# Genetic reductionist approach for studing the two-component signaling system in *Staphylococcus aureus*

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# Genetic reductionist approach for studing the two-component signaling system in *Staphylococcus aureus*

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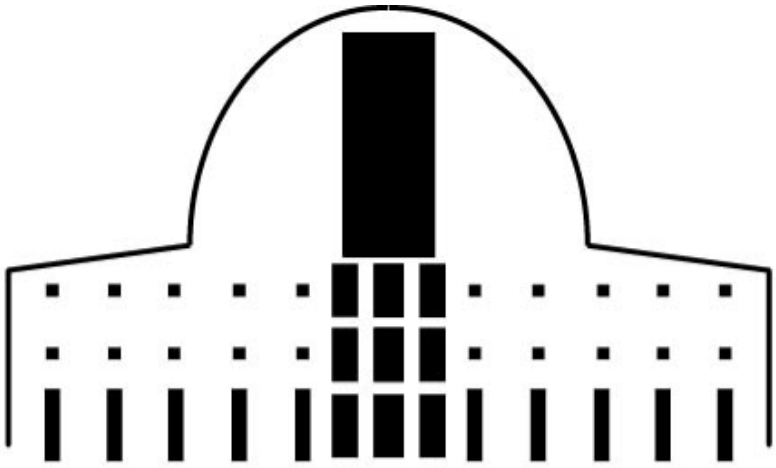

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INFORMAN:

Que la presente memoria de Tesis Doctoral "Genetic reductionist approach for studing the two-component signaling system in *Staphylococcus aureus*" elaborada por Doña **MAITE VILLANUEVA SAN MARTIN** ha sido realizada bajo su dirección y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor.

Y para que así conste, firma la presente en Pamplona, a 26 de junio de 2014.

Fdo. Íñigo Lasa Uzcudun Fdo. Alejandro Toledo Arana

"*La verdadera ciencia enseña, por encima de todo, a dudar y a ser ignorante".*

**Miguel de Unamuno**

*"A veces sentimos que lo que hacemos es tan sólo una gota en el mar, pero el mar sería menos si le faltara esa gota".*

**María Teresa de Calcuta**

*A mi familia y amigos*

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# CONTENTS

# *RESUMEN*

## RESUMEN

*Staphylococcus aureus* es una bacteria ubicua capaz de colonizar una gran variedad de ambientes. En el hombre, *S. aureus* coloniza las fosas nasales, piel de las axilas, ingles, garganta o incluso el tracto intestinal. Se calcula que un 20% de las personas adultas son portadores nasales de *S. aureus*. En determinadas circunstancias, la bacteria es capaz de atravesar la barrera epitelial y alcanzar los órganos internos. Cuando esto ocurre, *S. aureus* se convierte en un patógeno muy versátil capaz de causar enfermedades muy diversas, que pueden ir desde infecciones leves como forúnculos o abscesos hasta enfermedades graves como endocarditis, osteomielitis, neumonía o síndrome del shock tóxico.

El desarrollo de *S. aureus* en distintos ambientes requiere que la bacteria sea capaz de sensar las condiciones ambientales, transmitir los estímulos al citoplasma y activar los cambios necesarios para adecuar la fisiología a dicho ambiente. El principal mecanismo para sensar y responder a las señales ambientales en bacterias son los sistemas de dos-componentes (TCSs). Los TCSs están formados por un sensor de membrana o *histidine kinase* (HK) y un regulador de respuesta citoplásmico (RR). En el proceso de activación, el sensor recibe su señal específica y se autofosforila en un dominio histidina. A continuación el fosfato es transferido al residuo aspártico del RR que se encuentra en el citoplasma. De esta forma, el RR se activa y desencadena una respuesta que será acorde a la señal recibida.

Normalmente, una bacteria posee varios TCSs, siendo su número proporcional al tamaño del genoma, al número de ambientes distintos en las que es capaz de crecer y a la complejidad de su diferenciación celular.

Así, bacterias que viven en ambientes muy constantes, como las bacterias intracelulares estrictas carecen de TCSs, mientras que bacterias que viven en ambientes diversos pueden poseer cientos de ellos. En relación con el número y la función de los TCSs existen varias preguntas que hasta ahora no han sido analizadas: ¿cuántos TCSs necesita una bacteria de vida libre? ¿Son necesarios los TCSs cuando la bacteria crece en un ambiente constante? ¿Existe activación cruzada entre TCSs distintos *in vivo*?

Para responder a estas preguntas y realizar un estudio global de los procesos celulares controlados por los TCSs, en esta tesis hemos realizado una aproximación genética reduccionista usando como modelo dos cepas genéticamente no relacionadas de *S. aureus*. El trabajo ha consistido en la deleción completa de los 15 TCSs no esenciales que posee *S. aureus* y la mutación del sensor (WalK) del TCS *walKR*, cuya deleción completa resulta letal. Las bacterias resultantes carecen del sistema sensorial y su obtención demuestra que en condiciones ambientales constantes estos sistemas son dispensables para la vida de *S. aureus*.

Los mutantes deficientes en los TCSs muestran niveles de crecimiento indistinguibles a los de la cepa salvaje a 37ºC y 44ºC y un patrón metabólico similar. En cambio, los mutantes tienen deficiencias en el crecimiento a 28ºC, pierden la capacidad de reducción de nitratos, muestran mayor sensibilidad al Tritón X-100 así como una menor capacidad para sobrevivir en el ambiente e invadir células. Así mismo, los mutantes tienen reducida su virulencia y capacidad de colonizar órganos en un modelo de infección de ratón. Todos los fenotipos del mutante deficiente en los TCSs podían ser restaurados por la expresión ectópica de un único TCS indicando que cada uno de los fenotipos depende de un único TCS.

Finalmente, la cepa deficiente en los TCSs ha sido utilizada como una plataforma para el estudio de la especificidad de transmisión de señal *in vivo*, un concepto que en inglés se denomina "*cross-talk*" y que hasta ahora había sido estudiada *in vitro*. Para ello, hemos establecido una sencilla metodología que consiste en la complementación del mutante deficiente en TCSs con una colección de plásmidos que contienen una combinación de la familia de HKs y un RR. El análisis de las cepas complementadas nos ha permitido identificar la existencia de activación cruzada entre GraS y ArlR. Esta activación cruzada tiene lugar incluso en presencia de sus correspondientes parejas, la HK ArlS y el RR GraR. Teniendo en cuenta que durante este análisis global sólo hemos detectado activación cruzada entre estos TCSs, la conclusión de nuestro estudio es que la activación cruzada entre los TCSs puede ocurrir *in vivo*, pero no es frecuente.

En el futuro las cepas deficientes en los TCSs, o cepas derivadas conteniendo únicamente uno de ellos, servirán para identificar el regulón que controla cada TCS o para identificar nuevos fármacos que bloqueen específicamente a los TCSs.

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# *SUMMARY*

## SUMMARY

*Staphylococcus aureus* is a Gram-positive bacterium adapted to live in a wide variety of environmental niches. In humans, *S. aureus* colonizes the nose, skin, axilla, groin, throat or intestinal tract. Approximately 20% of the anterior nares of healthy human adults are persistently colonized with *S. aureus*. In some circumstances, the bacterium is able to traverse the epithelial barrier reaching internal organs. When this occurs, *S. aureus* can cause a variety of diseases, ranging from minor skin infections, such as furuncles or abscesses, to severe infections, such as endocarditis, osteomyelitis, pneumonia or toxic shock syndrome.

*S. aureus* needs to recognize environmental signals, transmit stimuli to the cytoplasm and activate the necessary changes to adapt the bacterial physiology to the conditions of each environmental niche. The main mechanism to sense and respond to environmental signals in bacteria is the two-component transduction system (TCSs). TCSs comprise a membrane sensor histidine kinase (HK) and a cytoplasmic response regulator (RR). During the activation process, the sensor receives a specific signal and autophosphorylates itself on a conserved histidine residue. The phosphate is then transferred to an aspartate residue of the cytoplasmic cognate RR. The phosphorylated RR triggers a specific response in accordance with the signal.

The genome of a single bacterial species usually encodes for multiple signal transducers: the number often proportional to the genome size, the diversity of environments in which organisms live and the complexity in

cellular differentiation. Bacteria inhabiting relatively stable host environments, such as obligate intracellular parasites, encode for few or even no TCS signalling systems, while ubiquitous bacteria that are able to live in a variety of environments encode high numbers of TCSs. In relation with the number and the function of TCSs in bacteria, several questions remain open: How many TCSs does a free-living bacterium need to live? Are TCSs necessary when bacteria live in a constant environment? Does cross-activation between different TCSs exist *in vivo*?

With the aim to answer these questions and to carry out a global analysis of the cellular processes controlled by TCSs, we generated *S. aureus* mutants devoid of the TCS signalling networks by using a genetic reductionist approach on two genetically unrelated *S. aureus* strains. The work consisted in the sequential deletion of the 15 non-essential TCSs of *S. aureus* as well as the deletion of the sensor (WalK) of the *walKR* TCS, whose complete deletion is lethal. The resulting mutants lacking the TCSs demonstrate that under constant environmental conditions these systems are dispensable for *S. aureus* survival.

Phenotypic analyses of the mutants devoid of TCSs revealed growth levels indistinguishable from the wild type at 37 and 44ºC, and similar metabolic capacities. However, mutants devoid of TCSs lose the capacity to reduce nitrate to nitrite, show lower growth rates at 28ºC and capacity to survive in the environment and higher sensitivity to detergents. Moreover, in the absence of TCSs, *S. aureus* is unable to invade eukaryotic cells and colonize organs, rendering the bacteria avirulent in a mouse infection model. Phenotypes associated to the TCS-deficient mutant can be restored

by the ectopic expression of single TCSs, indicating that each phenotype is most likely modulated by a single TCS.

The TCS-deficient strain was then used as a platform for studying signal transduction specificity (cross-talk) *in vivo*. For that purpose, we developed a simple method based on the complementation of the TCS-deficient mutant with plasmids that containing a combination of the HK family and a RR. Analysis of these complemented strains allowed the identification of cross-talk between GraS and ArlR. The cross-talk occurs even in the presence of the corresponding ArlS HK and GraR RR. Taking into account that our systematic analysis only found cross-activation between these two TCSs, we conclude that cross-activation between TCSs can occur *in vivo*, but it is rare.

We anticipate that the strains lacking the TCSs, or the set of strains containing single TCSs, will be extremely useful to identify the regulon of each TCS or for finding antimicrobials that specifically block TCS functions.

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- I. Ruiz de los Mozos, M. Vergara-Irigaray, V. Segura, M. Villanueva, N. Bitarte, M. Saramago, S. Domingues, C. M. Arraiano, P. Fechter, P. Romby, J. Valle, C. Solano, I. Lasa and A. Toledo-Arana (2013). Base Pairing Interaction Between 5'- and 3'-UTRs Controls *icaR* mRNA Translation in *Staphylococcus aureus*. PLOS Genetics, 9(12): e1004001
- I. Lasa and M. Villanueva (2014). Overlapping transcription and bacterial RNA removal. PNAS, 111(8): 2868-2869.
- M. Villanueva et al. System-based analysis of two-component signaling network in *Staphylococcus aureus*. Manuscript in preparation.

# *INTRODUCTION*

# INTRODUCTION

## The Genus *Staphylococcus*

Members of the genus *Staphylococcus* are Gram positive, AT-rich cocci (0.5 to 1.5 μm in diameter), non-motile, non-spore forming, usually with limited capsule formation, which are often arranged in grape-like clusters. Most species are facultative anaerobes able to survive in unfavorable environmental conditions, such as desiccation and high salt concentration. Different species of the genus *Staphylococcus* are regular and abundant colonizers of the human skin and mucous membranes. The species of the genus *Staphylococcus* are divided into two groups, based on the ability to produce the blood clotting enzyme coagulase. The coagulase-negative species (CNS) maintain a benign relationship with their host and are considered pathogenic only in certain situations, for example, when the natural barrier such as the skin is damaged via trauma, inoculation or implantation of medical devices. In contrast, the coagulase-positive *S. aureus* is a well-established pathogen due to the production of a number of virulence factors such toxins and degradative exoenzymes (Lowy, 1998).

## *Staphylococcus aureus*, a versatile pathogen

*S. aureus* is a ubiquitous yellow-pigmented bacterium that resides mainly in the upper human respiratory tract and on the skin. The ability of this pathogen to persist and replicate in different environments, together with the capacity to express a great array of virulence factors, gives to *S.*

*aureus* the versatility to cause a great variety of infections that affect humans and animals. Humans are the main reservoir of *S. aureus* and it has been estimated that approximately 20% of adults are persistent nasal carriers of the bacteria (Wertheim *et al.*, 2005), and 60% carry *S. aureus* intermittently (Kluytmans *et al.*, 1997). *S. aureus* is harmless in these locations, but it turns into an extremely threatening pathogen when it traverses the epithelial barrier and gains access to internal tissues from where it can infect almost any organ and cause a broad spectrum of infections including abscesses, pneumonia, endocarditis, osteomyelitis, sepsis and infections associated with foreign-body implants (Gordon and Lowy, 2008). *S. aureus* infections are the leading cause of nosocomial infections worldwide, especially among immune-suppressed patients (Wu *et al.*, 2003; Yarwood *et al.*, 2004). In 2007, based on data collected in 2005, the Centers for Disease Control and Prevention (CDC) published a report stating that *S. aureus* is the most significant cause of serious infectious diseases leading to deaths in the United States (Klevens *et al.*, 2007). The treatment of infections produced by *S. aureus* is becoming a serious public health problem owing to the widespread appearance of strains resistant to multiple antibiotics, called methicillin resistant *S. aureus* strains (MRSA) (Kennedy *et al.*, 2008). These strains are sensitive to glycopeptide antibiotics such as vancomycin and teicoplanin. However, in the last few years, vancomycin resistant *S. aureus* strains have been also isolated, probably due to the increasing use of this antibiotic to treat MRSA associated infections (Jones, 2008).

## Pathogenesis of *Staphylococcus aureus*

The versatility of *S. aureus* as a pathogen has its origin in the fact that *S. aureus* strains have a varying repertoire of virulence factors that can confer the following properties:

### Cell and tissue adhesion

*S. aureus* expresses a multitude of surface proteins that are covalently bound to bacterial cell wall peptidoglycan (CWA, cell wall-anchored proteins). The precise repertoire of CWA proteins varies among strains. *S. aureus* can express up to 24 different CWA proteins, whereas coagulase-negative staphylococci such as *S. epidermidis* and *Staphylococcus lugdunensis* express a smaller number (McCarthy and Lindsay, 2010). A big group of CWA proteins comprise what is termed the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) family. These proteins promote adhesion to the extracellular matrix of host tissues (Foster and Höök, 1998). Very often, different MSCRAMMs proteins are able to bind the same receptor; for example, at least five CWA proteins bind the plasma glycoprotein fibrinogen and several promote adhesion to squamous epithelial cells, indicating the existence of redundancy in their function (Foster *et al.*, 2014).

### Tissue damage and spreading

Superantigens (SAg) are a class of antigens that allow the non-specific interaction of the class II major histocompatibility complex MHC II with T cell

receptors. This interaction causes non-specific activation of T-cells resulting in proliferation and massive cytokine release that causes the toxic shock syndrome, a life-threatening condition. *S. aureus* strains can produce a wide array of exotoxins such as α-toxin or α-hemolysin, enterotoxins or TSST-1 (toxic shock syndrome toxin 1) that cause cytolytic effect on the host cells (Otto, 2014). Other toxins have cytolytic effects (leukocidin, ©-toxin) and can cause the lysis of leukocytes by forming pores in their cytoplasmic membranes (Prévost *et al.*, 1995; Labandeira-Rey *et al.*, 2007). In addition, *S. aureus* also produces proteins that are secreted to the media such as proteases, hyaluronidases, lipases or elastases. These proteins participate in the destruction of host tissues favoring dissemination to the internal tissues.

**Protection against the immune defense system**

*S. aureus* is highly resistant to the action of the lysozyme, a bactericidal protein that is a fundamental part of the innate immune defenses (Keshav *et al.*, 1991). *S. aureus* is able to inhibit the neutrophil chemotaxis secreting the chemotaxis inhibitory protein (CHIP) (de Haas *et al.*, 2004) and the Eap (extracellular adherence protein) (Chavakis *et al.*, 2002). Besides, *S. aureus* can survive in neutrophil phagosomes interfering with endosome fusion (Gresham *et al.*, 2000). *S. aureus* has also the ability to avoid phagocytosis due to (i) the expression of surface proteins such as Protein A (Peterson *et al.*, 1977) or Clumping factor A (Palmqvist *et al.*,

2004), (ii) the expression of a capsular polysaccharide (Thakker *et al.*, 1998) and (iii) the inactivation of the complement (Rooijakkers *et al.*, 2005).

### Biofilm formation on indwelling medical devices

Patients with indwelling medical devices, such as catheters, artificial heart valves, or bone and joint prostheses, can easily develop staphylococcal infections, due to the ability of *S. aureus* to colonize the implant surface as a biofilm (Costerton *et al.*, 1995). Inside the biofilm, bacteria are enclosed in a self-produced, hydrated polymeric matrix that confers increased resistance to desiccation and increased tolerance to antimicrobial agents and the host immune response by mechanisms that are still somewhat unclear (Høiby *et al.*, 2010). The biofilm formation process is believed to occur as sequential steps where initially, non-motile bacterial cells attach irreversibly to a surface. Subsequently, the bacteria interact with each other, proliferate and accumulate in multilayered cell clusters embedded in a self-produced extracellular matrix. Finally, some bacteria can detach from the biofilm into the media. The importance of biofilm detachment for infection resides in the dissemination of infection from the surface of medical device to other distant sites via the lymph and bloodstream. Detached biofilm bacteria may establish secondary biofilm infections elsewhere, possibly with increased severity, such as for example endocarditis. Additionally, detached bacteria may cause acute, nonbiofilm infections, such as sepsis (Otto, 2013).

