MUSCLE algorithm in geneious software.

7.4 *spA* promoter reporter system

The promoter and the 5'UTR of *spA* gene were fused to mCherry in a pOS1 plasmid backbone [136]. The primers NWMN_0055_-266_EcoRI_F/ NWMN_0055_-1_R were used to amplify the *spA* promoter and 5'UTR region from Newman genomic DNA, while StamCh.R/StamCh.F primers were used to amplify the mCherry gene. The two amplicons were fused through fusion PCR thanks to the complementary sequences of StamCh.F and NWMN_0055_-1_R. The resulting fusion of spA promoter and 5'UTR-mCherry was cloned into the pOS1 vector using EcorI-PstI restriction sites generating the pOS1pspA. The pOS1pspA was modified to obtain the variant of the *spA* promoter lacking of the RBS by whole plasmid PCR. The primers RBS_KO_pspA_F and RBS_KO_pspA_R were designed to anneal on the region to be modified, but carrying the RBS mutation. The PCR product was digested by DpnI and transformed into *E. coli* DH5α competent cells and several clones were sequenced to identify plasmid containing the mutation, named pOS1pspARBS. DH5α clones carrying the empty pOS1 plasmid, the wt promoter 5'UTR-mCherry fusion and the variant containing the RBS mutation were grown overnight in LB + 100µg/ml ampicillin, and the fluorescence levels were measured at 610nm in three independent experiments.

7.5 RNA extraction and cDNA synthesis

Samples for RNA extraction were collected from *S. aureus* isolates grown in liquid to the needed growth phase and stabilized using RNAprotect Bacteria Reagent (QUIAGEN, Germany) according to the manufacturer's instructions. Bacterial pellets were then either directly processed or stored at-80°C. The bacterial pellet was resuspended in 1 ml of Trizol reagent (Ambion) and lysed in a FastPrep-24 homogenizer (MP Biomedicals) using three cycles of 60 s at 6.5 m s^{-2} followed by 5 min incubation on ice after each cycle. Chloroform was then added in 1:5 ratio, the suspension mixed and centrifuged for 15'. The resulting aqueous upper-phase was retrieved and the RNA was purified using the PureLink kit (Ambion) applying an on-column DNase digestion step using the RNase-free DNase kit (QIAgen) according to the manufacturer's instructions. Residual DNA was removed by a second DNase treatment using RQ1 DNase (Promega) followed by a second RNA purification using the PureLink kit. RNA quality was assessed by gel electrophoresis and the absence of residual DNA was confirmed by qRT-PCR. cDNA synthesis was performed with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen-Life Technologies) according to the manufacturer's instructions, using random hexamer primer for reverse transcription (RT) on 300 to 2000 ng of total RNA.

7.6 Virulence factor transcription profile

The virulence factors transcription profile was assessed using the high-throughput qRT-PCR system BIOMARK HD (Fluidigm), with 83 TaqMan assays specific to virulence related genes (Table 3). To carry out the experiment in the BIOMARK system is necessary to perform a preamplification step in order to have samples with enough concentrated DNA template. Preamplification of the samples was performed with pooled primers relative to the genes to be tested in the assays. 2,5 ng of cDNA were amplified using the following cycling parameters: 95°C for 10 min; 10 cycles of 95°C for15s and 60°C for4 min. Each sample was diluted 1:5 using TE buffer and loaded onto a 48.48 Dynamic Array IFC (Fluidigm), following manufacturer instructions. Two Chips containing different sets of assays were used, both containing two technical replicates of the housekeeping gene *gyrB*. Two controls with TE and Tris diluted preamp mix respectively were included in each Chip. A Tris control was also included in the assays set. Raw data were manually checked for signals in the negative controls. *gyrB* was used as housekeeping reference gene, and each assay was normalized to

the mean of the *gyrB* replicates for each Chip. For the transcription kinetic profiles all the samples were normalized to the early stationary phase using the ΔΔct method. Clusterization of genes or samples was performed using the Multiple array viewer (Mev) application with Hierarchical Clustering, Pearson correlation as distance metric.

Comparative transcriptional analysis between SpA^+ and SpA^- strains was performed using GraphPad Prism 7 software as follows: the difference between mean Δct for the SpA^+ and SpA^- strains was calculated for each gene at each time points; two-way ANOVA was used to establish the significance of the difference comparing values at same time points, and correcting for multiple comparisons by Sidak correction.

7.7 qRT-PCR

qRT-PCR was done using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen-Life Technologies) using ROX as internal control on a STRATAGEN Mx3000P QPCR system using the following cycling parameters: 95°C for 10 min; 45 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s; 95°C for 1 min, 55°C for 30 s and finally 95°C for 30 s. Final data were analysed with the Δct method, normalizing samples to the expression levels of *gyrB*. Statistical significance was determined by t-test using GraphPad Prism 7 software.

7.8 Capsule immunoblot

S. aureus isolates were grown to OD_{600}=12, and then pelletted. The bacterial pellets were resuspended in 0.5% SDS, 5mM DTT, 100mM Tris and treated with proteinase K for 1h at 45°C. Serial dilutions were loaded onto a nitrocellulose membrane, using a dot blot apparatus. The membrane was blocked with PBS-Milk 10%-Tween20 0.05% and then incubated with CP5-specific rabbit antiserum, followed by goat anti-rabbit HRP. The blots were developed using the Pierce ECL Western Blotting substrate (ThermoFisher), the image acquired using Chemidoc (BIORAD) and analysed with Imagelab (BIO RAD). Statistical significance was determined by t-test using GraphPad Prism 7 software.