### Persistence and intracellular survival

Small-colony variants (SCVs) of *S. aureus* are found in chronic and antibiotic-refractory infections such as osteomyelitis, cystic fibrosis and device-related infections (Proctor *et al.*, 1995; Proctor *et al.*, 2006). It has been proposed that this phenotypic variation provides a survival advantage to persist within eukaryotic cells by protecting them from host defences and antibiotics (Eiff *et al.*, 1997; Löffler *et al.*, 2014). Phenotypically, SCVs grow more slowly than regular *S. aureus* colonies, leading to colonies that are one-tenth the size of "normal" *S. aureus.* SCVs are non-pigmented, non-hemolytic colonies and show increased resistance to aminoglycosides. The biochemical basis of this phenotypic abnormality is most often an auxotrophy for either menadione, hemin or thymidine. Any of these defects results in reduced tricarboxylic acid cycle metabolism and consequent reduced electron transport, yielding the typical SCV phenotype.

### Signal transduction systems in *S. aureus*

*S. aureus* possesses a series of surface-exposed sensors that typically comprised transmembrane (TM) proteins able to monitor environmental parameters and transmit the signal from the sensory module to an intracellular response regulator. According to the census of signal transduction proteins encoded in bacterial genomes at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/Complete_Genomes/SignalCensus.html) (Galperin, 2010), *S. aureus* contains the following signal transduction proteins: (i) 16 two-component

signal transduction systems (TCSs) that comprises a sensor membrane protein and a transcriptional regulator (Laub and Goulian, 2007; Gao and Stock, 2009). The number of TCSs differs enormously from species to species and seems to correlate with the variety of environmental cues bacteria is exposed to (Jung *et al.*, 2012); (ii) a single diguanylate cyclase (GGDEF domain containing protein) and a second protein with a highly modified GGDEF domain (Tu Quoc *et al.*, 2007; Holland *et al.*, 2008). Diguanylate cyclases convert internal or external signals into cyclic diguanylate (c-di-GMP) levels. The resulting c-di-GMP modulates the function of c-di-GMP binding molecules, proteins or RNA, which ultimately modifies the physiology and behaviour of the cell (Jenal and Malone, 2006; Römling and Amikam, 2006; Mills *et al.*, 2011); (iii) a single c-di-AMP cyclase that synthesize c-di-AMP. Similar to c-di-GMP, c-di-AMP interacts with a specific set of target proteins and upon binding alters their activity or function; (iv) two Ser/Thr protein-kinases and two Ser/Thr protein-phosphatases that upon receiving an stimulus from the environment can bind and modify the phosphorylation status of specific targets (Helmann, 2002).

In the next section I will summarize the most relevant information related to the signal transduction mechanisms. Although TCS are the most important sensorial system in *S. aureus*, I will first describe the other signaling systems.

**Cyclic dinucleotides (c-di-GMP and c-di-AMP) signal transduction pathways**

c-di-GMP is a universal secondary messenger in bacteria, regulating the transition between planktonic and biofilm lifestyle (Jenal, 2004; D'Argenio and Miller, 2004). Our knowledge about c-di-GMP signaling derives almost exclusively from studies in Gram-negative bacteria and implies that increases in c-di-GMP levels lead to inhibition of motility and promotion of biofilm formation whereas decreased c-di-GMP levels induce biofilm disassembly and bacterial motility (Jenal and Malone, 2006; Römling and Amikam, 2006; Tamayo *et al.*, 2007).

The intracellular pool of c-di-GMP depends on the activity of diguanylate cyclases (GGDEF-domain proteins) that synthesize the compound and by specific phosphodiesterases (EAL-, HD_GYP domain proteins) that degrade it (Galperin *et al.*, 2001; Ausmees *et al.*, 2001; Tamayo *et al.*, 2005). The number of diguanylate cyclases and phosphodiesterases encoded in bacterial genomes is highly variable. Most bacterial species have an intermediate number, some have a high number (more than 40) and few species have none (http://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html). *S. aureus* encodes only one conserved GGDEF domain protein, named GdpS (Tu Quoc *et al.*, 2007; Holland *et al.*, 2008). Biochemical and genetic studies indicate that GdpS is unable to synthesize c-di-GMP at least under the environmental conditions tested, indicating that *S. aureus* has only remnants of a c-di-GMP signaling pathway. This conclusion is also supported by the absence in the genome

of *S. aureus* with neither EAL or HD-GYP domain proteins nor c-di-GMP receptors (PilZ domain proteins, riboswitch). However, as GdpS still maintains the capacity to activate the synthesis of PIA/PNAG exopolysaccharide and biofilm development, it is assumed that the regulation proceeds through mechanisms independent of c-di-GMP.

Recent studies indicated that Gram-positive bacteria use another cyclic dinucleotide, c-di-AMP, as a signaling molecule to regulate essential biological processes. c-di-AMP is synthesized by proteins with a DAC domain, (DAC, PF02457 in the Pfam database) and degraded by proteins with phosphodiesterase DHH domain. *S. aureus* contains a single diadenylate cyclase protein (SA1967) and also a single protein with c-di-AMP specific phosphodiesterase activity, named GdpP (GGDEF modified phosphodiesterase). High c-di-AMP levels confer resistance to cell wall active antimicrobials such as oxacillin, penicillin G and lysostaphin and allow *S. aureus* to grow in the absence of LTA (Corrigan *et al.*, 2011). Screens for essential genes indicated that similarly to *Listeria monocytogenes* (Woodward *et al.*, 2010) and *B. subtilis* (Yun Luo and Helmann, 2012), *S. aureus* cannot grow in the absence of c-di-AMP, showing that c-di-AMP controls essential cellular pathways (Chaudhuri *et al.*, 2009). It is assumed that, similar to c-di-GMP signaling molecules, c-di-AMP interacts with a specific set of target proteins and upon binding alters their activity or function. Corrigan *et al*. have recently identified c-di-AMP receptors using a combination of pull-down, direct binding to genome-wide ORFome and bioinformatic approaches (Corrigan *et al.*, 2013). The four proteins able to

bind c-di-AMP in *S. aureus* are the potassium transporter component KtrA, a predicted cation/proton antiporter (CpaA), the HK protein KdpD and a protein of unknown function, renamed as PtsA. Because three of these proteins (KtrA, CpaA, and KdpD) have been implicated in potassium transport in other bacteria, it appears that c-di-AMP signaling regulates the potassium transport in *S. aureus*.

**Ser/Thr/Tyr kinases and phosphatases**

*S. aureus* has a functional serine/threonine kinase designated Stk1 (also known as PknB or PrkC) with three predicted extracellular PASTA domains; Stk1 is associated with a corresponding phosphatase named Stp1. Recent studies have described Stk1 and Stp1 as modulators of purine and pyrimidine biosynthesis, cell wall metabolism, autolysis and glutamine synthesis. Moreover, Stk1 is able to phosphorylate the global regulator MgrA, which controls partially the expression of the multi-drug efflux pump NorA (also HU, SarA, and VraR.)(Donat *et al.*, 2009; Ohlsen and Donat, 2010; Liebeke *et al.*, 2010) Cluzel *et al* demonstrated that Stk1 is able to also phosphorylate the AI-2 producer protein LuxS (Cluzel *et al.*, 2010). Finally, Stk1 has been shown to play a role in virulence in a mouse pyelonephritis model (Débarbouillé *et al.*, 2009).

Some MRSA strains have a second protein with eukaryotic-like tyrosine kinase domain encoded on the SCC*mec* element (SA0077). This putative protein kinase does not contain the extracellular PASTA domains and its function is unknown (Donat *et al.*, 2009).

*S. aureus* possess another Ser/Thr phosphatase named *rsbU*, a positive regulator of the general stress-response factor of gram positive organisms, named SigB. The phosphatase dephosphorylates RsbV anti-anti-sigma factor under stress-conditions and allows it to bind to RsbW (anti-sigma) releasing free SigB. Consequently, SigB can associate with core RNA polymerase and directly activate the transcription of genes comprising the SigB regulon.

## Two-component signal transduction systems (TCSs)

### Basic structure of TCSs

The prototypical TCS consists of a membrane-bound sensor histidine kinase (HK), which responds to extracytosolic signals, and a mobile cytosolic response regulator (RR), which in most cases triggers the bacterial response, altering the expression of target genes (Mascher *et al.*, 2006). In the classical two-component signalling pathway, activation of a HK leads to autophosphorylation on a conserved histidine residue followed by transfer of the phosphoryl group to an aspartate residue of the cognate RR. Phosphotransfer to the RR results very often in oligomerization and a conformational change that elicits the specific response (Gao and Stock, 2009). The phosphorylation state of the HK is the critical parameter that determines the transfer of the phosphate group toward its cognate RR and the final modulatory readout (Stock *et al.*, 2000).

The majority of HK are homodimeric membrane receptors. They have a variable, N-terminal, extracellular sensor domain that is connected via

transmembrane helices to the C-terminal cytoplasmic portion, which contains the transmitter domain with the catalytic machinery (Fig. 1). In class I HKs, which predominate in prokaryotes, the catalytic element contains the dimerization and histidine phosphotransfer domain (DHp), which is the site for autophosphorylation and phosphodonor reactions, and the catalytic and ATP-binding domain (CA) (Stock *et al.*, 2000).

The autophosphorylation reaction occurs in *cis* in some HKs (that is, the ATP bound to one subunit phosphorylates the His residue of the same subunit), while *trans*-autophosphorylation takes place in others (Casino *et al.*, 2009; Casino *et al.*, 2010). Class II HKs contain, instead of the DHp domain, a histidine phosphotransfer (HPt) domain separated from the CA domain by both an input and a dimerization domain (Fig. 1) (Bilwes *et al.*, 1999; Dutta *et al.*, 1999). Frequently the HK also catalyzes the dephosphorylation of its phosphorylated RR partner. Thus, the HK can act as both an RR kinase and phosphatase. Phosphatase reaction is not merely the reversal of RR-phosphorylation since it is not absolutely dependent on the presence of the His residue of the HK, although this residue strongly accelerates this process (Hsing and Silhavy, 1997). The functional switch between autophosphorylation, phosphotransfer and phosphatase activities implies different conformational states of individual CA and DHp domains and, most importantly, corresponding three-dimensional arrangements of these domains. These rearrangements are potential check points upon which environmental signals can act.

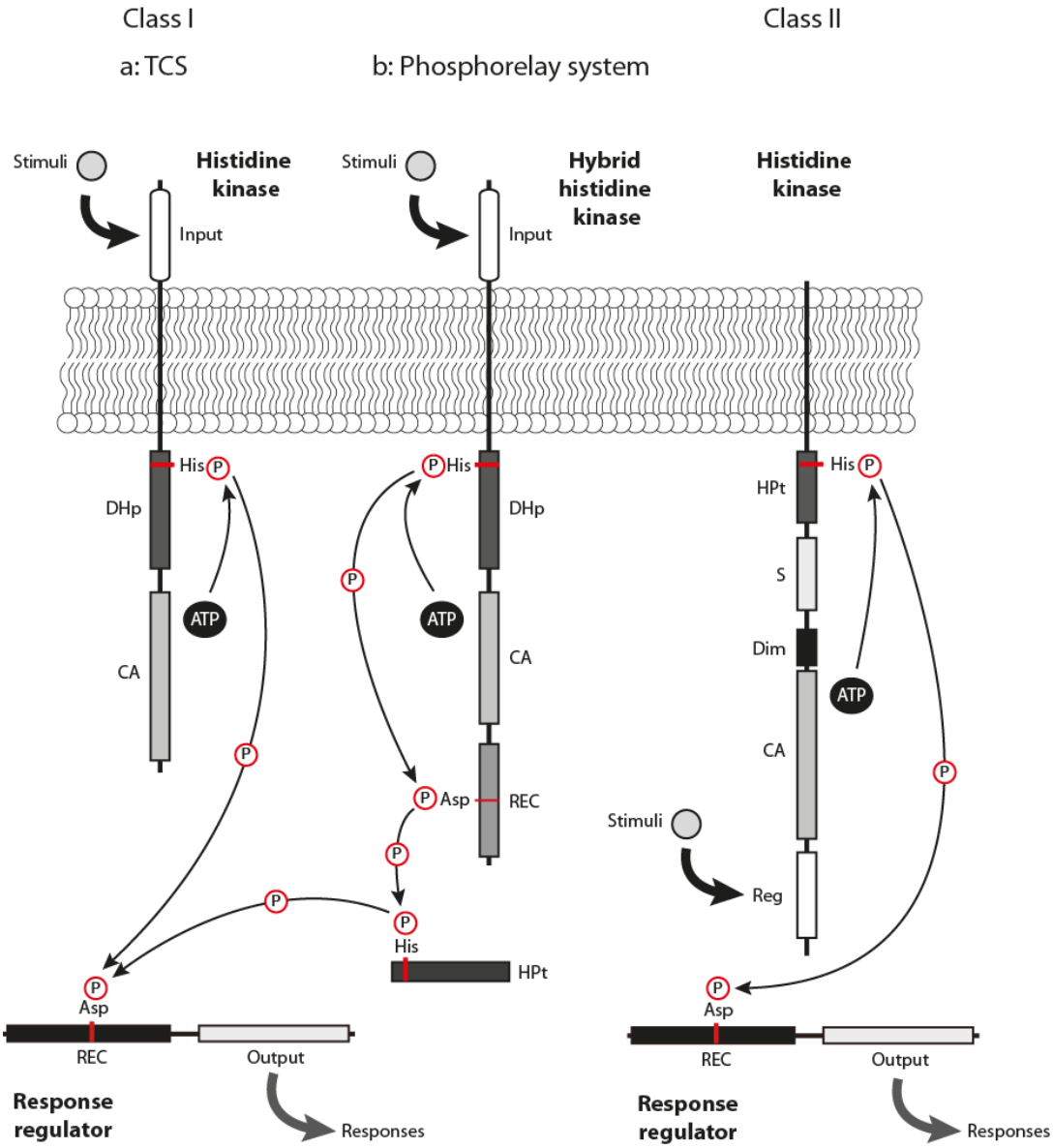


**Figure 1. Schematic representation of the prototypical two-component and phosphorelay system, dissecting the modules by which they are formed.** (a) The prototypical TCS pathway comprises a conserved phosphoryl transfer between the highly conserved kinase core (DHp and CA) and the receiver (REC) domain. (b) Phosphorelay scheme is formed by hybrid histidine kinase (HK) involving additional REC and histidine phosphotransfer (HPt) domains for multiple phosphotransfer events. The intermediate HPt domain can either be an independent protein or a domain linked to the HK. Class I HKs contain the dimerization and histidine phosphotransfer domain (DHp), which is the site for autophosphorylation and phosphodonor reactions, and the catalytic and ATP-binding domain (CA). Class II HKs contain, instead of the DHp domain, a histidine phosphotransfer (HPt) domain separated from the CA domain by both an input and a dimerization domain.

RRs typically consist of a highly conserved receiver domain (REC) that hosts the phosphoacceptor aspartate and an effector domain that interacts with targets (Stock *et al.*, 2000) (Fig. 1). The effector domain can interact with different targets including DNA (frequency of occurrence, 63%), protein (3%), RNA (1%), or alternatively, it can display an enzymatic activity (13%).

A common variant of the TCS pathway is the so-called phosphorelay. These pathways are usually initiated by a hybrid HK that autophosphorylates and then transfers its phosphoryl group intramolecularly to a RR-like receiver domain. The phosphoryl group is then transferred to an HPt domain (independent protein or linked to the HK) and subsequently to a terminal RR that finally controls gene expression (Fig. 1).

**Classification of two-component proteins**

In both HK and RR, modular domains with conserved structures and biochemical activities are present and determine the functionality of the two-component system pathways (Fig. 2).

Sensors can be grouped on the basis of their mechanism of stimulus perception. The largest group, the periplasmic (or extracellular)-sensing HKs, includes proteins with an extracellular sensory domain that is framed by at least two transmembrane (TM) helices. The kinase is localized in the cytoplasm (as for all other HKs). Thus, sensory and kinase domains are located in two different cellular compartments which are separated by a membrane. This type of membrane topology is typical for sensing solutes and nutrients. The second group contains HKs with sensing mechanisms

associated with the membrane-spanning helices. The unifying feature of this highly diverse group of sensor HKs is the presence of 2 to 20 TM regions implicated in signal perception, connected by very short intra- or extra-cellular linkers. These sensors lack an apparent extracellular input domain. Stimuli from the membrane include the mechanical properties of the cell envelope (such as turgor or mechanical stress) or are derived from membrane-bound enzymes or other membrane-integral components. Other membrane-related stimuli include ion or electrochemical gradients, transport processes, and the presence of compounds that affect cell envelope integrity. The third (and second-largest) group of sensory HKs, the cytoplasmic-sensing HKs, includes either membrane-anchored or soluble proteins with their input domains inside the cytoplasm. This class of sensor proteins detects the presence of cytoplasmic solutes or of proteins signaling the metabolic or developmental state of the cell or of the cell cycle. Other cytoplasmic TCSs respond to diffusible or internal stimuli, such as $O_2$ or $H_2$, or stimuli transmitted by TM sensors (Mascher, 2006).

HKs can be also classified according to their DHp domain. The Pfam database delineates five DHp sequence subfamilies. The HisKA subfamily (pfam00512), exemplified by EnvZ and NtrB, comprises the majority of sequences, whereas the HisKA_3 subfamily (pfam007730) covers about 10% of DHp domain sequences. Well studied examples of HisKA_3 members include *E. coli* NarX; *S. aureus* VraS; and *B. subtilis* DesK (Huynh *et al.*, 2010). There are also other subfamilies called HisKA_2 (pfam07568), His_kinase (pfam06580) and HWE HK (pfam07536).

RRs have a conserved regulatory or receiver domain (REC) and a variable effector domain, so RRs can be classified based on their differing output domain. The majority of RRs (63%) contain DNA binding domains, dominated by a small number of structural families named after extensively characterized members. These subfamilies include the OmpR/PhoB winged-helix domain (30% of all RRs) (Martínez-Hackert and Stock, 1997), the NarL/FixJ helix-turn-helix domain (17%) (Milani *et al.*, 2005), the NtrC/DctD AAA+ ATPase domain (10%) (Batchelor *et al.*, 2008), and the recently characterized LytR/AgrA domain with an unusual, predominantly β fold (3%) (Sidote *et al.*, 2008) (Fig. 2). RNA binding domains are found in only 1% of RRs and are mostly of the ANTAR family, working as antitermination factors (O'Hara *et al.*, 1999). Enzymatic domains are found in 13% of RRs. Most of these RRs are involved in regulation of c-di-GMP (Römling *et al.*, 2005) acting as diguanylate ciclases (GGDEF) or phosphodiesterases (EAL or HD-GYP). The remaining enzymatic subfamilies, in order of prevalence, are chemotaxis methylesterase CheB domains; HK domains; and protein phosphatase domains of PP2C family. A small and diverse group of effector domains that mediate interactions with other proteins or ligands occur in 3% of RRs. Approximately half of these RRs correspond to chemotaxis CheV-like proteins containing CheW domains.

### Functions of the TCSs in *S. aureus*

The core genome of *S. aureus* strains contains 16 TCSs (Table 1). This number is constant in the *S. aureus* strains with the exception of some MRSA strains that carry an additional TCS homologous to the chromosomal copy of *kdpDE* in the staphylococcal cassette chromosome *mec* (SCC*mec*), a mobile element (M Kuroda *et al.*, 2001; Hanssen and Ericson Sollid, 2006).

If we classify the *S. aureus* TCSs according to the RRs, there are four Pfam families based on their output domains. Specifically, nine RRs belong to the OmpR family, four to the NarL family, two to the LytR family, and one to the YesN family (Fig. 2). In contrast to other bacterial species, all of these output domains contain DNA-binding motifs, suggesting that all of them function as transcriptional regulators. Moreover, *S. aureus* TCSs can be also classified based on the DHp domain of the HKs. There are eight HisKA subfamily members, four HisKA_3 subfamily members, two His_kinase subfamily members and two undefined HKs (Fig. 2). It is also important to notice that there are two *S. aureus* HKs lacking TM domains, AirS and NreB, which are classified into the cytoplasmic-sensing HKs, while at least two are membrane-sensing HKs, such as GraS and BraS, and the others, such as ArlS or HssS, are considered as extracellular-sensing HKs (Fig.2).

All *S. aureus* HK and RR pairs are cotranscribed in operons and there is no orphan HK or RR. The composition of these TCSs operon, the known signals activating HKs, the function of the accessory proteins, and the regulons modulated by TCSs are described below.

**Table 1.** Decription of the sixteen two-component systems present in *S. aureus* genome.

| TCS | Sensing | Function | References |
|---|---|---|---|
| *walKR* | | Viability and virulence | Martin *et al.*, 1999<br>Dubrac and Msadek, 2004<br>Delaune *et al.*, 2012 |
| *kdpED*-like | | ATP-dependent potassium transport | Hanssen and E. Sollid, 2006 |
| *tcs3* | | | |
| *lytSR* | $\Delta\Psi$ | Autolysis and biofilm | Brunskill and Bayles, 1996a<br>Rice *et al.*, 2007<br>Sharma-Kuinkel *et al.*, 2009 |
| *graXRS* | CAMP | CAMP resistance | Min Li, Lai, *et al.*, 2007 |
| *saePQRS* | Alteration within the membrane,<br>e.g. $\alpha$-defensins | Virulence factor production | Giraudo *et al.*, 1994<br>Liang *et al.*, 2006<br>Voyich *et al.*, 2009 |
| *tcs7* | | | |
| *arlRS* | | Virulence factor production, autolysis, biofilm and agglutination | Fournier and Hooper, 2000<br>Fournier *et al.*, 2001<br>Toledo-Arana *et al.*, 2005<br>Walker *et al.,* 2013 |
| *srrAB* | $O_2$<br>NO | Virulence and biofilm under anaerobic conditions and NO detoxification | Yarwood *et al.*, 2001<br>Pragman *et al.*, 2004<br>Martina Ulrich *et al.*, 2007<br>Kinkel *et al.*, 2013 |
| *phoPR* | | | |
| *airSR* | Oxidation signals:<br>$O_2$, $H_2O_2$, NO, etc | Anaerobic respiration and cell wall metabolism | Fei Sun, Ji, *et al.*, 2012<br>Haipeng Sun *et al.*, 2013 |
| *vraSR* | Cell wall stress | Cell wall stress stimulon (CWSS) regulation | Makoto Kuroda *et al.*, 2003 |
| *agrBDCA* | Autoinducing peptide (AIP)<br>Oxidation | Quorum-sensing, oxidative stress resistance and virulence | Novick and Muir, 1999<br>Dunman *et al.*, 2001<br>Queck *et al.*, 2008<br>Fei Sun, Liang, *et al.*, 2012 |
| *kdpDE* | $K^+$ | Quorum-sensing, virulence and c-di-AMP sensing | Xue *et al.*, 2011<br>Zhao *et al.*, 2010<br>Corrigan *et al.*, 2013 |
| *hssRS* | Heme | Heme sensing and virulence | Stauff and Skaar, 2009<br>Torres *et al.*, 2007 |
| *nreABC* | $O_2$ | Nitrogen regulation | Schlag *et al.*, 2008 |
| braRS | Cell-wall damage | Antibiotic resistance and cell envelope damage sensing | Yoshida *et al.*, 2011<br>Hiron *et al.*, 2011<br>Kolar *et al.*, 2011 |

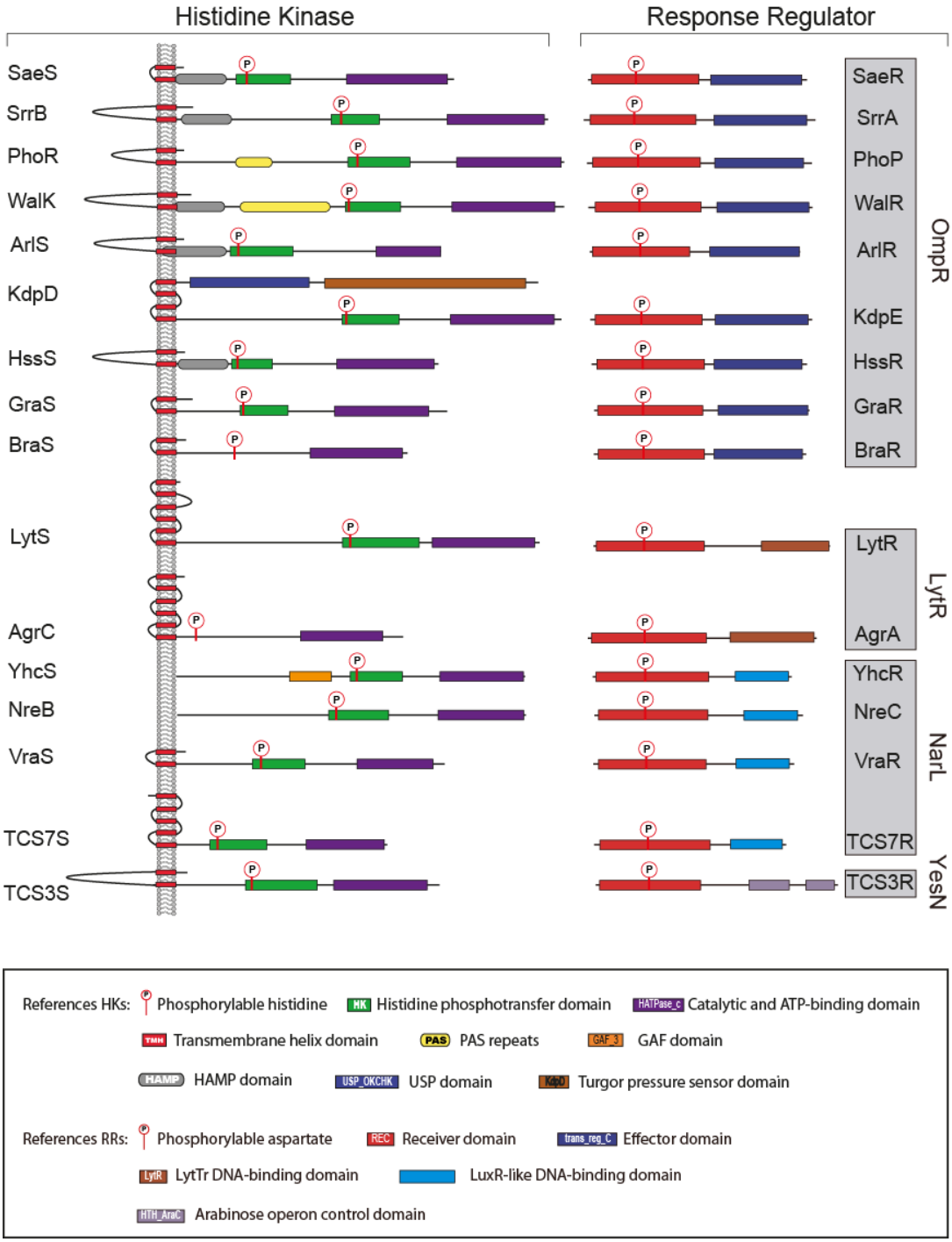


**Figure 2. Domain organization and topology of the 16 *S. aureus* histidine kinases and response regulators.** Domains are based on NCBI Conserved Domain search and transmembrane helix (TMH) domains are predicted by TMHMM Server v. 2.0.

The essential *walKR* system: the regulator of cell wall metabolism

The *walKR* (also know as *yycGF* or *vicKR*) is the only TCS which is essential for *S. aureus* growth and its presence is highly conserved among low G+C % Gram-positive bacteria (Martin *et al.*, 1999; Dubrac and Msadek, 2004; Dubrac *et al.*, 2008). It is encoded in a four-gene operon, where the function of those auxiliary proteins remains unknown (Winkler and Hoch, 2008). The WalKR system plays a major role in *S. aureus* peptidoglycan metabolism by controlling the expression of most cell wall hydrolases (Dubrac *et al.*, 2007). However, as none of the cell wall hydrolases is essential for *S. aureus* growth, it appears that it is the simultaneous absence of expression of several members of the family which is detrimental in the WalKR mutant (Vollmer *et al.*, 2008). In *B. subtilis*, YycG colocalized with FtsZ, the tubulin-like master regulator of cell division, and it has been related with cell division in this bacterium (Fukushima *et al.*, 2008). Other studies have shown that overexpression of two genes of WalKR regulon, LytM or SsaA, are able to restore cell viability of a WalKR conditional mutant. LytM is a glycyl-glycyl endopeptidase that hydrolyses the pentaglycine interpeptide bridge whereas SsaA belongs to the CHAP amidase family that cleaves the bond between the peptide and the glycan strand (Ramadurai *et al.*, 1999; Lang *et al.*, 2000). Again, as *lytM* and *ssaA* genes are not essential in the presence of WalKR, these results suggest that relaxation in peptidoglycan crosslinking is a major reason to explain the WalKR essentiality (Delaune *et al.*, 2011). Recent studies revealed that strains producing constitutively active WalR RR are strongly diminished in

their virulence due to enhanced recruitment of neutrophils and triggering of proinflammatory cytokine production associated with higher levels of released peptidoglycan fragments (Delaune *et al.*, 2012).

The *lytSR* system

The LytSR system plays a role in murein hydrolase activity and bacterial autolysis during planktonic growth (Brunskill and Bayles, 1996a; Brunskill and Bayles, 1996b; Groicher *et al.*, 2000). Using transcriptome analysis Sharma-Kuinkel *et al*. found that *lytS* mutation resulted in the downregulation of more than 400 genes and the upregulation of seven genes (Sharma-Kuinkel *et al.*, 2009). The gene whose transcription was more dramatically affected by the *lytS* mutation was the *lrgAB* operon, which is an inhibitor of the murein hydrolase activity. In the absence of LytSR system, higher expression of murein hydrolases caused cell lysis and released of DNA to the extracellular (eDNA) which enhances biofilm formation capacity (Patton *et al.*, 2006; Rice *et al.*, 2007; Sharma-Kuinkel *et al.*, 2009). LytS regulates *lrgAB* expression in response at least to changes in membrane potential (Patton *et al.*, 2006).

The Glycopeptide Resistance Associated system: *graSR*

GraSR (also known as ApsSR for Antimicrobial Peptide Sensor) belongs to the intramembrane-sensing HK subfamily. For the signalling pathway, this TCS requires the participation of the accessory protein, GraX (Falord *et al.*, 2011). GraSR controls the resistance to cationic antimicrobial

peptides (CAMP) through D-alanylation of teichoic acids, as well as MprF-dependent lysylination of phosphatidylglycerol, which increases the bacterial surface positive charge and prevents CAMP binding by electrostatic repulsion (Herbert *et al.*, 2007; Min Li, Cha, *et al.*, 2007; Otto, 2009). GraSR can also affect CAMP resistance indirectly by regulating the expression of the *vraFG operon*, which encodes an ABC transporter (Meehl *et al.*, 2007; Falord *et al.*, 2012). Other studies have shown that GraSR is also related with resistance to vancomycin as well as tolerance to high temperature and oxidative stress (Meehl *et al.*, 2007; Falord *et al.*, 2011). It has been recently described that the GraR RR undergoes specific phosphorylation by Stk1, a serine/threonine kinase (Fridman *et al.*, 2013).

The *staphylococcal* accessory protein effector system: *saeRS*

The SaeS (HK) and SaeR (RR) proteins are encoded in an operon that includes four ORFs (*saeP, saeQ, saeR* and *saeS*) (Giraudo *et al.*, 1994). The sensor SaeS is classified as an intramembrane HK because the short amino acid sequence that connects the two TM domains present in the protein seem to be insufficient to bind a ligand. SaeS does not possess phosphatase activity and requires the aid of SaeP and SaeQ auxiliary proteins to dephosphorylate SaeR and deactivate the expression of the corresponding target gene (Jeong *et al.*, 2012). SaeS is activated by perturbations of the bacterial membrane allowing the pathogen to react to phagocytosis related effector molecules (HNPs) (Geiger *et al.*, 2008). The SaeRS plays a key regulatory role in the production of important virulence

factors, such as α-hemolysin (*hla*), β-hemolysin (*hlb*), coagulase (*coa*), DNAse, Protein A (*spa*), fibronectin-binding protein A (*fnbA*), extracellular adherence protein (*eap*) and capsular polysaccharide (*cap*) probably through direct interaction with the target genes (Giraudo *et al.*, 1994; Steinhuber *et al.*, 2003; Liang *et al.*, 2006; Hiroko Kuroda *et al.*, 2007), indicating that SaeRS system is involved in host-pathogen interactions.

The autolysis-related locus system: *arlRS*

ArlRS was described as a TCS involved in the regulation of adhesion, autolysis and extracellular proteolytic activity (Fournier and Hooper, 2000). Following studies demonstrated that ArlR represses the production of virulence factors and some secreted proteins (α-toxin, β-haemolysin, coagulase, lipase, Protein A) (Fournier *et al.*, 2001). The regulation of some of these factors by the *arl* locus occurs indirectly via the *agr* and/or *sarA* regulatory pathway. A recent study has shown that ArlRS regulates bacterial autolysis differently in methicillin-sensitive (MSSA) and MRSA strains. Thus, the inactivation of *arlRS* increases bacterial autolysis in MSSA while it does not cause any effect in MRSA strains. The differences in the regulatory behaviour are partially due to difference in the *agr* expression between the two strain sets (Memmi *et al.*, 2012). Furthermore, deletion of *arlRS* bestows on *S. aureus* the capacity to form a biofilm in a chemically defined medium by a mechanism independent of the presence of the PIA/PNAG exopolysaccharide (Toledo-Arana *et al.*, 2005). ArlRS also regulates the agglutination mechanism during exposure to human plasma or fibrinogen,

by modulating the levels of the giant *staphylococcal* surface protein (*ebh*) (Walker *et al.*, 2013).

The staphylococcal respiratory response (*srrAB*) system: *srrAB*

SrrAB is homologue of the *resDE* TCS in *B. subtilis* (Birkey 1998). It is widely assumed that the sensor kinase SrrB responds to environmental oxygen to activate or repress target gene transcription. SrrAB is considered a major regulatory system for anaerobic gene regulation (Yarwood *et al.*, 2001). However, how SrrAB is controlled at molecular level still remains a mystery. The SrrAB repress toxic shock syndrome toxin (TSST-1) and Protein A expression under microaerobic/anaerobic conditions and modulate the expression of RNAIII, the effector molecule of the global quorum-sensing system *agr* (Pragman *et al.*, 2004). SrrAB is important for protection from killing by neutrophils under anaerobic conditions and it is also responsible for activating *icaADBC operon* expression and PIA/PNAG exopolysaccharide production in anaerobic conditions (Martina Ulrich *et al.*, 2007). In addition to oxygen limitation, the SrrAB TCS responds to nitric oxide and regulates the expression of *hmp*, an NO detoxification enzyme (Kinkel *et al.*, 2013).