7.9 Knock out strain generation

N315 and Newman Δ*spA* strains were created by allelic exchange using the pIMAY system [137]. This system was created to obtain clean gene deletions, and is based on the integration of the plasmid in the target DNA region and its following excision. The origin of replication of the pIMAY plasmid is highly temperature sensitive in staphylococci, as it allows the replication of the plasmid at temperatures below 30°C, while at 37°C plasmid integrants are selected. The excision of the plasmid at 28°C can generate two different alternative sequences: the wt sequence or a clean deletion of the target sequence. Anhydrotetracycline-mediated induction of the *secY* antisense RNA prevents the growth of cells that retain the integrated plasmid and selects for the clones that lost the pIMAY [138]. For the generation of the *spA* mutant the upstream and downstream regions of the *spA* gene were amplified using two pairs of primers (spA_ko_DS_F/spA_ko_DS_R and spA_ko_US_R/spA_ko_US_F), fused together by fusion PCR and cloned into pIMAY vector using KpnI and SacI restriction sites. The plasmid was amplified in DC10B *E.coli* strain at 37°C and transformed into the intermediate *S. aureus* RN4220 at 28°C [137]. The plasmid was then amplified in the RN4220 background at 28°C and transformed into N315 and Newman strain. The transformants were grown overnight in TSB + 10 µg ml^{-1} chloramphenicol at 28°C for plasmid amplification. The positive clones were inoculated in new pre-warmed TSB + 10 µg ml^{-1} chloramphenicol medium and grown overnight at 37°C to allow the integration of the plasmid. The cultures were plated onto TSA plates + 10 µg ml^{-1} chloramphenicol and grown overnight at 37°C. The resulting clones were inoculated in TSB medium without antibiotic, grown overnight at 28°C, and plated onto TSA plates + 1µg ml^{-1} anhydrotetracycline. The bigger colonies (the ones that did not show growth inhibition) were purified and tested for *spa* deletion by colony PCR using the spA-verif_F/ spA-verif_R pair of primers. The positive clones were then tested by western blot for the absence of SpA production.

7.10 Biofilm formation test

The strains to be analyzed were inoculated in 200µl of TSB, with a 1:40 dilution from an overnight liquid growth in a 96 well plate. The plate was left at 37°C for 24h to allow biofilm formation. The plate was washed three times in water, and then dried. The samples were incubated 3' in 100ul of crystal violet, and then the plate was rinsed 3 times in water, and dried. The dye was dissolved in 200ul of ethanol/acetone (80/20) and the absorbance was measured at 595nm using Tecan. Three independent replicates of the experiment were performed, with three technical replicates for each experiment.

7.11 Phagocytic uptake experiment

Phagocytic uptake was measured using a protocol similar to the one already described by Nordenfelt et al. [139].Overnight cultures were fixed in 1% paraformaldehyde and counted by flow cytometry (SOS1). Fixed bacteria were stained with FM-64fx (ThermoFisher) for 15' in the dark, excess of fluorochrome was removed by washing in PBS.10^7 stained bacteria were incubated with rabbit anti capsule serum, 1% guinea pig complement and 10^6 HL60 differentiated cells for 30' in the dark. Cells were then washed in PBS and fixed in 1% paraformaldehyde for 20'. The samples were then washed and incubated in PBS 1%BSA and 1:100 anti-S*taphylococcus aureus* polyclonal antibodies (ThermoFisher) for 1h 4°C in the dark. After a wash in PBS BSA, the samples were incubated in Rhodamine (TRITC) F(ab')2 Fragment Goat Anti-Rabbit IgG (Jackson immunoresearch), for 30' at 4°C. After a wash in PBS BSA and a second wash in PBS, the samples were re-suspended in PBS and read at FACS SOS1. Cells population was gated using forward scatter versus side scatter and then singlets were gated with SSCA vs SSC. Neutrophils with only internalized bacteria were gated selecting for FM64FX positive and TRITC negative events. For each sample, 10000 events were analyzed using FlowJo software. In each sample, the delta geometric mean of the positive and negative populations was multiplied by the percentage of positive neutrophils, giving an estimate of the bacteria internalized for each sample.