The anaerobic iron-sulfur cluster-containing redox sensor regulator system: *airSR*

Initial studies on AirSR (formerly YhcSR) suggested that this TCS system was essential for growth, because overexpression of an antisense

RNA in a clinical isolate *S. aureus* WCUH29 inhibits bacterial growth *in vitro* (Junsong Sun *et al.*, 2005; Yan *et al.*, 2011). However, in a previous study we were able to generate mutants of YhcSR in *S. aureus* 15981 clinical strain indicating that this TCS is not essential for *S. aureus* (Toledo-Arana *et al.*, 2005). Furthermore, the presence of ΔYhcS and ΔYhcR mutants at the Nebraska transposon mutant library unambiguously confirmed that YhcSR is not essential for *S. aureus*. YhcSR has been recently renamed as AirSR, for anaerobic iron-sulfur cluster-containing redox sensor regulator (Fei Sun, Ji, *et al.*, 2012). This study revealed that AirS is an oxygen sensor that only exhibits an efficient kinase activity with an oxidized $[2Fe\text{-}2S]^{2+}$ motif. Thus, prolonged exposure of AirS to $O_2$ or strong oxidants such as $H_2O_2$ and NO abolishes the kinase activity. Accordingly, the analysis of the global gene expression profiles of AirR mutant using microarrays showed that AirR does not affect gene expression in aerobic conditions whereas a total of 67 (midexponential growth phase) or 355 genes (stationary phase) were differentially expressed under anaerobic conditions (Fei Sun, Ji, *et al.*, 2012). AirR-regulated genes include other TCS such as Agr and SaeRS, anti-sigma factors (RsbU and RsbW), cell wall metabolism-associated genes, as well as key virulence factors such as capsular polysaccharide, γ-hemolysin and Protein A.

The vancomycin-resistance-associated system: *vraSR*

The *vraSR* system belongs to an operon of four genes (*orf1, yvqF, vraS* and *vraR*). The YvqF transmembrane protein seems to be involved in

sensing the stress signal that triggers the signal transduction (McCallum *et al.*, 2011). VraSR controls the cell wall stress stimulon that protects against cell envelope damaging agents. VraR RR regulates genes associated with cell wall biosynthesis such as *pbp2, murZ, sgtB* and *fmtA* (Makoto Kuroda *et al.*, 2003; Utaida, 2003; Gardete *et al.*, 2006 s; Sobral *et al.*, 2007).

The accessory gene regulator system: *agrBDCA*

Quorum sensing (QS) is a widespread cell-cell communication mechanism to coordinate the expression of multiple genes in a cell density-dependent manner. For this purpose bacteria secrete and sense small diffusible molecules called autoinducers (AIs). The Agr system is the best-characterized QS system in *S. aureus*. The *agr* system is activated during transition from the exponential to the stationary growth phase by an autoregulatory mechanism involving a secreted autoinducing peptide (AIP) that senses the estate of cell-density (Novick and Muir, 1999).

The *agr* locus consists of four genes *agrBDCA* that are cotranscribed (RNAII) by P2 promoter and a genetic region which is transcribed in the opposite direction by P3 promoter and encodes the *agr* system effector molecule (RNAIII) and the virulence factor δ-toxin (*hld*) (Peng *et al.*, 1988; Janzon *et al.*, 1989). RNAII comprises a typical bacterial TCS consisting of the sensor HK AgrC and the RR AgrA. In addition, it encodes AgrD, the precursor of the quorum signal that can further be processed and exported as a thiolactone-containing oligopeptide autoinducer (autoinducing peptide, AIP) by the cotranscribed AgrB (Zhang and Ji, 2004; Qiu *et al.*, 2005;

Kavanaugh *et al.*, 2007). When AIP reaches a critical concentration, it interacts to the extracellular sensory domain of AgrC, AIP activates the kinase activity of AgrC, subsequently leading to phosphorylation of the RR AgrA. AgrA is a DNA-binding protein that after activation induces the transcription of both promoters P2 and P3 (Koenig *et al.*, 2004). On the other hand, RNAIII promotes the upregulation of exoprotein production (proteases, lipases, nucleases) and the downregulation of cell surface protein transcription during the late-logarithmic and stationary phases of growth (Dunman *et al.*, 2001). The regulatory RNAIII acts by direct base pairing with target mRNAs (*spa, coa*, *fib, ssa*-like) or can also operate indirectly, via the translational repression of *rot* (*hla, hld, geh, ssp* etc) (Boisset *et al.*, 2007).

Besides, an RNAIII-independent mechanism for gene regulation by AgrA has also been recently described (Queck *et al.*, 2008). Phosphorylated AgrA downregulates genes involved in carbohydrate and amino acid metabolism and upregulates the expression of phenol-soluble modulins (PSM) by direct binding (Queck *et al.*, 2008).

Sun *et al.* (Fei Sun, Liang, *et al.*, 2012) recently discovered that *agr* system has integrated an intrinsic oxidation-sensing mechanism through a redox-active Cys-199 in the RR AgrA. The oxidized AgrA forms an intramolecular disulfide bond and dissociates from its cognate DNA, leading to down-regulation of RNAIII expression and up-regulation of *bsaA* (glutathione peroxidase), essential for bacterial resistance to oxidative stress (Fei Sun, Liang, *et al.*, 2012). Moreover, the system is modulated by

nutrient availability, monitored by the transcriptional regulator CodY (Roux *et al.*, 2014).

*The kdpDE system*

The HK KdpD and its cognate RR KdpE belong to a family of TCS that sense potassium ($K^+$) concentration (Freeman *et al.*, 2013). The KdpDE system was first characterized in *E. coli*, in which proteins KdpD and KdpE regulate the production of the high-affinity $K^+$ transporter Kdp ATPase (Polarek *et al.*, 1992). The Kdp ATPase is an efficient $K^+$ scavenging system that is expressed when cells can not meet the cellular requirements for $K^+$. Because Kdp ATPase system works as a high-affinity $K^+$ transporter in several bacteria and the genome organization of the Kdp ATPase operon is highly conserved, it was assumed that KdpDE system was also mainly dedicated to $K^+$ homeostasis in *S. aureus*. By contrast, evidence suggests that $K^+$ availability has little to do with KdpD/KdpE activity. The expression of *kdpDE operon* is up-regulated and its deletion results in attenuated survival when *S. aureus* is grown in human blood, despite the fact that blood is rich in $K^+$. Additionally*, S. aureus kdpDE* transcription is enhanced during biofilm formation and in response to microbicidal neutrophil extracts, situations in which $K^+$ is not necessarily limited (Beenken *et al.*, 2004; Palazzolo-Ballance *et al.*, 2008). Recent studies indicated that the main function of KdpDE is associated with transcriptional regulation for a series of virulence factors (*spa, cap, hla, aur, geh*, and *hlgB*) in response to $K^+$ concentrations and population density, connected to AI-2 quorum sensing system (Zhao *et*

*al.*, 2010; Xue *et al.*, 2011). Also, Corrigan *et al*. have found that KdpD is a receptor of c-di-AMP, though the regulatory mechanisms underlying this interaction remain unknown (Corrigan *et al.*, 2013). Intriguingly, a paralogous KdpED system is present in certain *S. aureus* strains that possess the SCC-*mec* type II element (Hanssen and Ericson Sollid, 2006). The function of this additional KdpED system remains unknown.

The heme-sensing system: *hssRS*

HssRS system allows *S. aureus* to sense heme. Upon sensing heme, HssRS directly regulates the expression of the heme-regulated ABC transporter HrtAB, which plays a main role in the intracellular heme homeostasis (Friedman *et al.*, 2006; Torres *et al.*, 2007; Stauff *et al.*, 2007). The inability to sense or respond to heme increases the virulence of *S. aureus* (Torres *et al.*, 2007).

The nitrate respiration system: *nreABC*

The *nreBC* is essential for the transcriptional activation of genes involved in dissimilatory reduction and transport of nitrate and nitrite. The operon plays a crucial role in the bacterial survival in anoxic environments (Schlag *et al.*, 2008). NreA is an accessory protein consisting almost exclusively of a GAF domain, which is one of the largest families of cyclic nucleotide-binding regulatory domains (Martinez *et al.*, 2002). This system has been deeply studied in *S. carnosus* and the cytoplasmic histidine

sensor kinase responds to $O_2$ by the $[4Fe\text{-}4S]^{2+}$ containing PAS domain (Kamps *et al.*, 2004; Müllner *et al.*, 2008).

The bacitracin resistance associated system: *braRS*

The *braRS* (also known as *nsaRS or bceRS*) TCS also belongs, with GraRS, to the intramembrane-sensing HK family, which is conserved within the firmicutes (Mascher, 2006). The system is essential for bacitracin and nisin resistance and its function is directly related with the activation of two operons encoding ABC transporters, *braDE* and *vraDE* (Blake *et al.*, 2011; Yoshida *et al.*, 2011; Hiron *et al.*, 2011). Other studies showed that BraRS is important in sensing cell envelope damage produced by several antimicrobial agents (Kolar *et al.*, 2011).

TCSs with unknown functions: *phoPR*, *tcs3* and *tcs7*

The function of TCSs 3, 7 and PhoPR (Fig. 2 and Table 1) remain unknown. Considering the distance between genes, it appears that the *tcs3* is an operon composed by 3 ORFs (MW0198, MW0199 and MW0200). Based on sequence homologies, the RR (MW0198) belongs to the AraC family while the HK (MW0199) to the His_kinase family (pfam06580) (Fig. 2). The third ORF (MW0200) encoded the periplasmic iron-binding accessory protein, BitC.

The *tcs7* appears to be an operon of 4 ORFs (MW1206, MW1207, MW1208 and MW1209). Sequence homologies predict that MW1206 and MW1207 proteins encode for an ABC transporter ATP-binding protein and

permease protein, respectively. The HK MW1208 belongs to HisKA_3 family while the RR MW1209 belongs to the NarL family.

Finally, the *S. aureus* PhoPR is homologous to the *B. subtilis* PhoPR, which mediates the phosphate-deficiency response. PhoPR regulon seems to be related with ResDE system (homologous to SrrAB) in *B. subtilis*.

**Specificity in two-component signal transduction**

HKs and RRs each comprise paralogous gene families and the members of each family share significant homology at both the primary sequence level and the structural level. Moreover, all TCS share a common mechanism of phosphotransfer. The similarity of these signalling proteins raises the possibility of cross-talk between different TCS pathways. Cross-talk is defined as the communication between two pathways that, if eliminated, would leave intact two distinct, functioning pathways. In general, cross-talk between distinct pathways must be kept to a minimum, otherwise an organism would not be able to evoke the necessary response to a specific input stimulus. However, under some conditions, it may be advantageous to an organism to permit or use cross-talk as a means of either integrating multiple signals or diversifying the response to a single input; we refer to such cases, where cross-talk benefits the organism, as “cross-regulation” to distinguish them from detrimental, unwanted cross-talk (Wanner, 1992). We also distinguish cross-talk and cross-regulation from pathways that are inherently, or necessarily, branched. For example, cases

where the topology of a signalling pathway includes one-to-many or many-to-one relationships that are required for an organism to mount a proper response to a given stimulus (Laub and Goulian, 2007).

Although most species encode dozens, if not hundreds, of these signalling pathways, there is relatively little cross-talk, indicating that individual pathways are well insulated and highly specific (Podgornaia and Laub, 2013). Bacteria have several mechanisms that ensure the specificity and insulation of these pathways. First, a general phenomenon responsible of preventing cross-talk is the phosphatase activity of bifunctional HKs for their cognate RRs. This mechanism of negative control termed transmitter phosphatase activity eliminates unwanted phosphorylation of the receiver by sources other than the cognate transmitter module (Gao and Stock, 2009). However, even for systems with monofunctional HKs, there may be additional phosphatases that protect against undesired cross-talk (Stock *et al.*, 2000). Indeed, the absence of a bifunctional sensor is a prerequisite for *in vivo* cross-talk and *in vitro* non-cognate phosphotransfer. Whereas the phosphotransfer reaction can occur *in vivo* between certain non-cognate HKs and RRs, transmitter phosphatase activity appears to be specific for a cognate pair (Siryaporn and Goulian, 2008; Groban *et al.*, 2009).

Second, there is evidence that RR competition also helps to limit cross-talk between some signalling pathways. The cognate RR normally out-competes the non-cognate RR, and this competition effect is enhanced by the low abundance *in vivo* of HKs relative to RRs. Consequently, deleting

a given RR can lead to inappropriate cross-talk from its cognate HK to other RR (Siryaporn and Goulian, 2008; Groban *et al.*, 2009).

Third, specificity could also arise through temporal or spatial restriction of pathways, thereby ensuring that only one of the two systems is expressed at a given time or localizing HKs and RRs subcellularly in bacteria (Laub and Goulian, 2007).

Most cross-talk analysis has been carried out *in vitro* or exploiting isolated TCS couples and making simple, double or triple mutants. However, a system-based analysis to determine the possible functionality of the cross-talk process *in vivo* is still missing.

Several studies have demonstrated that HKs typically harbor a global and strong kinetic preference for their cognate RRs *in vitro* (Skerker *et al.*, 2005). This ability to discriminate cognate from non-cognate partners in the absence of other cellular components indicates that specificity is encoded primarily at the molecular level. Analyses of amino acid coevolution in cognate signaling proteins identified the key specificity-determining residues in histidine kinases and response regulators (Skerker *et al.*, 2008; Casino *et al.*, 2009). Those amino acids are located at or near the presumed molecular interface formed during phosphotransfer, on the surface of an $\alpha$ helix in each protein and are represented in Figure 3 (Huynh *et al.*, 2010; Willett and Kirby, 2012). Moreover, this residues conferring phosphotransfer and phosphatase specificity determine binding affinity, another critical parameter for preventing cross-talk (Willett *et al.*, 2013).

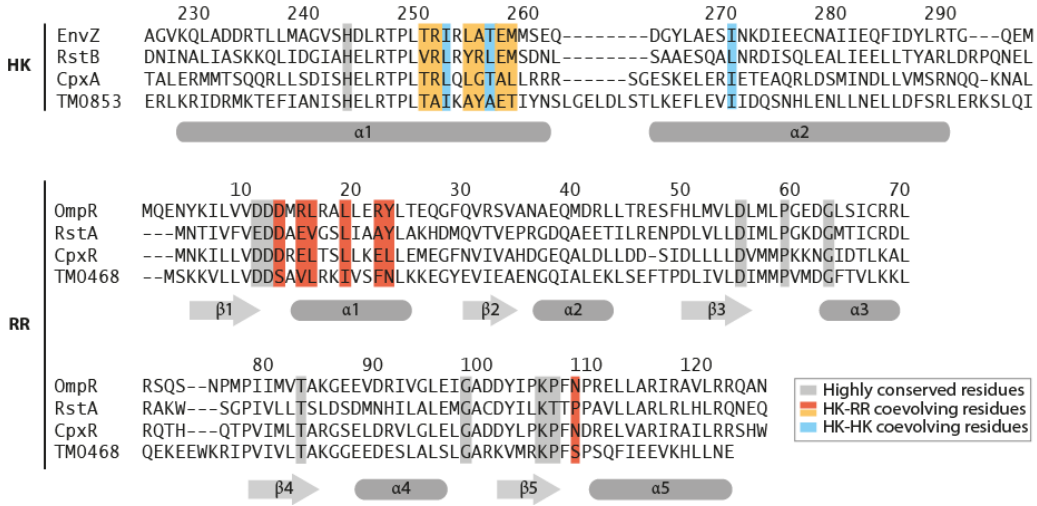


**Figure 3. Specificity residues in two-component signaling proteins.** The picture shows a sequence alignment of TM0853, a *Thermotoga maritima* kinase, with three *E. coli* kinases, EnvZ, RstB, and CpxA, and an alignment of TM0468, a *Thermotoga maritima* response regulator with three *E. coli* regulators, OmpR, RstA, and CpxR. Secondary structure elements are indicated beneath the primary sequence. Coevolving residues on the histidine kinase and response regulator are coloured orange and red, respectively. Residues in histidine kinases that coevolve strongly with other kinase residues are coloured cyan. Highly conserved residues are coloured grey (Capra *et al.*, 2012)

Phylogenetic analyses indicate that, for most bacterial species, the majority of new TCS pathways emerge through gene duplication. Immediately after duplication of a HK-RR pair, the two signaling pathways are identical, such that each HK can interact with each RR. After the pathways diverge with respect to signal inputs and downstream outputs, there is a need to avoid the residual cross-talk via changes in the specificity residues of one or both of the duplicated HKs. Such mutations must be compensated through corresponding changes in their cognate regulators. This intermolecular coevolution provides the insulation between the two new pathways while maintaining phosphotransfer within each system (Capra *et al.*, 2012). However, not all mutational trajectories have these characteristics of maintaining phosphotranfer and avoiding cross-talk, raising the possibility that sequence evolution following duplication is constrained or that natural selection may have favoured certain trajectories over others.