Table 1 . Staphylococcal strain used in this study

Strain	Description	Reference
Newman	Clinical strain, MSSA, CC8, ST254, CP5	Baba *et al. J Bacteriol* 2008
LAC	Clinical strain, MRSA, USA300, SCCmec, IV, CC8, ST8, CP5	Miller *et al. N Engl J Med* 2005
MW2	Clinical strain, MRSA, USA400, SCCmec, IV, CC1, ST1, CP8	Baba *et al. Lancet* 2002
Mu50	Clinical strain, HA-VR-MRSA, USA100, SCCmec, II, CC5, ST5, CP5	Hiramatsu *et al. J Antimicrob Chemo*
Staph19	Clinical strain, MRSA, SCCmec, IV, ST80, CP8	Bagnoli *et al. PNAS* 2015
NRS216	Clinical Strain, MSSA, ST30, CC30, CP8	Bagnoli *et al. PNAS* 2015
Reynolds	Laboratory strain, MSSA, CC25, ST25, CP5	Karakawa *et al. J Clin Microbiol* 19
Wright	Laboratory strain, MSSA, CP8	Bagnoli *et al. PNAS* 2015
Becker	Laboratory strain, MSSA, CP8	Cook *et al. Hum Vaccin* 2009
ATCC6538	Laboratory strain, MSSA, CC97, ST467, CP5	Bagnoli *et al. PNAS* 2015
Lowenstein	Laboratory strain, MSSA, CC25, ST25, CP5	Fattom *et al. Infect Immun* 1990
BD1686	Clinical strain, HA-MRSA, USA100, CC5, ST5, CP5	Bagnoli *et al. PNAS* 2015
BD1534	Clinical strain, HA-MRSA, USA200, CC30, ST36, CP8	Bagnoli *et al. PNAS* 2015
BD1449	Clinical strain, CA-MRSA, USA1000, CC59, ST59, CP8	Bagnoli *et al. PNAS* 2015
NRS382	Clinical strain, HA-MRSA, USA100, CC5, ST5, CP5	Bagnoli *et al. PNAS* 2015
NRS248	Clinical strain, MRSA, CC1, ST1, CP8	Bagnoli *et al. PNAS* 2015
NRS252	Clinical strain, MSSA, CC30, ST30, CP8	Bagnoli *et al. PNAS* 2015
Staph 15	Clinical strain, CA-MRSA, ST30, CC30, CP8	Bagnoli *et al. PNAS* 2015
Staph 17	Clinical strain, CA-MRSA, ST8, CC8, CP5	Bagnoli *et al. PNAS* 2015
Staph 18	Clinical strain, CA-MRSA, ST8, CC8, CP5	Bagnoli *et al. PNAS* 2015
Staph 21	Clinical strain, CA-MRSA, SCCmec, IV, ST80, CP8	Bagnoli *et al. PNAS* 2015
MSSA 94 ISS	Clinical strain, MSSA, CC22, ST22, CP5	Bagnoli *et al. PNAS* 2015
IT-SA1	Clinical strain from infective endocarditis, CC30, ST30, CP8	Rindi *et al. Eur J Clin Invest* 2006
IT-SA2	Clinical strain from infective endocarditis, CP8	Rindi *et al. Eur J Clin Invest* 2006
IT-SA3	Clinical strain from infective endocarditis, CC101, ST101, CP8	Rindi *et al. Eur J Clin Invest* 2006
IT-SA4	Clinical strain from infective endocarditis, CC30, ST34, CP8	Rindi *et al. Eur J Clin Invest* 2006
IT-SA5	Clinical strain from infective endocarditis, CC8, ST8, CP5	Rindi *et al. Eur J Clin Invest* 2006
IT-SA6	Clinical strain from infective endocarditis, CC5, ST5, CP5	Rindi *et al. Eur J Clin Invest* 2006
IT-SA7	Clinical strain from infective endocarditis, CC20, ST20, CP5	Rindi *et al. Eur J Clin Invest* 2006
IT-SA8	Clinical strain from infective endocarditis, CC15, ST15, CP8	Rindi *et al. Eur J Clin Invest* 2006
IT-SA9	Clinical strain from infective endocarditis, CC121, ST120, CP8	Rindi *et al. Eur J Clin Invest* 2006
IT-SA10	Clinical strain from infective endocarditis, CC15, ST15, CP8	Rindi *et al. Eur J Clin Invest* 2006
IT-SA11	Clinical strain from infective endocarditis, CC5, ST5, CP8	Rindi *et al. Eur J Clin Invest* 2006
IT-SA12	Clinical strain from infective endocarditis, CC72, ST72, CP5	Rindi *et al. Eur J Clin Invest* 2006
IT-SA14	Clinical strain from infective endocarditis, CC5, ST5, CP5	Rindi *et al. Eur J Clin Invest* 2006
IT-SA15	Clinical strain from infective endocarditis, CC45, ST45, CP8	Rindi *et al. Eur J Clin Invest* 2006
IT-SA16	Clinical strain from infective endocarditis, CC72, ST72, CP8	Rindi *et al. Eur J Clin Invest* 2006
IT-SA17	Clinical strain from infective endocarditis, CC121, ST120, CP8	Rindi *et al. Eur J Clin Invest* 2006
IT-SA18	Clinical strain from infective endocarditis, CC5, ST5, CP5	Rindi *et al. Eur J Clin Invest* 2006
IT-SA19	Clinical strain from infective endocarditis, CC5, ST5, CP5	Rindi *et al. Eur J Clin Invest* 2006
SW-ST239-III	Clinical strain, MRSA, SCCmec, III, CC8, ST239, CP8	Bagnoli *et al. PNAS* 2015
SW-ST80-IV-PVL	Clinical strain, MRSA, SCCmec, IV, pvl+, ST80, CP8	Bagnoli *et al. PNAS* 2015
SW-ST5-IV-PVL	Clinical strain, MRSA, SCCmec, IV, pvl+, CC5, ST5, CP5	Bagnoli *et al. PNAS* 2015

Table 1 (continued). Staphylococcal strain used in this study

Strain	Description	Reference
SW-ST30-PVL	Clinical strain, MRSA, pvl+, CC30, ST30 CP8	Bagnoli *et al. PNAS* 2015
SW-ST398	Clinical strain, MRSA, CC398, ST398, CP5	Bagnoli *et al. PNAS* 2015
SW-ST228-I	Clinical strain, MRSA, CC5, ST288, CP5	Bagnoli *et al. PNAS* 2015
SW-ST8-IV	Clinical strain, MRSA, CC8, ST8, CP5	Bagnoli *et al. PNAS* 2015
SW-ST88	Clinical strain, MRSA, CC88, ST88, CP8	Bagnoli *et al. PNAS* 2015
SW-ST45	Clinical strain, MRSA, CC45, ST45, CP8	Bagnoli *et al. PNAS* 2015
SW-ST42	Clinical strain, MSSA, Singleton, ST42, CP8	Bagnoli *et al. PNAS* 2015
SW-ST152	Clinical strain, MRSA, CC152, ST152, CP5	Bagnoli *et al. PNAS* 2015
SW-ST59	Clinical strain, MRSA, CC59, ST59, CP8	Bagnoli *et al. PNAS* 2015
BW01	Clinical strain, HA, USA 100	this study
CF Serology 29	Clinical strain, HA, USA 100	this study
CF Serology 33	Clinical strain, HA, USA 100	this study
CI 1434	Clinical strain, HA, USA 100	this study
CI 1438	Clinical strain, HA, USA 100	this study
CI 1492	Clinical strain, HA, USA 100	this study
CI 334	Clinical strain, USA 100	this study
CI 394	Clinical strain, HA, USA 100	this study
CI 398	Clinical strain, HA, USA 100	this study
CI 482	Clinical strain, USA 100	this study
CI 683	Clinical strain, HA, USA 100	this study
CI 697	Clinical strain, USA 100	this study
CI 709	Clinical strain, HA, USA 100	this study
CI 755	Clinical strain, USA 100	this study
CI 846	Clinical strain, HA, USA 100	this study
CO01	Clinical strain, USA 100	this study
DH1	Clinical strain, HA, USA 100	this study
DL1	Clinical strain, HA, USA 100	this study
DL2	Clinical strain, HA, USA 100	this study
DLBAL-L	Clinical strain, HA, USA 100	this study
DLBAL-R	Clinical strain, HA, USA 100	this study
GM1	Clinical strain, HA, USA 100	this study
GM2	Clinical strain, HA, USA 100	this study
JMH1	Clinical strain, HA, USA 100	this study
M016 LIM	Clinical strain, CA, USA 100	this study
MB01	Clinical strain, HA, USA 100	this study
MM1	Clinical strain, HA, USA 100	this study
MM2	Clinical strain, HA, USA 100	this study
RT1	Clinical strain, HA, USA 100	this study
SGBAL	Clinical strain, HA, USA 100	this study
CF Serology 22	Clinical strain, CA, USA 300	this study
CF Serology 26	Clinical strain, CA, USA 300	this study
CR01	Clinical strain, HA , USA 300	this study
DB1	Clinical strain, HA, USA 300	this study

Table 1 (continued). Staphylococcal strain used in this study

Strain	Description	Reference
KC1	Clinical strain, HA, USA 300	this study
LH01_Hem	Clinical strain, HA, USA 300	this study
MB02_Hem	Clinical strain, HA, USA 300	this study
MG	Clinical strain, HA, USA 300	this study
MM01	Clinical strain, HA, USA 300	this study
RF01	Clinical strain, HA, USA 300	this study
TH1	Clinical strain, HA, USA 300	this study
M 299 LIM	Clinical strain, CA, USA 700	this study
SG4NQ	Clinical strain, CA, USA 700	this study
SG6NH	Clinical strain, CA, USA 700	this study
SG6NM	Clinical strain, CA, USA 700	this study
SG9NS	Clinical strain, CA, USA 700	this study
WB9nm	Clinical strain, CA, USA 700	this study
WB9NO	Clinical strain, CA, USA 700	this study
WB9Nq	Clinical strain, CA, USA 700	this study
WB4NT	Clinical strain, CA, USA 1100	this study
TR10NK	Clinical strain, CA, USA 1100	this study
TR10NL	Clinical strain, CA, USA 1100	this study
Serology 2	Clinical strain, CA, USA 300	this study
Serology 12	Clinical strain, CA, USA 100	this study
Serology 16	Clinical strain, CA, USA 100	this study
Serology 21	Clinical strain, CA, USA 500	this study
Serology 22	Clinical strain, CA, USA 200	this study
Serology 23	Clinical strain, CA, USA 500	this study
Serology 27	Clinical strain, CA, USA 100	this study
Serology 28	Clinical strain, CA, USA 300	this study
Serology 36	Clinical strain, CA, USA 200	this study
CF Serology 1	Clinical strain, HA, USA 200	this study
CF Serology 2	Clinical strain, HA, USA 400/700	this study
CF Serology 5	Clinical strain, HA, USA 200	this study
CF Serology 10	Clinical strain, HA, USA 300	this study
CF Serology 11	Clinical strain, HA, USA 400	this study
CF Serology 16	Clinical strain, HA, USA 900	this study
CF Serology 25	Clinical strain, HA, USA 1000	this study
CF Serology 30	Clinical strain, HA, USA 400	this study
CF Serology 31	Clinical strain, HA, USA 300/200	this study
CF Serology 32	Clinical strain, HA, USA 800	this study
CF Serology 34	Clinical strain, HA, USA 400	this study
RN4220	Laboratory strain, restriction-deficient mutant	Kreiswirth *et al. Nature* 1983
N315 Δ*spA*	N315 derivative, lacking *spA* gene	this study

Strains from BW01 to CF Serology 34 were provided by Isaac Thomsen and C. Buddy Creech from Vanderbilt University School of Medicine.

Table1. plasmid used in this study

Name	Description	Antobitic resistance	Reference
pOS1	Ori+ for Gram-positive strains; Ori− (pBR322) plasmid replication in Gram-negative bacteria	chloramphenicol/ ampicillin	Schneewind *et al.*EMBO J
pOS1pspA	pOS1 derivative harboring spA promoter and 5'UTR fusion with mCherry reporter gene	chloramphenicol/ ampicillin	this study
pOS1pspARBS	pOS1pspA derivative carriyng the 5'UTR variant lacking of the RBS	chloramphenicol/ ampicillin	this study
pIMAY	plasmid vector for allelic replacement; Temperature-sensitive Gram-positive replicon, tetracyclin inducible *secY* antisense	chloramphenicol	Monk *et al.* MBio, 2012
pIMAYspAUSDS	pIMAY derivative for *spA* deletion	chloramphenicol	this study

Table 2. Oligonucleotides used in this study. Low case letters shows restriction sites, underlined letters correspond to annealing regions in fusion PCR