# *OBJECTIVES*

# OBJECTIVES OF THE THESIS

Bacteria depend on their ability to sense and respond to external signals to survive in different niches. Two-component signal transduction proteins are the prevalent mechanism for transmitting changes in environmental conditions to the proteins and other components that respond to signals. While the molecular mechanisms underlying the two-component signal transduction cascade have been elucidated, the consequences of the removal of the complete TCS sensorial network and global pleiotropic effects of this signaling network remain unknown, primarily because most bacteria encode dozens, even hundreds, of TCSs. In this study, we take advantage of the moderate complexity of the TCS signaling network in *S. aureus* to perform the first system-based analysis of this signaling network in bacteria.

The specific objectives of this thesis are:

1. Transcriptomic analysis to determine the expression of the TCSs of *S. aureus* using tiling arrays and RNA sequencing.
2. Generation of a *S. aureus* strain devoid of the complete two-component sensorial system.
3. Phenotypic characterization of the consequences of the absence of the two-component sensorial system for *S. aureus*.
4. Identification of the phenotypes regulated by individual TCSs.
5. System-based analysis of cross-talk between non-cognate HKs and RRs *in vivo*.

# *MATERIALS AND METHODS*

# MATERIALS AND METHODS

## Oligonucleotides, plasmids, bacterial strains and culture conditions.

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 2, Table 3 and Table 4 respectively (see Tables at the end of this section). *Staphylococcus aureus* strains were grown in trypticase soy broth supplemented with 0.25% glucose (TSB-gluc) (Pronadisa), B2 medium (1% casein hydrolysate, 2.5% yeast extract, 2.5% NaCl, 0.1% $K_2HPO_4$, and 0.5% glucose [wt/vol]) and chemically defined medium HHWm (Hussain-Hastings-White modified medium) (Toledo-Arana *et al.*, 2005). *Escherichia coli* was grown in LB broth (Pronadisa). When required for selective growth, medium was supplemented with appropriated antibiotics at the following concentrations: erythromycin (Em), 1.5 μg $ml^{-1}$ and 10 μg $ml^{-1}$; ampicillin (Amp), 100 μg $ml^{-1}$, chloramphenicol (Clo), 10 μg $ml^{-1}$ and 20 μg $ml^{-1}$. (Baba *et al.*, 2002; McDougal *et al.*, 2003; Úbeda *et al.*, 2007).

## RNA extraction

Bacteria were grown in TSB-gluc at 37ºC under shaking conditions (250 rpm) until the culture reached an $OD_{650nm}$ of 0,8. Total RNA from bacterial pellets was extracted using TRIzol reagent method as previously described (Toledo-Arana *et al.*, 2009). Briefly, bacterial pellets were resuspended into 400 µl of solution A (Glucose 10%, Tris 12.5 mM pH7.6, EDTA 10 mM), and mixed to 60 µl of 0.5M EDTA. Resuspended cells were

transferred into Lysing Matrix B tubes (MP Biomedicals) containing 500 µl of acid phenol (Ambion) and mixed. Bacteria were mechanically lysed using the Fastprep apparatus (BIO101) at speed 6.0 during 45 sec at 4ºC. After lysis, tubes were centrifuged 10 min at 14,000 rpm at 4ºC. The aqueous phase was transferred to 2-ml tubes containing 1 ml TRIzol, mixed and incubated for 5 min at room temperature. 100 µl of chloroform was added, mixed gently and incubated for 3 min at room temperature. Tubes were centrifuged for 10 min at 14,000 rpm at 4°C. The aqueous phase was transferred into a 2-ml tube containing 200 µl of chloroform, mixed and incubated for 5 min at room temperature. Tubes were centrifuged for 5 min at 14,000 rpm at 4°C. RNA contained in the aqueous phase was precipitated by addition of 500 µl of isopropanol and incubated 15 min at room temperature. Tubes were centrifuged for 15 min at 14,000 rpm at 4°C. RNA pellets were washed with 75% ethanol. Dried RNA pellets were resuspended in DEPC-treated water. RNA concentrations were quantified and RNA qualities were determined using Agilent RNA Nano LabChips (Agilent Technologies). RNAs were stored at -80°C until needed.

**RNA sequencing**

Total RNA was extracted after lysing bacteria in the Fastprep homogenizer (Bio101) using TRIzol reagent (Invitrogen). The RNA fraction shorter than 50 nt was purified from total RNA (100 mg) through electrophoresis at 75V constant voltage during 16 min with flashPAGE fractionator (AMBION). Purified RNA fraction was incubated with 2U of TAP

(Epicenter) for 2h at 37ºC to convert 5' triphosphate groups to monophosphates. The TAP treated short RNA fraction was ligated to the 3' ModBan oligonucleotide (Table 4) by using the truncated T4 RNA ligase2. This was followed by a second gel purification on 10% TBE-UREA gel to remove the non-ligated 3' ModBan oligonucleotide. The extracted product was ligated to 5' RNA Illumina adapter by using T4 RNA ligase. cDNA was produced using SuperScript III and the RT oligonucleotide, complementary to the ModBan 3' linker. The resulting RT product was then amplified using Sol_5_SBS3 and Sol_3_ModBan adapters, purified and sent for Illumina sequencing.

**cDNA synthesis, labelling and tiling array hybridization**

Before cDNA synthesis, RNA integrity from each sample was confirmed on Agilent RNA Nano LabChips (Agilent Technologies). 10 µg of total RNA were reverse transcribed using SuperScript II reverse transcriptase (Invitrogen Life Technologies) and processed following the protocol of the Affymetrix GeneChip Expression Analysis Technical Manual (P/N 702232 Rev. 2) in the presence of 6 ng $ml^{-1}$ Actinomycin D to avoid spurious second-strand cDNA synthesis during reverse transcription reaction (Perocchi *et al.*, 2007). Sense RNA corresponding to *B. subtilis* poly-A *lys*, *phe*, *thr*, *trp*, *dap* genes were spiked into sample RNA as control for labelling and hybridization steps. cDNA was digested by DNase I (PIERCE) in 10X DNase I buffer (USB-Affymetrix) and the size of digestion products was analysed in the Agilent Bioanalyser 2100 using RNA Nano

LabChips to ensure that the fragmentation resulted in a majority of products in the range of 50 to 200 base-pairs. The fragmented cDNA was then biotinylated using terminal deoxynucleotidyl transferase (Promega) and the GeneChip DNA labelling reagent (Affymetrix) following the manufacturer's recommendations. Biotinylated cDNA (5 microgram per array) was hybridized on custom *S. aureus* tiling microarrays designed as described (Segura *et al.*, 2012). Hybridization was carried out during 16 h according to the Affymetrix protocol in a total volume of 200 µl per hybridization chamber. Following incubation, the arrays were washed and stained in the Fluidics station 450 (Affymetrix) using the protocol n°FS450_0005. Scanning of the arrays was then performed using the GeneChip scanner 3000 (Affymetrix). A first scan of the chip was carried out with gene expression sub-array parameters followed by a second scan with tiling sub-array parameters. Intensity signals of each probe cells were computed by the GeneChip operating software (GCOS) and stored in cell intensity files (.CEL extension) before preprocessing and analysis.

**Microarray data analysis**

Data analysis of the tiling sub-array was performed using the Tiling Analysis Software (TAS) from Affymetrix (http://www.affymetrix.com). Output bar files containing probe signal values were converted in graphic type files (.gr extension file) to be loaded at the *S. aureus* transcriptome browser (http://staph.unavarra.es/).

**cDNA libraries for RNA-seq, read mapping and statistics analysis**

Deep sequencing of RNAs from *S. aureus* 15981 strain was performed as previously described (Lasa *et al.*, 2011). Mapped reads were included in .wig files to be loaded at the *S. aureus* transcriptome browser (http://staph.unavarra.es/).

**Electrocompetent *Staphylococcus* cells**

*Staphyloccocal* electrocompetent cells were generated as previously described (Schenk and Laddaga, 1992). Briefly, bacteria were grown in 200 ml of B2 broth at 37°C under shaking conditions (200 rpm) until the culture reached an $OD_{600nm}$ of 0.5. Culture was incubated for 15 min on ice and then it was centrifuged and the pellet was washed three times with sterile water. A final washing step was done with 30 ml of ice-cold 10% glycerol. The pellet was resuspended into 15 ml of ice-cold 10% glycerol and incubated for 15 min at 20°C. Culture was centrifuged and bacterial pellet was resuspended into 200 µl of ice-cold 10% glycerol. Aliquots of 50 µl were stored at -80°C. Plasmids were transformed into staphylococci by electroporation, using a previously described protocol (Cucarella *et al.*, 2001).

**Preparation of ϕ80 transducing lysates**

For the donor lysate preparation, 50 ml of the *S. aureus* RN4220 overnight culture (containig pMAD plasmid to be transduced) were diluted in 5 ml of the phage broth (containing $CaCl_2$). 200 ml of phage dilutions $10^5$

$10^6$ $10^7$ $10^8$ (in phage broth containing $CaCl_2$) were added to 300 ml of the donor cells and the mixture was incubated at room temperature for 30 min. 10 ml of molten phage top agar (containing $CaCl_2$) at 55ºC were added to the tubes and immediately the liquid was poured over the surface of 2 phage base plates (containing $CaCl_2$). Plates were incubated overnight at 37ºC. Next day the top layer containing the phage plaques was scraped of with a Pasteur pipette into a falcon of 50 ml. Falcons were centrifuged and the supernatant was filtered through 0.45 mm filter and stored al 4ºC.

**Phage Transduction**

For the phage transduction, the recipient bacteria were grown in 20 ml of TSB overnight. Culture was centrifuged and resuspended in 1 ml of TSB. 500 μl of the culture were mixed with 1 ml of LB (containing CaCl2) and 500 ml of the phage lysate. There was also a control sample that had no phages. Mixture was incubated at 37ºC for 25 min on a water bath and then at 37ºC for 15 min in a orbital shaker. Reaction was stopped on ice and 1 ml of cold 0.02M Na citrate was added. The tube was centrifuged and the pellet was resuspended in 1 ml of 0.02M Na citrate and incubated on ice for 2 h. The bacterial solution was spread onto five TSA plates (200 μl per plate) containing Em 1.5 μg $ml^{-1}$ and Na citrate.

**Allelic exchange of chromosomal genes**

To generate deletion mutants, we amplified by PCR two fragments of 500 bp that flanked the left (primers A and B, Table 4) and right sequences

(primers C and D, Table 4) of the region targeted for deletion. The PCR products were purified and cloned separately in the pGEM-T Easy vector (Promega). Fragments were then fused by ligation into the shuttle vector pMAD (Arnaud *et al.*, 2004). The resulting plasmid was transformed into *S. aureus* MW2 strains by electroporation or transduction. Homologous recombination experiments were performed as described (Valle *et al.*, 2003). Erythromycin sensitive white colonies, which no longer contained the pMAD plasmid, were tested by PCR using primers E and F (Table 4).

**Genome sequencing of the parental and mutant strains by Illumina**

Genomic DNA was prepared from an overnight culture of parental (MW2 and 8325r) and mutant (MW2 ΔXV and 8325r ΔXV) strains grown in TSB-gluc at 37°C, as described previously (Marmur 1961) and sequenced on an Illumina Hi-Seq 2000 instrument (Genomics Platform of CIBIR, La Rioja, Spain). Reads (6M-9M 150-bp) were assembled *de novo* using SOAPdenovo2 algorithm (Ruibang Luo *et al.*, 2012). The reads were assembled into 466 contigs (>100 bp in size) corresponding to 2864441 bp in length, with a N50 value of 16063 and a GC content of 32.67 % for MW2, 236 contigs (>100 bp in size) corresponding to 2856644 bp in length, with a N50 value of 50702 and a GC content of 32.76 % for MW2 ΔXV, 283 contigs (>100 bp in size) corresponding to 2839577 bp in length, with a N50 value of 60685 and a GC content of 32.80 % for 8325r and 292 contigs (>100 bp in size) corresponding to 2807518 bp in length, with a N50 value

of 79329 and a GC content of 32.80 % for 8325r ΔXV. Subsequently, contigs were ordered with Mauve (Rissman *et al.*, 2009). Variant base calling of the mutant XV relative to MW2 and 8325r was performed with SAMTOOLS (Heng Li *et al.*, 2009). Finally genomic reorganization, insertions and deletions were studied with the Breseq pipeline (Barrick *et al.*, 2009).

**Construction of the insertional mutation of *walKR* operon**

*S. aureus* strains were transformed with plasmid pSD3-3 (Dubrac and Msadek, 2004), a derivative of pDH88. Integration of the plasmid through a single crossover event generated the strain ΔXV P*spac-walRK* (ΔXVI*), in which the entire *walRK* operon is under the control of the P*spac* iso-propyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. *S. aureus* strains were grown in Trypticase soy broth (TSB) supplemented with chloramphenicol (10 μg $ml^{-1}$).

**Construction of plasmids expressing HKs and/or RRs genes**

To construct the plasmids expressing RRs, we amplified them by PCR using forward and reverse primers and the MW2 chromosomal DNA as template (Table 4). Forward primers carried SalI-XhoI tail and reverse primers carried BamHI-XmaI-KpnI tail. The PCR products were amplified with Phusion® High-Fidelity DNA Polymerase (Fermentas-Thermo Scientific), purified and cloned in pCR®-Blunt II TOPO vector (Invitrogen). Fragments were then ligated using SalI and KpnI enzymes in the vectors

pEW ($P_{blaZ}$ promoter) and pEI ($P_{cad}$ promoter). These plasmids were constructed by combination of pCN40, pCN47 and pCN51 (Charpentier *et al.*, 2004).

Similarly, the histidine kinases (HK) were amplified by PCR with the forward and reverse primers and the MW2 chromosomal DNA as template (Table 4). Forward primers carried BamHI tail and reverse primers carried XmaI-AscI tail. The PCR products were amplified with Phusion® High-Fidelity DNA Polymerase (Fermentas-Thermo Scientific), purified and cloned in pCR®-Blunt II TOPO vector (Invitrogen). Fragments were then ligated using BamHI and AscI enzymes in the vectors pCW ($P_{blaZ}$ promoter) and pCI ($P_{cad}$ promoter). These compatible plasmids were constructed by combination of pCN40, pCN47, pCN51 (Charpentier *et al.*, 2004) and pCU1 (Augustin *et al.*, 1992) plasmids.

For the construction of the plasmids expressing a combination of HKs and RRs, TOPO HKn plasmids were digested with BamHI and XmaI enzymes and the BamHI/XmaI module, containing the histidine kinase, was transferred to the corresponding plasmid, pEW RRn or pEI RRn. Modules containing RRs and HKs can be moved together from one plasmid to another by digestion with SalI or XhoI and XmaI or KpnI.

It is important to notice that the pEI inducible plasmid has a basal expression without cadmium. All the experiments on this thesis in which the pEI plasmid is used, were carried out without cadmium.

**Antibiotic Resistance**

Antibiotimicrobial susceptibility tests were performed at the University Clinic of Navarra. Antibiotics routinely used in clinic for treatment of *S. aureus* infections were tested by VITEK 2 system.

**Growth kinetics analysis**

In order to compare growth kinetics, overnight cultures of the strains were serially diluted ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$) in TSB-gluc and 5 μL of no diluted, $10^{-2}$, $10^{-4}$ and $10^{-6}$ diluted cultures were spotted onto TSA plates. The plates were then incubated at 28, 37 and 44ºC overnight and representative pictures were taken with a digital camera.

**Biochemical characterization**

API Staph galleries (Biomerieux) were used to analyze bacterial metabolic patterns, according to the manufacturer´s instructions. Homogeneous bacterial suspensions with a turbidity equivalent to 0.5 McFarland were prepared using the API Staph Medium. The microtubes were then filled with the bacterial suspensions and the strip was incubated inside the incubation box at 37ºC for 24 h. The changes in the bacterial metabolic patterns were analyzed comparing the corresponding mutants with the wild type *S. aureus* strain.

**Quantification of nitrite production**

Extracellular concentrations of nitrite were determined colorimetrically by the Griess Reagent System (Promega) according to the manufacturer´s instructions. Conditions of decreased oxygen tension were created by growing the bacterial strains in 15-ml Falcon tubes that were completely filled with medium (Schlag *et al.*, 2008). Potassium nitrate ($KNO_3$) was added to the medium at 20 mM. A purple/magenta color is being indicative of the presence of nitrites in the media. Representative pictures were taken with a digital camera.

**Desiccation Experiments**

The desiccation experiment was adapted from a protocol as described (White *et al.*, 2006). Briefly, 100 µl from overnight cultures grown in TSB-gluc medium at 37°C were tested immediately (initial numbers) or air dried and stored in 24-well tissue culture plates at room temperature for 21 days. After rehydration of bacteria in 500 µl TSBgluc, the number of viable cells remaining in each sample was determined by serially diluting cell mixtures and plating in duplicate. The average and SD of three independent assays were recorded.

**Triton resistance test**

Gradient plates (0 to 0,5%) were used to compare resistance phenotypes for Triton X-100 (USB) (McCallum *et al.*, 2011). OmniTray single well plates (nunc) were filled with triton containing agar and a slope

was created when the media was still in liquid state. The triton gradient was obtained upon filling the plate with trypticase soy agar. Overnight cultures of the test strains were diluted in TSB-gluc to $OD_{650nm}$ = 0.4 and the cell suspensions were swabbed across agar plates containing triton concentration gradients. Plates were incubated at 37°C for 24 hours. Triton sensitivity is observed as a growth deficiency, primarily pronounced on the concentrated area of the plate.

**Quantification of Protein A expression**

Overnight cultures of the strains were diluted in TSB-gluc to an $OD_{650nm}$ = 0.1 and 50 μL of the diluted cultures were mixed with 1950 μL of chemically defined medium HHWm (Hussain-Hastings-White modified medium) to inoculate sterile 24-well polystyrene microtitre plates (Costar). Plates were incubated at 37ºC for 24 hours, trying to mimic biofilm forming conditions. Eight ml of bacterial cultures were centrifuged and pellets were resuspended in 60 μl PBS. Then, 2 μl of Lysostaphin 1 mg $ml^{-1}$ (Sigma) and 3 μl of DNase I 1mg $ml^{-1}$ (Sigma) were added. After 2 h of incubation at 37ºC cell lysates were centrifuged and supernatants were collected. Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad). Samples were adjusted to 3-5 μg of total protein and one volume of Laemmli buffer was added. Total protein extracts were denatured by boiling at 100ºC for 5 min. Proteins were separated on 10% SDS-polyacrylamide gels and stained with 0.25% Coomassie brilliant blue R250 (Sigma) as loading controls. For Western blotting, proteins were transferred onto

Hybond-ECL nitrocellulose membranes (Amersham Biosciences) by semi-dry electroblotting. Membranes were blocked overnight with 5% skimmed milk in phosphate-buffered saline (PBS) with 0.1% Tween 20, and incubated with goat anti-mouse secondary antibodies labelled with phosphatase alkaline (Sigma) diluted 1:2500 for 2 h at room temperature. Protein A was detected with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

**Cell adhesion and invasion assay**

Human hepatic Hep3B and epithelial A549 cells were used for this study. Adherence and invasion experiments were performed as described previously (Valle *et al.*, 2012). Briefly, prior to use, wells were seeded with 0.3 x $10^6$ cells in 6-well tissue culture plates and 0.5 x $10^5$ cells in 24-well tissue culture plates. Once cells were confluent (1.2 x $10^6$ or 0.2 x $10^6$ cells per well) the culture medium was removed and cells were washed with Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) plus 10% heat-inactivated fetal bovine serum. For adherence assays, overnight bacterial cultures were mixed vigorously and added to the monolayer cells in a multiplicity of infection of 10 in DMEM. Incubation was carried out 1 hour at 37ºC in 5% $CO_2$. To remove non-adherent bacteria, cells were washed three times with sterile PBS. Eukaryotic cells were lysed with 0.1% Triton X-100. Before plating extracts were mixed vigorously by vortexing and sonication. The number of adherent bacteria were determined by serial dilution and plating. For invasion assays, bacteria were added to the

monolayer cells in a multiplicity of infection of 40 in DMEM. Incubation was carried out for 1 hour at 37ºC in 5% $CO_2$. To kill extracellular bacteria, media was replaced with 2 ml of DMEM containing 50 µg $ml^{-1}$ of gentamicin (SIGMA) for 2 hour. Cell monolayers were washed three times with sterile PBS and lysed with 0.1% Triton X-100. Before plating extracts were mixed vigorously by vortexing and sonication. The number of intracellular bacteria was determined by serial dilution and plating. Experiments were performed in triplicate and repeated four times for each cell line.

**Mouse infection model: kidney colonization assay**

MW2 wild type and MW2 ΔXV *S. aureus* strains were cultured overnight in TSB plates and a single colony was resuspended into 5 mL of PBS to an optical density at $650_{nm}$ of 0,2 (1 X $10^8$ cfu/mL). For the colonization assay, CD1 mice were inoculated by eye vain injection with 100 µl (1 X $10^7$ cells) of these cultures of staphylococcal strains. Seven mice were used for each strain group. After one week mice were euthanized. Kidneys were removed, homogenized in PBS (9 mL per gram), and plated on tryptic soy agar for determination of the number of colony-forming units (CFU).

**Mouse infection model: survival assay**

MW2 wild type and MW2 ΔXV *S. aureus* strains were cultured overnight in TSB plates and a single colony was resuspended into 5 mL of PBS to an optical density at $650_{nm}$ of 0,2 (1 X $10^8$ cfu/mL). For the survival

assay, groups of 7 CD1 female mice were inoculated by eye vain injection with 100 µl (1 X $10^7$ cells) of each staphylococcal strain cultures. The death of any mice was monitored every day for seven weeks.

**Statistical analysis**

The statistical analysis was performed with the GraphPad Prism program. Data corresponding to adhesion, invasion and kidney colonization assay were compared using the Mann-Whitney U tests. However, data corresponding to dessication resistance were compared usin Unpaired Student´s t-test. Finally data corresponding to the survival assay were compared using Log-rank (Mantel-Cox) test. All the tests were two-tailed and the significance level was 5%.

**Table 2.** Strains used in this study

| Strains | Relevant characteristics | MIC[a] | Reference |
|---|---|---|---|
| ***Staphylococcus aureus*** | | | |
| 15981 | Clinical isolate | 532 | (Valle *et al.*, 2003) |
| 15981 Δ*rnc::cat86* | 15981 with deletion of the *rnc* gene | 1327 | (Lasa *et al.*, 2011) |
| RN10359 | RN450 lysogenic for 80α phage | 3337 | (Úbeda *et al.*, 2007) |
| ISP479r | ISP479c with *rsbU* gene restored | 1680 | (Toledo-Arana *et al.*, 2005) |
| MW2 | Typical community-acquired strain of MRSA, which was isolated in 1998 in North Dakota, USA. | 3566 | (Baba *et al.*, 2002) |
| MW2 Δ3 | MW2 strain harbouring ΔMW0199-MW0198 | 4032 | This study |
| MW2 Δ4 | MW2 Δ*lytSR* | 2964 | This study |
| MW2 Δ5 | MW2 Δ*graRS* | 11 | This study |
| MW2 Δ6 | MW2 Δ*saeRS* | 2965 | This study |
| MW2 Δ7 | MW2 ΔMW1207-MW1208 | 4033 | This study |
| MW2 Δ8 | MW2 Δ*arlRS* | 4034 | This study |
| MW2 Δ9 | MW2 Δ*srrAB* | 2966 | This study |
| MW2 Δ10 | MW2 Δ*phoRP* | 4035 | This study |
| MW2 Δ11 | MW2 Δ*yhcSR* | 3670 | This study |
| MW2 Δ12 | MW2 Δ*vraRS* | 4036 | This study |
| MW2 Δ13 | MW2 Δ*agrBDCA* | 4037 | This study |
| MW2 Δ14 | MW2 Δ*kdpDE* | 4038 | This study |
| MW2 Δ15 | MW2 Δ*hssRS* | 2979 | This study |
| MW2 Δ16 | MW2 Δ*nreABC* | 2967 | This study |
| MW2 Δ17 | MW2 Δ*nsaRS* | 4039 | This study |
| MW2 ΔI | MW2 Δ*yhc* | 3670 | This study |
| MW2 ΔII | MW2 ΔI Δ*tcs3* | 3713 | This study |
| MW2 ΔIII | MW2 ΔII Δ*lyt* | 3717 | This study |
| MW2 ΔIV | MW2 ΔIII Δ*gra* | 66 | This study |
| MW2 ΔV | MW2 ΔIV Δ*sae* | 371 | This study |
| MW2 ΔVI | MW2 ΔV Δ*tcs7* | 768 | This study |
| MW2 ΔVII | MW2 ΔVI Δ*hss* | 1336 | This study |
| MW2 ΔVIII | MW2 ΔVII Δ*nre* | 1345 | This study |
| MW2 ΔIX | MW2 ΔVIII Δb*ra* | 1379 | This study |
| MW2 ΔX | MW2 ΔIX Δ*kdp* | 2955 | This study |
| MW2 ΔXI | MW2 ΔX Δ*vra* | 2956 | This study |
| MW2 ΔXII | MW2 ΔXI Δ*pho* | 2957 | This study |
| MW2 ΔXIII | MW2 ΔXII Δ*arl* | 2958 | This study |
| MW2 ΔXIV | MW2 ΔXIII Δ*agr* | 2960 | This study |
| MW2 ΔXV | MW2 ΔXIV Δ*srr* | 2961 | This study |
| 8325r (RN1 *rsbU*+) | 8325 with *rsbU* gene restored | 2968 | R. Novick |
| 8325r ΔXV | 8325r ΔXIV Δ*agr* | 2969 | This study |
| MW2 ΔXVI* | MW2 ΔXV *Pspac-walKR* | 2975 | This study |
| ST1000 | RN4220 P*spac-walKR* | | (Dubrac *et al.*, 2007) |
| MW2 ΔXVI* *walR* | MW2 ΔXV P*spac-walKR* carrying pEI *walR* plasmid | 4674 | This study |
| MW2 ΔXVI* *walR* D52A | MW2 ΔXV *Pspac-walKR* carrying pEI *walR* D52A plasmid | 4797 | This study |
| MW2 ΔXV pEI *arlRS* | MW2 ΔXV carrying pEI *arlRS* plasmid | 4539 | This study |
| MW2 Δ8 pEI *arlR* | MW2 Δ*arl* carrying pEI *arlRS* plasmid | 4890 | This study |
| MW2 Δ8 pEI *arlRS* | MW2 Δ*arl* carrying pEI *arlRS* plasmid | 4538 | This study |
| MW2 ΔXV pEI *vraSR* | MW2 ΔXV carrying pEI *vraRS* plasmid | 4676 | This study |
| MW2 Δ12 pEI *vraR* | MW2 Δ*vra* carrying pEI *vraRS* plasmid | 4889 | This study |
| MW2 Δ12 pEI *vraSR* | MW2 Δ*vra* carrying pEI *vraRS* plasmid | 4675 | This study |
| MW2 ΔXV pEI *srrAB* | MW2 ΔXV carrying pEI *srrAB* plasmid | 4680 | This study |
| MW2 Δ9 pEI *srrB* | MW2 Δ*srr* carrying pEI *srrAB* plasmid | 4679 | This study |
| MW2 Δ9 pEI *srrAB* | MW2 Δ*srr* carrying pEI *srrAB* plasmid | 4677 | This study |
| MW2 ΔXV pEI *nreBC* | MW2 ΔXV carrying pEI *nreBC* plasmid | 4536 | This study |
| MW2 Δ16 pEI *nreBC* | MW2 Δnre carrying pEI *nreBC* plasmid | 4535 | This study |
| MW2 Δ16 pEW | MW2 Δ*nre* carrying pEW empty plasmid | 3893 | This study |
| MW2 Δ16 pEW *nreC* | MW2 Δ*nre* carrying pEW *nreC* plasmid | 3889 | This study |
| MW2 Δ16 pEW *nreBC* | MW2 Δ*nre* carrying pEW *nreBC* plasmid | 3892 | This study |
| MW2 Δ16 pEW *nreC* pCW *nreB* | MW2 Δ*nre* carrying pEW *nre*C and pCW *nreB* plasmids | 3925 | This study |
| MW2 ΔXV pEW | MW2 ΔXV carrying pEW plasmid | 3885 | This study |
| MW2 ΔXV pEW *nreC* | MW2 ΔXV carrying pEW *nreC* plasmid | 3888 | This study |
| MW2 ΔXV pEW *nreBC* | MW2 ΔXV carrying pEW *nreBC* plasmid | 3891 | This study |
| MW2 ΔXV pEW *nreC* pCW | MW2 ΔXV carrying pEW *nreC* and pCW empty plasmid | 4707 | This study |

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**Table 2.** Continued.

| Strains | Relevant characteristics | MIC[a] | Reference |
|---|---|---|---|
| MW2 ΔXV pEW *nreC* pCW *nreB* | MW2 ΔXV carrying pEW *nreC* and pCW *nreB* plasmids | 3927 | This study |
| MW2 ΔXV pEW *nreC* pCW *hk3* | MW2 ΔXV carrying pEW *nreC* and pCW *hk3* plasmids | 3962 | This study |
| MW2 ΔXV pEW *nreC* pCW *lytS* | MW2 ΔXV carrying pEW *nreC* and pCW *lytS* plasmids | 3963 | This study |
| MW2 ΔXV pEW *nreC* pCW *graS* | MW2 ΔXV carrying pEW *nreC* and pCW *graS* plasmids | 3964 | This study |
| MW2 ΔXV pEW *nreC* pCW *saeS* | MW2 ΔXV carrying pEW *nreC* and pCW *saeS* plasmids | 3965 | This study |
| MW2 ΔXV pEW *nreC* pCW *hk7* | MW2 ΔXV carrying pEW *nreC* and pCW *hk7* plasmids | 3968 | This study |
| MW2 ΔXV pEW *nreC* pCW *arlS* | MW2 ΔXV carrying pEW *nreC* and pCW *arlS* plasmids | 3969 | This study |
| MW2 ΔXV pEW *nreC* pCW *srrB* | MW2 ΔXV carrying pEW *nreC* and pCW *srrB* plasmids | 3970 | This study |
| MW2 ΔXV pEW *nreC* pCW *phoR* | MW2 ΔXV carrying pEW *nreC* and pCW *phoR* plasmids | 3971 | This study |
| MW2 ΔXV pEW *nreC* pCW *yhcS* | MW2 ΔXV carrying pEW *nreC* and pCW *yhcS* plasmids | 3972 | This study |
| MW2 ΔXV pEW *nreC* pCW *vraS* | MW2 ΔXV carrying pEW *nreC* and pCW *vraS* plasmids | 3973 | This study |
| MW2 ΔXV pEW *nreC* pCW *agrC* | MW2 ΔXV carrying pEW *nreC* and pCW *agrC* plasmids | 3974 | This study |
| MW2 ΔXV pEW *nreC* pCW *kdpD* | MW2 ΔXV carrying pEW *nreC* and pCW *kdpD* plasmids | 3975 | This study |
| MW2 ΔXV pEW *nreC* pCW *hssS* | MW2 ΔXV carrying pEW *nreC* and pCW *hssS* plasmids | 3976 | This study |
| MW2 ΔXV pEW *nreC* pCW *braS* | MW2 ΔXV carrying pEW *nreC* and pCW *braS* plasmids | 3977 | This study |
| MW2 ΔXV pEI *srrA hk3* | MW2 ΔXV carrying pEI *srrA hk3* plasmid | 4774 | This study |
| MW2 ΔXV pEI *srrA lytS* | MW2 ΔXV carrying pEI *srrA lytS* plasmid | 4775 | This study |
| MW2 ΔXV pEI *srrA graS* | MW2 ΔXV carrying pEI *vraR graS* plasmid | 4776 | This study |
| MW2 ΔXV pEI *srrA saeS* | MW2 ΔXV carrying pEI *srrA saeS* plasmid | 4777 | This study |
| MW2 ΔXV pEI *srrA hk7* | MW2 ΔXV carrying pEI *srrA hk7* plasmid | 4778 | This study |
| MW2 ΔXV pEI *srrA arlS* | MW2 ΔXV carrying pEI *srrA arlS* plasmid | 4779 | This study |
| MW2 ΔXV pEI *srrA phoS* | MW2 ΔXV carrying pEI *srrA phoS* plasmid | 4780 | This study |
| MW2 ΔXV pEI *srrA yhcS* | MW2 ΔXV carrying pEI *srrA yhcS* plasmid | 4781 | This study |
| MW2 ΔXV pEI *srrA vraS* | MW2 ΔXV carrying pEI *srrA yhcS* plasmid | 4782 | This study |
| MW2 ΔXV pEI *srrA agrC* | MW2 ΔXV carrying pEI *srrA agrC* plasmid | 4783 | This study |
| MW2 ΔXV pEI *srrA kdpD* | MW2 ΔXV carrying pEI *srrA kdpD* plasmid | 4784 | This study |
| MW2 ΔXV pEI *srrA hssS* | MW2 ΔXV carrying pEI *srrA hssS* plasmid | 4785 | This study |
| MW2 ΔXV pEI *srrA nreB* | MW2 ΔXV carrying pEI *srrA nreB* plasmid | 4786 | This study |
| MW2 ΔXV pEI *srrA hk17* | MW2 ΔXV carrying pEI *srrA hk17*plasmid | 4787 | This study |
| MW2 ΔXV pEI *vraR hk3* | MW2 ΔXV carrying pEI *vraR hk3* plasmid | 4745 | This study |
| MW2 ΔXV pEI *vraR lytS* | MW2 ΔXV carrying pEI *vraR lytS* plasmid | 4746 | This study |
| MW2 ΔXV pEI *vraR graS* | MW2 ΔXV carrying pEI *vraR graS* plasmid | 4747 | This study |
| MW2 ΔXV pEI *vraR saeS* | MW2 ΔXV carrying pEI *vraR saeS* plasmid | 4748 | This study |
| MW2 ΔXV pEI *vraR hk7* | MW2 ΔXV carrying pEI *vraR hk7* plasmid | 4749 | This study |
| MW2 ΔXV pEI *vraR arlS* | MW2 ΔXV carrying pEI *vraR arlS* plasmid | 4750 | This study |
| MW2 ΔXV pEI *vraR srrA* | MW2 ΔXV carrying pEI *vraR srrA* plasmid | 4751 | This study |
| MW2 ΔXV pEI *vraR phoS* | MW2 ΔXV carrying pEI *vraR phoS* plasmid | 4752 | This study |
| MW2 ΔXV pEI *vraR yhcS* | MW2 ΔXV carrying pEI *vraR yhcS* plasmid | 4753 | This study |
| MW2 ΔXV pEI *vraR agrC* | MW2 ΔXV carrying pEI *vraR agrC* plasmid | 4754 | This study |
| MW2 ΔXV pEI *vraR kdpD* | MW2 ΔXV carrying pEI *vraR hk3* plasmid | 4755 | This study |
| MW2 ΔXV pEI *vraR hssS* | MW2 ΔXV carrying pEI *vraR hssS* plasmid | 4756 | This study |
| MW2 ΔXV pEI *vraR nreB* | MW2 ΔXV carrying pEI *vraR nreB* plasmid | 4757 | This study |
| MW2 ΔXV pEI *vraR hk17* | MW2 ΔXV carrying pEI *vraR hk17*plasmid | 4758 | This study |
| MW2 ΔXV pEI *vraR* pCI | MW2 ΔXV carrying pEI *vraR* and pCI empty plasmids | 4798 | This study |
| MW2 ΔXV pEI *vraR* pCI *vraS* | MW2 ΔXV carrying pEI *vraR* and pCI *vraS* plasmids | 4800 | This study |
| MW2 ΔXV pEI *vraR* pCI *yhcS* | MW2 ΔXV carrying pEI *vraR* and pCI *yhcS* plasmids | 4799 | This study |
| MW2 ΔXV pEI *vraR* pCI *yhcRS* | MW2 ΔXV carrying pEI *vraR* and pCI *yhcRS* plasmids | 4801 | This study |
| MW2 ΔXV pEI *vraSR* H156A pCI | MW2 ΔXV carrying pEI *vraSR* H156A and pCI empty plasmids | 4859 | This study |
| MW2 ΔXV pEI *vraSR* H156A pCI *yhcS* | MW2 ΔXV carrying pEI *vraSR* H156A and pCI *yhcS* plasmids | 4860 | This study |