Name	Sequence	Restriction site	Application	Reference
StamCh.F	ATGGTGTCAAAAGGTGAAGAAGATAATATG	N/A	amplification for fusion to Staph promoters	this study
StamCh.R	GCTTGGctgcagTTATTTGTATAATTC	PstI	amplification for fusion to Staph promoters	this study
NWMN_0055_-266_EcoRI_F	ATCCGGgaattcGAAATTAAACCTCAGCACATTCAAAG	EcoRI	SpA promoter for mCherry fusion	this study
NWMN_0055_-1_R	CTTCTTCACCTTTTGACACCATATTAATACCCCCTGTATG TATTTGTAAAG	N/A	SpA promoter for mCherry fusion	this study
RBS_KO_pspA_F	ACAAATACATAATTAATATGGTGTCAAAAGGTGAAGAA G	N/A	RBS deletion pOS1pspA	this study
RBS_KO_pspA_R	CATATTAATTATGTATTTGTAAAGTCATCATAATATAAC G	N/A	RBS deletion pOS1pspA	this study
spA_ko_DS_notI_F	TATATAgcggccgcATTTAATTGGTGCAACTGGGAC	NotI	*spA* KO	this study
spA_ko_US_notI_R	ATATATgcggccgcTTGCAGATCAAAGTGAATCACAG	NotI	*spA* KO	this study
pIMAY_notI_F	ATATAgcggccgcCAGCTTTTGTTCCCTTTAGTGAGG	NotI	pIMAY linearization	this study
pIMAY_notI_R	ATATAgcggccgcCAATTCGCCCTATAGTGAGTCG	NotI	pIMAY linearization	this study
capA_rt_F	TATCAACATCCAAGTTAAAAGTGG	N/A	qRT PCR *capA*	this study
capA_rt_R	TCCAATATAACTGTATTCACCAATG	N/A	qRT PCR *capA*	this study
cap5H_rt	GAAAAACCAGTCCTCTAAAGAATC	N/A	qRT PCR *cap5H*	this study
capA	GGTGCGACTTTAACTGCTG	N/A	qRT PCR *cap5H*	this study

Table 3. TaqMan assays used in this study

Assay name	Newman gene	Oligo function	Sequence	Function-notes
adsA	NWMN_0022	Forward primer Reverse primer Probe	CATCACTTTCACCACGAATGTTTG GCGTCTAACATTAAATCAGGCTTTTC TTACATACAAATGATATCC	adenosine synthase A
agrA	NWMN_1946	Forward primer Reverse primer Probe	CTCGCAACTGATAATCCTTATGAGG GTAACGAAAATAATGTTACCAACTGGG GATATTCAACTTTCAACTG	AgrA - accessory gene regulator A
ahpC	NWMN_0372	Forward primer Reverse primer Probe	GAAATCTTACCATTTACAGCGCAAG GCCTAATTTTTGTAATTCTTCATATTGG CTATCCTGCTGACTTCTC	Alkyl hydroperoxide reductasesubunit C
asp23	NWMN_2086	Forward primer Reverse primer Probe	CAAGCATACGACAATCAAACTGGTG GCAGCGATACCAGCAATTTTTTC CGTCAAAAACAACAAGAAC	alkaline shock protein 23
atl	NWMN_0922	Forward primer Reverse primer Probe	CGAAACAGCACCAACGGATTACTTA CAGCATAGTTATTCATTGAACGTGCAA ACTGCACCGACACCC	autolysin
aur	NWMN_2536	Forward primer Reverse primer Probe	GAGCACTTTATCACCAGCAGCATTAG GTTTTTACATCAGTAACAGCGTAATCTTG GAGGTGACTCAAAAGAG	zinc metalloproteinase aureolysin
cap5H	NWMN_0102	Forward primer Reverse primer Probe	GCTGAAAAACCAGTCCTCTAAAGAATC CAAATCCAATATAACTGTATTCACCAATG TAAGATTCATCGCTTGG	capsular polysaccharide 5
capA	NWMN_0095	Forward primer Reverse primer Probe	CAACTTATCAACATCCAAGTTAAAAGTGG TTTGGTGCGACTTTAACTGCTG CCGAAGATTATGAGTG	capsular polysaccharide synthesis enzyme
capA2	NWMN_2563	Forward primer Reverse primer Probe	GCCTGACAAATATACTGCTTCTACTC GACTGCAAACTACTTTGAACATTTTGG GTCCTCAAGTGATTTAG	capsular polysaccharide biosynthesis protein capA2
chp	NWMN_1877	Forward primer Reverse primer Probe	GGAATCAGTACACACCATCATTCAG ATTTCTCAAACGTTCATCTAATTTTCC CCGTTTCCTACAAATG	chemotaxis-inhibiting protein CHIPS
clfA	NWMN_0756	Forward primer Reverse primer Probe	CAACGAATCAAGCTAATACACCG GTTGTTGAAACATTTTCCGCATTTG GTGAATCAAACAAGTAATG	clumping factor A, fibrinogen-binding protein A
clfB	NWMN_2529	Forward primer Reverse primer Probe	GGATAGGCAATCATCAAGCACAAG GCTATCTACATTCGCACTGTTTGTG CAATATGATAGAAACACC	clumping factor B
coa	NWMN_0166	Forward primer Reverse primer Probe	GAAATAAAACCACAAGGTACTGAATCAACG GCTTCATATCCAAATGTTCCATCG CAATTTAACAAAACACC	staphylocoagulase
eap	NWMN_1872	Forward primer Reverse primer Probe	CGAAAATAAAGCTAAAAGAAACTATCAAG GCTTTCTTAGCATATTTTAAATCTTGTTCAC CATTTTCAAATAAACCTTG	MHC class II analog protein
ebpS	NWMN_1389	Forward primer Reverse primer Probe	AAAGGTGCAGCGATCGGT GCGGCAGAAGCACTTTTACTTG CTCCAGCCAAACCTG	elastin binding protein
esaA	NWMN_0220	Forward primer Reverse primer Probe	GTTGCTGAGTCTGGTTTGAAAAATGG GATATTTTCGATGGTGTTTTAGCGTC CATGATTGTTATCCCAG	essA, TVIISS
esaB	NWMN_0221	Forward primer Reverse primer Probe	CAGCACGTAAAAGTAACATTTGATTTTAC TTTCAACAAGTAATTGACCTTTCGTC ATAATTACGGCACATATG	esaB, TVIISS
essA	NWMN_0221	Forward primer Reverse primer Probe	CGACTCGCTTGAATGAAACTAAAAAAGTG CCCCTACAGACATCAAAATGTACG AAGACTTCGGAGAGTG	essA, TVIISS

Table 3 (continued). TaqMan assays used in this study

Assay name	Newman gene	Oligo function	Sequence	Function-notes
essB	NWMN_0222	Forward primer Reverse primer Probe	GATTCCTAAGTCTTCAATTAAACCAGAAC GGTGTATGATTGTCATTAATGTCATAATG CCATATTTTATAGATGCTG	essB, TVIISS
essC	NWMN_0223	Forward primer Reverse primer Probe	TTCGCCAAGGATTATTCACCGTG CTAATGGCGGTATAATGGAACG CAATACAGAAGAACAATAC	essC, TVIISS
esxA	NWMN_0219	Forward primer Reverse primer Probe	GAGTCCAGAGGAAATCAGAGCAAAA CCTTGTGCACGTGTTAAATCAGATAAAA CTTGCCCGTAAGATTG	esxA secreted protein
esxB	NWMN_0225	Forward primer Reverse primer Probe	TCGCTGAGTATATCGAAGGTAGTGA CGGTTGTACTAATTCTTCTTGAAACTTTGC TTGGCGAACTGTCCTTC	esxB secreted protein
eta	NWMN_1082	Forward primer Reverse primer Probe	GCTTTCTTGATTTGGATTCACCTTTTATG GCCAGACATGAAAAATGTTGTGAACAC AATAATGTGAAAGAACAAT	exfoliative toxin A
fbp	NWMN_1119	Forward primer Reverse primer Probe	CACAAAATCAATCAACCTGATAATGAC AATCTTGAAAAGTTTGGATGGATTGAC AAAATAGACAAAACCATC	fibronectin/fibrinogen-binding protein
fhuD2	NWMN_2185	Forward primer Reverse primer Probe	CCAACAGTAGTTGTTGACTATAATAAGCA GCAGTTGTTTCTTCCCAATCTTTCTT CTTTACCAACAATTTTCC	hydroxamate siderophore binding lipoprotein
FLIPr	NWMN_1067	Forward primer Reverse primer Probe	GCCAAGGTGATGTGAAGAAAGCAG CGAGTCGATTTCACCGTTTTTAACAAC GTCCTTTAGAAGAGAACAG	FLIPr
fnbA	NWMN_2399	Forward primer Reverse primer Probe	ATTGAAACAATAGAAGAAACGGATTCATC CTTCAAAGTCAATTGGATTTGATTCCTC CCATACTGCTGTGGATAG	fnbA, C-term truncation in Newman
fnbB	NWMN_2397	Forward primer Reverse primer Probe	GCGACATCAACTGAGCAACCATC GAAGTTTCTACTTTTGGTGCTTGCACAG TAACAACAGAAGAAGCAC	fnbB, C-term truncation in Newman
geh	NWMN_0262	Forward primer Reverse primer Probe	CACATCAAATGCAGTCAGGAAAGC CTTGTCGTTCAGAATCTTGCTTTACTTG GTGGAACAGTGACAGAAG	triacylglycerol lipase
gyrB	NWMN_0004	Forward primer Reverse primer Probe	GGTGACTGCATTGTCAGATGTAAAC CTGCTTCTAAACCTTCTAATACTTGTATTTG CCCAGCACCATAATTA	DNA gyrase subunit B
hla	NWMN_1073	Forward primer Reverse primer Probe	TATAGTCAGCTCAGTAACAACAACA TGCATGCCATTTTCTTTATCATAAGTGAC ATGCCGCAGATTCT	alpha-hemolysin precursor
hlb	NWMN_1926	Forward primer Reverse primer Probe	GTAATATTCAATGAAGCATTTGATAATGG GGATATTTACTTACAATCGCTACGCC CTGAAGGTAGCTACTCATC	beta-hemolysin
hld	NWMN_2624	Forward primer Reverse primer Probe	TTAAGGAAGGAGTGATTTCAATGG TGAATTTGTTCACTGTGTCGATAATC GATATCATTTCAACAATC	delta-hemolysin, RNAIII
hlgA	NWMN_2318	Forward primer Reverse primer Probe	GCAGAAAATAAGATAGAAGATATCGG TCAAATTGAATGTTTTGAGTTATAGC GTGCAGAAATCATCAAAAG	gamma-hemolysin component A
hlgB2	NWMN_2320	Forward primer Reverse primer Probe	GCTACTGGGAATATTAACTCAGGCTTTG GTGCATAATCAACGACGTTTACTGAATC GGGAGCTAAATACAATG	gamma hemolysin, component B
hlgC	NWMN_2319	Forward primer Reverse primer Probe	GATACTGAAGACATCGGTAAAGGAA AGAGCTAATGAATCCTTGCATCTTTAA TTATCAAAAGGACAGAAG	gamma-hemolysin component C