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**Table 2.** Continued.

| Strains | Relevant characteristics | MIC[a] | Reference |
|---|---|---|---|
| MW2 ΔXV pEI *vraSR* H156A pCI *yhcRS* | MW2 ΔXV carrying pEI *vraSR* H156A and pCI *yhcRS* plasmids | 4861 | This study |
| MW2 ΔXV pEI *arlR hk3* | MW2 ΔXV carrying pEI *arlR hk3* plasmid | 5041 | This study |
| MW2 ΔXV pEI *arlR lytS* | MW2 ΔXV carrying pEI *arlR lytS* plasmid | 5042 | This study |
| MW2 ΔXV pEI *arlR graS* | MW2 ΔXV carrying pEI *arlR graS* plasmid | 5043 | This study |
| MW2 ΔXV pEI *arlR saeS* | MW2 ΔXV carrying pEI *arlR saeS* plasmid | 5044 | This study |
| MW2 ΔXV pEI *arlR hk7* | MW2 ΔXV carrying pEI *arlR hk7* plasmid | 5045 | This study |
| MW2 ΔXV pEI *arlR srrA* | MW2 ΔXV carrying pEI *arlR srrA* plasmid | 5046 | This study |
| MW2 ΔXV pEI *arlR phoS* | MW2 ΔXV carrying pEI *arlR phoS* plasmid | 5047 | This study |
| MW2 ΔXV pEI *arlR yhcS* | MW2 ΔXV carrying pEI *arlR yhcS* plasmid | 5048 | This study |
| MW2 ΔXV pEI *arlR vraS* | MW2 ΔXV carrying pEI *arlR vraS* plasmid | 5049 | This study |
| MW2 ΔXV pEI *arlR agrC* | MW2 ΔXV carrying pEI *arlR agrC* plasmid | 5050 | This study |
| MW2 ΔXV pEI *arlR kdpD* | MW2 ΔXV carrying pEI *arlR kdpD* plasmid | 5051 | This study |
| MW2 ΔXV pEI *arlR hssS* | MW2 ΔXV carrying pEI *arlR hssS* plasmid | 5052 | This study |
| MW2 ΔXV pEI *arlR nreB* | MW2 ΔXV carrying pEI *arlR nreB* plasmid | 5053 | This study |
| MW2 ΔXV pEI *arlR hk17* | MW2 ΔXV carrying pEI *arlR hk17*plasmid | 5054 | This study |
| MW2 Δ8 pEI *arlR* D52A | MW2 Δ*arl* carrying pEI *arlR* D52A plasmid | 5001 | This study |
| MW2 Δ8 pEI *arlRS* H242A | MW2 Δ*arl* carrying pEI *arlRS* H242A plasmid | 4924 | This study |
| MW2 ΔXV pEI *arlR* D52A *arlS* | MW2 ΔXV carrying pEI *arlR* D52A *arlS* plasmid | 5079 | This study |
| MW2 ΔXV pEI *arlR* D52A *graS* | MW2 ΔXV carrying pEI *arlR* D52A *graS* plasmid | 5080 | This study |
| MW2 Δ8 Δ5 pEI *arlR* | MW2 Δ*arl* Δ*gra* carrying pEI *arlR* | 5102 | This study |

[a]Microbial Biofilm Laboratory strain collection number

**Table 3.** Plasmids used in this study

| Plasmids | Relevant characteristic(s) | Source or reference |
|---|---|---|
| pMAD | *E. coli - S. aureus* shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria. The vector contains the *bgaB* gene encoding a b- galactosidase under the control of a constitutive promoter as reporter of plasmid presence. $Ap^R$, $Em^R$. | (Arnaud *et al.*, 2004) |
| pMAD TCS3AD | pMAD plasmid containing the allele for deletion of the *tcs3* genes | This study |
| pMAD TCS4AD | pMAD plasmid containing the allele for deletion of the *lytRS* genes | This study |
| pMAD TCS5AD | pMAD plasmid containing the allele for deletion of the *graRS* genes | This study |
| pMAD TCS6AD | pMAD plasmid containing the allele for deletion of the *saeRS* genes | (Toledo-Arana *et al.*, 2005) |
| pMAD TCS7AD | pMAD plasmid containing the allele for deletion of the *tcs7* genes | (Toledo-Arana *et al.*, 2005) |
| pMAD TCS8AD | pMAD plasmid containing the allele for deletion of the *arlRS* genes | (Toledo-Arana *et al.*, 2005) |
| pMAD TCS9AD | pMAD plasmid containing the allele for deletion of the *srrAB* genes | (Toledo-Arana *et al.*, 2005) |
| pMAD TCS10AD | pMAD plasmid containing the allele for deletion of the *phoRP* genes | (Toledo-Arana *et al.*, 2005) |
| pMAD TCS11AD | pMAD plasmid containing the allele for deletion of the *yhcRS* genes | This study |
| pMAD TCS12AD | pMAD plasmid containing the allele for deletion of the *vraRS* genes | (Toledo-Arana *et al.*, 2005) |
| pMAD TCS13AD | pMAD plasmid containing the allele for deletion of the *agrBDCA* genes | (Valle *et al.*, 2003) |
| pMAD TCS14AD | pMAD plasmid containing the allele for deletion of the *kdpDE* genes | This study |
| pMAD TCS15AD | pMAD plasmid containing the allele for deletion of the *hssRS* genes | (Toledo-Arana *et al.*, 2005) |
| pMAD TCS16AD | pMAD plasmid containing the allele for deletion of the *nreBC* genes | This study |
| pMAD TCS17AD | pMAD plasmid containing the allele for deletion of the *braRS* genes | This study |
| pSD3-3 | Derivative of pDH88. P*spac-walKR* | (Dubrac and Msadek, 2004) |
| pCN51 | *E. coli - S. aureus* shuttle vector to express genes under the control of the $P_{cad}$ cadmium-inducible promoter. Low copy number (20 to 25 copies/cell). $Em^R$. Renamed pEI | This study |
| pEI *arlRS* | pCN51 plasmid expressing *arlRS* TCS | This study |
| pEI *arlR* | pCN51 plasmid expressing *arlR* RR gene | This study |
| pEI *srrAB* | pCN51 plasmid expressing *srrAB* TCS | This study |
| pEI *srrB* | pCN51 plasmid expressing *srrB* RR gene | This study |
| pEI *vraSR* | pCN51 plasmid expressing *vraSR* TCS | This study |
| pEI *vraR* | pCN51 plasmid expressing *vraR* RR gene | This study |
| pEI *nreBC* | pCN51 plasmid expressing *nreBC* TCS | This study |
| pCN40 | *E. coli - S. aureus* shuttle vector to express genes under the control of the $P_{blaZ}$ constitutive promoter. Low copy number (20 to 25 copies/cell). $Em^R$ | (Charpentier *et al.*, 2004) |
| pCN47 | *E. coli - S. aureus* shuttle vector to express genes under its own promoter. $Em^R$ | (Charpentier *et al.*, 2004) |
| pCU1 | Vector for complementation experiments. $Ap^r$ $Cm^r$. Renamed pCW | (Augustin *et al.*, 1992) |
| pEW | Derivative of pCN47 plasmid containing the $P_{blaz}$ promoter of pCN40. $Em^R$ | This study |
| pCW | Derivative of pCU1 plasmid containing the cloning module ($P_{blaz}$, MCS and TT) of pEW. $Ap^r$ $Cm^r$ | This study |
| pEW *nreC* | pEW plasmid constitutively expressing *nreC* RR gene | This study |
| pEW *nreBC* | pEW plasmid constitutively expressing *nreBC* TCS | This study |
| pCW *nreB* | pCW plasmid constitutively expressing *nreB* HK gene | This study |
| pCW *hk3* | pCW plasmid constitutively expressing *hk3S* HK gene | This study |
| pCW *lytS* | pCW plasmid constitutively expressing *lytS* HK gene | This study |
| pCW *graS* | pCW plasmid constitutively expressing *graS* HK gene | This study |
| pCW *saeS* | pCW plasmid constitutively expressing *saeS* HK gene | This study |
| pCW *hk7* | pCW plasmid constitutively expressing *hk7S* HK gene | This study |
| pCW *arlS* | pCW plasmid constitutively expressing *arlS* HK gene | This study |
| pCW *srrB* | pCW plasmid constitutively expressing *srrB* HK gene | This study |
| pCW *phoR* | pCW plasmid constitutively expressing *phoR* HK gene | This study |
| pCW *yhcS* | pCW plasmid constitutively expressing *yhcS* HK gene | This study |
| pCW *vraS* | pCW plasmid constitutively expressing *vraS* HK gene | This study |
| pCW *agrC* | pCW plasmid constitutively expressing *agrC* HK gene | This study |
| pCW *kdpD* | pCW plasmid constitutively expressing *kdpD* HK gene | This study |
| pCW *hssS* | pCW plasmid constitutively expressing *hssS* HK gene | This study |
| pCW *braS* | pCW plasmid constitutively expressing *braS* HK gene | This study |

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**Table 3**. Continued.

| Plasmids | Relevant characteristic(s) | Source or reference |
|---|---|---|
| pEI *srrA hk3* | pCN51 plasmid expressing *srrA* RR and *hk3* HK genes | This study |
| pEI *srrA lytS* | pCN51 plasmid expressing *srrA* RR and *lytS* HK genes | This study |
| pEI *srrA graS* | pCN51 plasmid expressing *srrA* RR and *graS* HK genes | This study |
| pEI *srrA saeS* | pCN51 plasmid expressing *srrA* RR and *saeS* HK genes | This study |
| pEI *srrA hk7* | pCN51 plasmid expressing *srrA* RR and *hk7* HK genes | This study |
| pEI *srrA arlS* | pCN51 plasmid expressing *srrA* RR and *arlS* HK genes | This study |
| pEI *srrA phoR* | pCN51 plasmid expressing *srrA* RR and *phoR* HK genes | This study |
| pEI *srrA yhcS* | pCN51 plasmid expressing *srrA* RR and *yhcS* HK genes | This study |
| pEI *srrA vraS* | pCN51 plasmid expressing *srrA* RR and *vraS* HK genes | This study |
| pEI *srrA agrC* | pCN51 plasmid expressing *srrA* RR and *agrC* HK genes | This study |
| pEI *srrA kdpD* | pCN51 plasmid expressing *srrA* RR and *kdpD* HK genes | This study |
| pEI *srrA hssS* | pCN51 plasmid expressing *srrA* RR and *hssS* HK genes | This study |
| pEI *srrA nreB* | pCN51 plasmid expressing *srrA* RR and *nreB* HK genes | This study |
| pEI *srrA braS* | pCN51 plasmid expressing *srrA* RR and *braS* HK genes | This study |
| pEI *vraR hk3* | pCN51 plasmid expressing *vraR* RR and *hk3* HK genes | This study |
| pEI *vraR lytS* | pCN51 plasmid expressing *vraR* RR and *lytS* HK genes | This study |
| pEI *vraR graS* | pCN51 plasmid expressing *vraR* RR and *graS* HK genes | This study |
| pEI *vraR saeS* | pCN51 plasmid expressing *vraR* RR and *saeS* HK genes | This study |
| pEI *vraR hk7* | pCN51 plasmid expressing *vraR* RR and *hk7* HK genes | This study |
| pEI *vraR arlS* | pCN51 plasmid expressing *vraR* RR and *arlS* HK genes | This study |
| pEI *vraR srrB* | pCN51 plasmid expressing *vraR* RR and *srrB* HK genes | This study |
| pEI *vraR phoR* | pCN51 plasmid expressing *vraR* RR and *phoR* HK genes | This study |
| pEI *vraR yhcS* | pCN51 plasmid expressing *vraR* RR and *yhcS* HK genes | This study |
| pEI *vraR agrC* | pCN51 plasmid expressing *vraR* RR and *agrC* HK genes | This study |
| pEI *vraR kdpD* | pCN51 plasmid expressing *vraR* RR and *kdpD* HK genes | This study |
| pEI *vraR hssS* | pCN51 plasmid expressing *vraR* RR and *hssS* HK genes | This study |
| pEI *vraR nreB* | pCN51 plasmid expressing *vraR* RR and *nreB* HK genes | This study |
| pEI *vraR braS* | pCN51 plasmid expressing *vraR* RR and *braS* HK genes | This study |
| pCI | Derivative of pCW containing the $P_{cad}$ cadmium-inducible promoter of pCN51. $Ap^r$ $Cm^r$. Renamed pCI | This study |
| pCI *vraS* | pCI plasmid expressing *vraS* HK gene | This study |
| pCI *yhcS* | pCI plasmid expressing *yhcS* HK gene | This study |
| pCI *yhcSR* | pCI plasmid expressing *yhcSR* TCS | This study |
| pCI *vraSR* H156A | pCI plasmid expressing *vraSR* H156A TCS | This study |
| pEI *arlR hk3* | pCN51 plasmid expressing *arlR* RR and *hk3* HK genes | This study |
| pEI *arlR lytS* | pCN51 plasmid expressing *arlR* RR and *lytS* HK genes | This study |
| pEI *arlR graS* | pCN51 plasmid expressing *arlR* RR and *graS* HK genes | This study |
| pEI *arlR saeS* | pCN51 plasmid expressing *arlR* RR and *saeS* HK genes | This study |
| pEI *arlR hk7* | pCN51 plasmid expressing *arlR* RR and *hk7* HK genes | This study |
| pEI *arlR srrB* | pCN51 plasmid expressing *arlR* RR and *srrB* HK genes | This study |
| pEI *arlR phoR* | pCN51 plasmid expressing *arlR* RR and *phoR* HK genes | This study |
| pEI *arlR yhcS* | pCN51 plasmid expressing *arlR* RR and *yhcS* HK genes | This study |
| pEI *arlR vraS* | pCN51 plasmid expressing *arlR* RR and *vraS* HK genes | This study |
| pEI *arlR agrC* | pCN51 plasmid expressing *arlR* RR and *agrC* HK genes | This study |
| pEI *arlR kdpD* | pCN51 plasmid expressing *arlR* RR and *kdpD* HK genes | This study |
| pEI *arlR hssS* | pCN51 plasmid expressing *arlR* RR and *hssS* HK genes | This study |
| pEI *arlR nreB* | pCN51 plasmid expressing *arlR* RR and *nreB* HK genes | This study |
| pEI *arlR braS* | pCN51 plasmid expressing *arlR* RR and *braS* HK genes | This study |
| pEI *arlR* D52A | pCN51 plasmid expressing *arlR* D52A RR gene | This study |
| pEI *arlRS* H242A | pCN51 plasmid expressing *arlRS* H242A TCS | This study |
| pEI *arlR* D52A *arlS* | pCN51 plasmid expressing *arlR* D52A RR and *arlS* HK genes | This study |
| pEI *arlR* D52A *graS* | pCN51 plasmid expressing *arlR* D52A RR and *graS* HK genes | This study |