Table 3 (continued). TaqMan assays used in this study

Assay name	Newman gene	Oligo function	Sequence	Function-notes
hlgC2	NWMN_2319	Forward primer Reverse primer Probe	GATAAAACAAGTAATAAATGGGGCGTGAC GAATGGCCATCGCATAGCTTTAAC GATATTAAAGATGCAAGG	gamma-hemolysin component C
hysA	NWMN_2106	Forward primer Reverse primer Probe	GGAATGGATTTTGAAAATCAGGAC GATGAATCAGTACTTTTAATGCCAGTTCC CATATTTCATATTAAACG	hyaluronate lyase
icaB	NWMN_2567	Forward primer Reverse primer Probe	GGATGGTCATCATATTGCAAATGCA AATTCGCTTTTCTTACACGGTGATAATTT CCAGAGCACTATTTTC	intercellular adhesion protein IcaB
isaA	NWMN_2469	Forward primer Reverse primer Probe	CGTTGATCAAGCACACTTAGTTGACTTAG GCTCCATGACCATGTAGTACCATTTGAAG CTCCAATCAAAGATGGTG	immunodominant antigen A
isaB	NWMN_2537	Forward primer Reverse primer Probe	GGCAAGGACTTGAAAAAAGAAAATGGT CGACAACTCTATTATGATCAACGACAAAC ACCGCTATCAGCTTCC	immunodominant antigen B
isdA	NWMN_1041	Forward primer Reverse primer Probe	GCAGTTGAACCTGGATATAAGAGCTTA TGCTTTTTCAAATTCCAAATGCGTAGT TCGTGCCACAAATTAA	iron-regulated heme-iron binding protein
isdB	NWMN_1040	Forward primer Reverse primer Probe	GGAGAAAATTTGAAGTTTATGAAGGTGACA TGTTTTCGCTTTTTTATATGGCGCTAA CAGTGCAGATAAATTC	iron-regulated heme-iron binding protein
isdC	NWMN_1042	Forward primer Reverse primer Probe	TAATTATCATCATCGCGACATTCAG CCATTTTTCTTAATGTACTTTGCCGG CAATACCAATGACACGTC	iron-regulated cell surface protein
isdG	NWMN_1047	Forward primer Reverse primer Probe	CGAGACATGGGATTGAAACATTAGAAG GGGCTACTTTCATCTTCATTTTTACTTC ACAGTTTGGAAATCAAA	cytoplasmic heme-iron binding protein
isdH	NWMN_1624	Forward primer Reverse primer Probe	GTTGCATCGGTCATTGTCAGTAC GTTGCATTATTATTTTGATTTTCCG CAAGCAGCAGAAAATAC	haptoglobin-binding surface anchored protein
ltaA	NWMN_0886	Forward primer Reverse primer Probe	CTGTAGCAATAACGTCTCTAGCATTTTC GGTGATGCTGGAAACCAAATAAC CAAAAATCGTTTTAACG	glycolipid permease LtaA
ltaS	NWMN_0687	Forward primer Reverse primer Probe	CCGTAATAACGATTACCTTGAAGACG TTTTTTGCCTTTAAAGAATAGGAACACAC TGAATCCTTATAGTTTAG	glycerol phosphate lipoteichoic acid synthase
lukD	NWMN_1718	Forward primer Reverse primer Probe	GAAAGTTACAGAACTACGATTGATAGAAAAACA ATTATTCATAATTTTGTGCGCCTCAACA CCCCAGCCAATTGA	leukocidin LukD subunit
lukE	NWMN_1719	Forward primer Reverse primer Probe	GATGTTGGTCAAACATTAGGATATAACATTG ATTGTTTTAGAATAATTAAATGAGCCATTGCCA CTGACTGGAAATTACC	leukocidin LukE subunit
lukS	NWMN_1928	Forward primer Reverse primer Probe	GCAGCAACGACTCAAGCAAATTC GTTTCAGTTCGTTTTGTGATTTTACCG GAACATGTTGATAAGTCTC	leukocidin LukS subunit
mgrA	NWMN_0655	Forward primer Reverse primer Probe	GCTCAAAGACAAGTTAATCGCTACTACTC GTGCTAATTCAGTTACGACTTTCTTGAC CCCACAATTTCTTGTC	MgrA - MarR family regulatory protein
mntA	NWMN_0603	Forward primer Reverse primer Probe	GTGTGGAACAAGTGATTTTATCAGG CACTTAATTCTGAAATTTGTCGATGAC GGATGGTTTAGACGACC	ABC transporter ATP-binding protein
mntH	NWMN_0971	Forward primer Reverse primer Probe	GGAAACTGGATAACATCAATGCAAG GTCGTGTCATTTGAGCTAAGTCCATAC GGCTATACTTTGCTATTC	Mn2+/Fe2+ transporter NRAMP family protein

Table 3 (continued). TaqMan assays used in this study

Assay name	Newman gene	Oligo function	Sequence	Function-notes
nuc	NWMN_1236	Forward primer Reverse primer Probe	CCTGTACAACCATTTGGCAAAGAAGC GCAAGTCCCTTTTCCACTAATTCC CGCTATGGTAGAACATTG	thermonuclease
NWMN_0677	NWMN_0677	Forward primer Reverse primer Probe	GTGAAACTGTTGAAGGTAAAGCTG ACCATTGCGATTTCTTTACC CAAATCATCAAAAGGTCC	hypothetical protein
NWMN_0851	NWMN_0851	Forward primer Reverse primer Probe	CAATTGCAGTAGATGGCATTATGGC CTTTCCAAGTAATCGTGTAAACGGCAG CCAAAAGATAGCCAATTA	putative surface protein
NWMN_1231	NWMN_1231	Forward primer Reverse primer Probe	GGTGCTGGAAAGTCAACGTTAATTG GAAACATCGTTTTTTGGAACATTATACTG AATTCTGGTGAGATATTTG	ABC transporter (ATP-binding protein) homolog
saeP	NWMN_0677	Forward primer Reverse primer Probe	CGTAGTCAACCATTGCGATTTC GTGAAACTGTTGAAGGTAAAGCTGAG GACGTATAAATCTGGACC	SaeRS auxiliary protein
sak	NWMN_1880	Forward primer Reverse primer Probe	GAGGTAAGTGCATCAAGTTCATTCGAC GTCCCAGGTTTAATAGGAAACTCGAC GATGGTAAATGTGACTGG	staphylokinase
sarA	NWMN_0588	Forward primer Reverse primer Probe	GAGTTGTTATCAATGGTCACTTATGCTG CTTTGTTTTCGCTGATGTATGTCAATAC GAATTTTCAATTAGCTTTG	SarA transcriptional regulator
sarR	NWMN_2195	Forward primer Reverse primer Probe	GTCAACGCAACATTTCAAGTTAAGAAG CTCTGAGCACTTAGCAATCTCTTTAGATG TCAATTTGAACTATGAAG	SarR transcriptional regulator
sarS	NWMN_0056	Forward primer Reverse primer Probe	GATGAGCGTAATACTTACATTTCAATATCTG CTATCTTTTGGTATCATCTGTGATTCAC CAGAACGTGTTACATTG	SarS transcriptional regulator
sarZ	NWMN_2286	Forward primer Reverse primer Probe	GGTTACATTGTTTTAATGGCGATTG CATCTTTCTCTTCACGTGTTCGAAC CTTAGATTCTGGAACACTG	SarZ - MarR family regulatory protein
sasA	NWMN_2553	Forward primer Reverse primer Probe	GCGACAAATTTACAACAAGTACAATTTGG CGATTGTCACGACTTGATCAACATTTC CTGCTGTTACACAAGTG	serine-threoinine rich antigen
sasB	NWMN_2061	Forward primer Reverse primer Probe	CTACTATGCAAACGAATAGTAAGCAAGG GTAATTCTTGAAGCATCAGCAACTGC GAATTAGCAACTGTAAATG	methicillin resistance determinant FmtB protein
sasC	NWMN_1649	Forward primer Reverse primer Probe	GGTTCAGGAGGACATCTAACTTTAAAGG TGCCGCACGTCTACTTCTCTTTTTC GAGCTAGTTGCAATTGC	similar to fmtB protein, cell wall anchored
sasD	NWMN_0078	Forward primer Reverse primer Probe	CCTTATGGCGGAGTAGTACCACAAG GCGTCGCATCATACAATTTCATATTATAG GCACAATATACTGAATTAG	similar to functionally unknown protein
sasF	NWMN_2545	Forward primer Reverse primer Probe	CATTGATTGATCAATCACAAGATAAGTCG CGATTTGATAATCCTTTATTCGTCC TTACAAACGAAATTAGG	similar to functionally unknown protein
sasG	NWMN_2392	Forward primer Reverse primer Probe	CGAGAAAATACCGCAAGGTCATAAA TGTCTGGATTCTTGATTCCTGGTTT CAGATCAAACGGAAAAAGTA	cell wall surface anchor family protein
sbi	NWMN_2317	Forward primer Reverse primer Probe	GAAGAACAACGTAACCAATACATCAAAAC GTAAAAAGCGTTTTGTTGTGCAACAC GAAGTATTCTCTGAATCAC	immunoglobulin G-binding protein Sbi
scn	NWMN_1876	Forward primer Reverse primer Probe	CTTGCCAACATCGAATGAATATCAAAAC GTCTTTTGACTTAAGAGCATACATTGC GATGAACTAAATGTTAATG	staphylococcal complement inhibitor SCIN

Table 3 (continued). TaqMan assays used in this study

Assay name	Newman gene	Oligo function	Sequence	Function-notes
scpA	NWMN_1403	Forward primer Reverse primer Probe	CAGAGCAGTATATGCAGTACGTTCATGC GATATTCTATTAAACGCCCAACTAAATC TACCACAATCAACATCAG	Staphopain A, cystein protease
sdrC	NWMN_0523	Forward primer Reverse primer Probe	ATGAATAATAAAAAGACAGCAACAAATAGA AGCAGTACCTACAGAATACTTTCTTATCGA AAAGGCATGATACCAAATCG	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrC
sirA	NWMN_0059	Forward primer Reverse primer Probe	CATGGACACAAAAACCGAAATTC CATTTCTAACTTTTGACGCGACAATTAAG CTAAGATTGTAGGTCAAG	siderophore compound ABC transporter binding protein
spa	NWMN_0055	Forward primer Reverse primer Probe	CAAACCTGGTCAAGAACTTGTTGTTG GCTAATGATAATCCACCAAATACAGTTG CATGCAGATGCTAAC	Immunoglobulin G binding protein A
sspA	NWMN_0918	Forward primer Reverse primer Probe	CTTATATTCAAGTTGAAGCACCTACTGG CTTTTAAAGCATGAGGATCACCGTG GGTGTAGTTGTAGGTAAAG	V8 protease, glutamyl endopeptidase precursor
sspB	NWMN_0917	Forward primer Reverse primer Probe	CAACAACAATTTGCTGGTTATGCTAAAG TAAACAATTTTACCGTCTTTTATAACTGG GTAATGCAAAAACTGG	cysteine protease, staphylopain
sta011-01	NWMN_0042	Forward primer Reverse primer Probe	GAAGATATGGTAGCTAAAGGCATGGTTC GTGCGGTTTTCCTTCATCC CTACTATGTCGATGTGACT	staphylococcal tandem lipoprotein
sta011-05	NWMN_0148	Forward primer Reverse primer Probe	CGCTTTATGAAATTGATGGTCACG CTTATCTACCTTTAACAACATACGGTCTTC CAGTTGTTGGATCAGATG	staphylococcal tandem lipoprotein
sta011-06	NWMN_0403	Forward primer Reverse primer Probe	GAACCGCTAAAGGCCATTATTTTGTTAC CTGCTTGAGCTTATCATCTTTTACTTC CCGGAATGGTAAACTAC	staphylococcal tandem lipoprotein
sta011-16	NWMN_2379	Forward primer Reverse primer Probe	GAACCACAAAAGGGTATTATTTTATAAGTG CTTGTCATTCGGTAGTGGCTTCG GTAACGGCAGACCAAAG	staphylococcal tandem lipoprotein
vWbp	NWMN_0757	Forward primer Reverse primer Probe	GAAAAATAAATTGCTAGTTTTATCATTGG TCCCCAGAAACCACTGCAC GTGTATCACAAATTTGG	secreted von Willebrand factor-binding protein

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