**Table 4.** Oligonucleotides used in this study

| Oligonucleotide | Sequence |
|---|---|
| **Small RNA library sequencing** | |
| 3′ ModBan | AMP-5′p-5′pCTGTAGGCACCATCAAT-ddC |
| | OH- |
| 5′ Illumina RNA adapter | rArCrArCrUrCrUrUrUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrC-OH |
| RT-PCR | ATTGATGGTGCCTACAG |
| Sol_5_SBS3 | AATGATACGGCGACCACCGAACACTCTTTCCCTACACGACG |
| Sol_3_ModBan | CAAGCAGAAGACGGCATACGATTGATGGTGCCTACAG |
| **Deletion of two-component systems** | |
| tcs3-E | ATCTACTCAAGTACTGCTTT |
| tcs3-F | TGTTTCAAACGTCTTTGTATA |
| lyt-E | GAAAAGAAAAATGTAGATTTGA |
| lyt-F | AATAACATCATTGGCAAATTG |
| gra-E | AAATGACTTGGATTCAGTGT |
| gra-F | AAAAAGATAGATGGCATAATG |
| sae-E | GTTGGTGATTTTAGACTTTTA |
| sae-F | ATAAGATTGTAGAGCACATAA |
| tcs7-E | AATTGCCGGAAGATGTTAAA |
| tcs7-F | TATCTTTGTAGGCTTTATCG |
| arl-E | AGTGATCTGAAACAATTCC |
| arl-F | AAGAATAGTAAATAAAACGC |
| srr-E | TTTGTACAAAGGTAGACTTG |
| srr-F | TGGTTACGGATTACTTTAAAG |
| pho-E | AATGAGACCATATGAAAGCC |
| pho-F | CAGGTGCAAACATTAATTATG |
| yhc-E | CATATAAAGGATCACCAATAA |
| yhc-F | CATAGTTATTCATTATACCAC |
| vra-E | TGACGAACAAGTGAAATGG |
| vra-F | CGTTCTATTATTGGGATGTG |
| agr-E | GGGGATGTTATTAATTATGAA |
| agr-F | TAGTCATTTATACGAAGGGA |
| kdp-E | TACTAATTAAACATGATAATGG |
| kdp-F | GAATTCGTTTTCAATAATTGATTCTCTG |
| hss-E | CATACATTGTGTCGTTTAAAA |
| hss-F | AACCAATGATTAAGCTAATAAA |
| nre-E | TTAAGTTCAGCGTCGGATAT |
| nre-F | AACTTTACATTATTACGATGAAA |
| nsa-E | TACTTTCTGCTTGTTACTGT |
| nsa-F | ACACAAGCGTATATTCAATC |
| **Constitutive expression of TCS** | |
| SaeS-Rv (XmaI AscI) | *GGCGCGCCCGGG*ATCGGATTATGACGTAATGT |
| SaeS-Fw (BamHI) | *GGATCC*ATTTGAAAGGAGCCGATAAT |
| SaeS int Rv | TTGTTGCGTAATTTCAGAAG |
| SaeR-Rv (BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*ATTATCGGCTCCTTTCAAAT |
| SaeR-Fw (XhoI SalI) | GTCGACATTGCTCGAGCGAACAGAGGTGAAAAAATAG |
| SaeR int Rv | CATTAACTTCTGGCATCATG |
| NreR-Rv (BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*AATTTCAAACTCTAAAACTCTA |
| NreR-Fw (XhoI SalI) | GTCGACATTGCTCGAGCATACATTGGGGGAATAAAA |
| NreR int Rv | GGCATACTTAAATCCATTAG |
| NreS-Rv (XmaI AscI) | *GGCGCGCCCGGG*GTATGTTTCAAATTGGAATGT |
| NreS-Fw (BamHI) | GGATCCAGATGAATTAGGGTGTAAGT |
| NreS int Rv | AATACTTGGAAATTCGTAGC |
| WalK(S)-Rv (XmaI AscI) | *GGCGCGCCCGGG*CCTTATTATTCATCCCAATC |
| WalK(S)-Fw (BamHI) | GGATCCATGAGTAGAGGTCGAAACG |
| WalR-Rv (BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*CTCTACTCATGTTGTTGGA |
| WalR-Fw (XhoI SalI) | GTCGACATTGCTCGAGTTAAGAAAAGAGGTTTATGC |
| WalK(S) int Rv | TCGTCGCAATAATAATTTGG |
| WalR int Rv | ACATGATATCTAGTAATACG |

Continued on following page

**Table 4.** Continued.

| Oligonucleotide | Sequence |
|---|---|
| **Constitutive expression of TCS** | |
| TCS3S-Rv (XmaI AscI) | *GGCGCGCCCGGG*TACCTTAAACATCTACATTC |
| TCS3S-Fw (BamHI) | *GGATCC*TTTTTGGAGATGATTCAATG |
| TCS3S int Rv | CTTGAAACGAATTATTATGC |
| TCS3R-Rv(BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*AATCTATTTTGCTTGCTTAC |
| TCS3R-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*TTCAAGGGGGAATGTAGAT |
| LytS-Rv (XmaI AscI) | *GGCGCGCCCGGG*TATTTATTCCTCCTCTTGTC |
| LytS-Fw (BamHI) | *GGATCC*AATTTACTGAGGTGCTATCG |
| LytS int Rv | GTGTGTTAGCTAAAGATACA |
| LytR-Rv (BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*ACTGTTAAAGTAACCCTATC |
| LytR-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*GACAAGAGGAGGAATAAATA |
| GraS-Rv (XmaI AscI) | *GGCGCGCCCGGG*CGCATGTTTAAAATGACAAA |
| GraS-Fw (BamHI) | *GGATCC*TAGGAAAAGGATATATGGCT |
| GraS int Rv | GTTTCCGCTAAATCTTTATG |
| GraR-Rv (BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*CAAATTATTCATGAGCCATA |
| GraR-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*AATGATATTGGGTGATATGG |
| TCS7S-Rv (XmaI AscI) | *GGCGCGCCCGGG*AAAGATGTCATGCTATTCCT |
| TCS7S-Fw (BamHI) | *GGATCC*AAAGGGCGGAATAAAATATG |
| TCS7 int Rv | ATTGGATGTACACTGAAAAC |
| TCS7R-Rv(BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*ATTTAGATCCAGCCTTTTTC |
| TCS7R-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*AACAGGAGGAATAGCATGA |
| ArlS-Rv (XmaI AscI) | *GGCGCGCCCGGG*GATTAAAATATGATTTTAAACG |
| ArlS-Fw (BamHI) | *GGATCC*TGGCGTTGGGTATGTGATA |
| ArlS int Rv | TACCTAAAGATGCATTCAAG |
| ArlR-Rv (BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*TTTGTCATCGTATCACATAC |
| ArlR-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*GTAATATGAGGTGTACAAAT |
| SrrB(S)-Rv (XmaI AscI) | *GGCGCGCCCGGG*CAATTTTATTCTGGTTTTGG |
| SrrB(S)-Fw (BamHI) | *GGATCC*ATTTGAGGTTAAATCTAATG |
| SrrB(S) inr Rv | ACCAGGATTTTCAATTAATG |
| SrrA(R)-Rv(BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*ACTATTTAGCCGGCTCATC |
| SrrA(R)-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*TGTGTGGGAGGTATGACC |
| PhoR(S)-Rv (XmaI AscI) | *GGCGCGCCCGGG*TTTTATTCTTTATAATCTTTTAG |
| PhoR(S)-Fw (BamHI) | *GGATCC*ATTGGAAAGACCTAAAGAAC |
| PhoR(S) int Rv | ATGCTTAGTTATCAATAAATC |
| PhoP-Rv (BamHIXmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*TCATCATTGTTCTTTAGGTC |
| PhoP(R)-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*ATAAGTTAGGGAGGCATAC |
| YhcS-Rv (XmaI AscI) | *GGCGCGCCCGGG*GCTATTTTATAGGAATTGTG |
| YhcS-Fw (BamHI) | *GGATCC*AAATGAATTGGAGCGATTTG |
| YhcR-Rv (BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*TTCTAAATCAACTTATTTTCC |
| YhcR-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*ATTTAAGGAGATAACCCATG |
| VraS-Rv (XmaI AscI) | *GGCGCGCCCGGG*CTTTAATCGTCATACGAATC |
| VraS-Fw (BamHI) | *GGATCC*GAGACGTAGAGGTGATTTAT |
| VraS inr Rv | TCCAATCATTTTGCTGATTG |
| VraR-Rv (BamHI XmaI KpnI) | GGTACCAATACCCGGGCAATGGATCCGAACTATTGAATTAAATTATGT |
| VraR-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*AAATAAGGAGGATTCGTATG |
| AgrC(S)-Rv (XmaI AscI) | *GGCGCGCCCGGG*GGCTAGTTGTTAATAATTTC |
| AgrC(S)-Fw (BamHI) | *GGATCC*TATAAGAGAAAGTGTGATAG |
| AgrC(S) int Rv | TAACCATTACTAAAAATACAG |
| AgrA-Rv(BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*TATCTTATTATATTTTTTTAACG |
| AgrA(R)-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*CATAAGGATGTGAATGTATG |
| KdpD(S)-Rv (XmaI AscI) | *GGCGCGCCCGGG*CATTATACGTCTCCTTCATT |
| KdpD(S)-Fw (BamHI) | *GGATCC*TATCGAGGTGAAGGTTATG |
| KdpD(S) int Rv | GGTCGACATCTAAATATTGA |
| KdpE-Rv(BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*ATTATTTCTCTTTCCACTGC |
| KdpE(R)-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*AATGAAGGAGACGTATAATG |
| HssS-Rv (XmaI AscI) | *GGCGCGCCCGGG*AGATTAAAGTGAATTATTTGG |
| HssS-Fw (BamHI) | *GGATCC*CAAGGCTATAAGGTGGAGA |
| HssS int Rv | CGGTCATTATTTGGTAATTC |
| HssR-Rv (BamHI XmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*TTTAAACATGATTCTCCACC |
| HssR-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*GATAAGGGAGTTTATAGCTA |

Continued on following page

**Table 4.** Continued.

| Oligonucleotide | Sequence |
|---|---|
| **Constitutive expression of TCS** | |
| BraS-Rv (XmaI AscI) | *GGCGCGCCCGGG*TTTTTATTCATCTGGAAATTG |
| BraS-Fw (BamHI) | *GGATCC*AGTATAGGGTGAATGCAATG |
| BraS int Rv | GTTCATTTTTAAGCTGATAC |
| BraR-Rv(BamHIXmaI KpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*CCCTATACTTTATATCCGACA |
| BraR-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*TTGAAGGAAGAAGATTATAG |
| SA0067(S)-Rv (XmaI AscI) | *GGCGCGCCCGGG*TCAACGACCAATAATGTTGT |
| SA0067(S)-Fw (BamHI) | *GGATCC*AAAAAAGAGGGAAACTTACC |
| SA0067(S) int Rv | TTTGTATGTGCTAGTTCATC |
| SA0066R-Rv(BamHI XmaIKpnI) | *GGTACC*AATA*CCCGGG*CAAT*GGATCC*GTTATTTATTCTAAGTGTTGC |
| SA0066R-Fw (XhoI SalI) | *GTCGAC*ATTG*CTCGAG*TGAAATGGAGGGAGCCAT |
| **Aminoacide substitution** | |
| WalR D53A Fw | GCAGATTTAAAAACACCTTTAGCAAGCA |
| WalR D53A Rv cola | TGCTTGCTAAAGGTGTTTTTAAATCTGCGGCTAAGTTTTGAATCAGTTC |
| ArlR D52A Fw | GCATTAATGTTGCCGTCAATTAATG |
| ArlR D52A Rv cola | CATTAATTGACGGCAACATTAATGCTAATATGATTAAATCATAGTA |
| ArlS H242A Fw | GCAGAATTACGAACACCATTACAAATTA |
| ArlS H242A Rv cola | TAATTTGTAATGGTGTTCGTAATTCTGCTGACGCATCTTCAACAAATTG |
| VraS H156A Fw | GCAGATTCTGTTAGTCAGCAACTTTTTG |
| VraS H156A Rv cola | CAAAAAGTTGCTGACTAACAGAATCTGCAAGTTCTCGAGCTAGTCTTTG |

*Italic*, restriction enzyme site included in the oligonucleotide.

# *RESULTS*

# RESULTS

## Global characterization of the TCS transcriptional units of *S. aureus*

The starting point of this thesis was the characterization of the transcriptional profile of the TCSs present in the *S. aureus* genome. This analysis was necessary to define the TCSs that were transcriptionally active under the standard growth conditions used in this study. We took advantage of a global study carried out in the laboratory to resolve the complete transcriptome map of *S. aureus*. Our transcriptome map was built based on the information obtained with two different techniques, RNA-seq and tiling arrays, which improves the accuracy and robustness of the data. The normalized tiling arrays signals and the mapped reads are integrated in a web repository that enables the visualization of the transcriptome information (*S. aureus* Transcriptome Browser, http://staph.unavarra.es/). The transcriptome map defines for each TCS (Figs. 4 and 5): the 5' transcriptional start site, 3' transcriptional termination site, the untranslated regions (UTRs), genes cotranscribed within the same transcriptional unit, the presence of alternative promoters and antisense transcripts and the level of transcription under the growth conditions tested (Table 5).

**Table 5.** Two component system transcriptional units of *S. aureus*

| | Name | Genes | Transcript size | 5´ UTR | 3´ UTR |
|---|---|---|---|---|---|
| TCS1 | *walKR* | walR | 4865 | 145 | |
| | | walK | | | |
| | | MW0020 | | | |
| | | MW0021 | | | 64 |
| TCS3 | *tcs3* | MW0200 | 3398 | 31 | |
| | | MW0199 | | | |
| | | MW0198 | | | 95 |
| TCS4 | *lytSR* | lytS | 2537 | 25 | |
| | | lytR | | | 15 |
| TCS5 | *graXRS* | graX | 3280 | 617 | |
| | | graR | | | |
| | | graS | | | 17 |
| TCS6 | *saePQRS* | saeP | 714 / 3067 | 27 | 247 |
| | | saeQ | 1950 | 140 | |
| | | saeR | ND[a] | ND[a] | |
| | | saeS | | | 69 |
| TCS7 | *tcs7* | MW1206 | ND[a] | ND[a] | |
| | | MW1207 | | | |
| | | MW1208 | ND[a] | ND[a] | |
| | | MW1209 | | | ND[a] |
| TCS8 | *arlRS* | arlR | 2234 | 33 | |
| | | arlS | | | 190 |
| TCS9 | *srrAB* | scpA | 2951 / 4695 | 40 | |
| | | scpB | | | |
| | | rluB | | | |
| | | srrA | 805 / 2549 | 23 | 57 |
| | | srrB | | | 69 |
| TCS10 | *phoPR* | phoP | 2508 | 88 | |
| | | phoR | | | 55 |
| TCS11 | *yhcSR* | yhcS | 1873 | 28 | |
| | | yhcR | | | 88 |
| TCS12 | *vraSR* | MW1827 | 3000 | 122 | |
| | | yvqF | | | |
| | | vraS | | | |
| | | vraR | | | 117 |
| TCS13 | *agrBDCA* | agrB | 2957 | 105 | |
| | | agrD | | | |
| | | agrC | | | |
| | | agrA | | | 94 |
| TCS14 | *kdpDE* | kdpD | ND[a] | ND[a] | |
| | | kdpE | | | 52 |
| TCS15 | *hssRS* | hssR | 2120 | 29 | |
| | | hssS | | | 51 |
| TCS16 | *nreABC* | narG | 8836 | 82 | |
| | | narH | | | |
| | | narJ | | | |
| | | narI | | | |
| | | nreA | | | |
| | | nreB | | | |
| | | nreC | | | 72 |
| TCS17 | *braRS* | MW2546 | 1897 | 40 | |
| | | braR | | | |
| | | braS | | | 68 |

[a]ND, not determined by RNAseq

The analysis of the 5' boundaries showed that two TCS transcripts have long 5'-UTRs*, walKR* and specially *graRS*, with a length of 145 and 617 nt respectively (Table 5). The long 5' UTR of the *gra* operon overlaps with the transcript produced from the neighbour MW0619 gene (Fig. 5). These overlapping regions generate double stranded RNA molecules that are then digested by the activity of RNase III, the only double stranded RNase produced by *S. aureus*. We also identified two TCS operons, *arlRS* and *vraSR,* with long 3´-UTRs, 190 and 117 nt respectively.

Regarding the presence of antisense transcription, RNA-seq data suggested the presence of non-coding antisense RNAs (asRNAs) at least in three different TCS: *yhc*, *agr* and *sae* (Figs. 5 and 6). The presence of asRNAs was further confirmed by the presence of similar amounts of short RNAs in those regions where overlapping transcriptions occurs. Interestingly, these short RNA fractions disappear in the RNase III mutant, strongly suggesting that a large majority of short RNA molecules are produced by the cleavage activity of double-stranded RNase III enzyme (Fig. 6).

The transcriptome analysis also showed that many TCSs are members of longer operons. For example, *nreBC* genes are transcribed together within the *nar* operon, encoding the nitrate reductase machinery. The transcription of *srrAB* locus is especially complex. The *srrAB* genes are transcribed as a bicistronic operon or they are also transcribed together with the *scpAB* genes, which are located upstream of TCS genes. Besides, the *srrA* gene si transcribed alone. The biological reasons explaining why the

expression of this TCS is sometimes linked to neighbour genes, whereas in other situations they are transcribed independently are unknown. Similar complex transcriptional patterns have beed described for the *sae* locus. In this case, the different transcriptional units generated from this locus have been deeply studied (Novick, 2003; Steinhuber *et al.*, 2003). In agreement with previous transcriptional studies, our transcriptome data revealed that *saeP* is transcribed as an operon together with *saeQRS*, but it is also transcribed alone as an mRNA with a long 3'-UTR of 247 nt (Figs. 4 and 5) (Table 5). Finally the quorum sensing TCS *agrCA* is cotranscribed in the four genes operon *agrBDCA* as previously described (Peng *et al.*, 1988; Janzon *et al.*, 1989) (Figs. 4 and 5).

Another conclusion from our transcriptome analysis is that most TCSs are transcribed constitutively in the four strains tested. Despite the fact that the RNA used for the transcriptome analysis was purified from bacteria grown in a specific environmental condition (TSB-gluc at 37°C under shaking conditions (200 rpm) until the culture reached an $OD_{600}$ of 0.8), high levels of expression for most TCS were detected (Figs. 4 and 5). Only the expression of TCS7 and TCS14 (*kdpDE*) was very low in this particular environmental condition. These results suggest that most TCSs are transcribed constitutively.

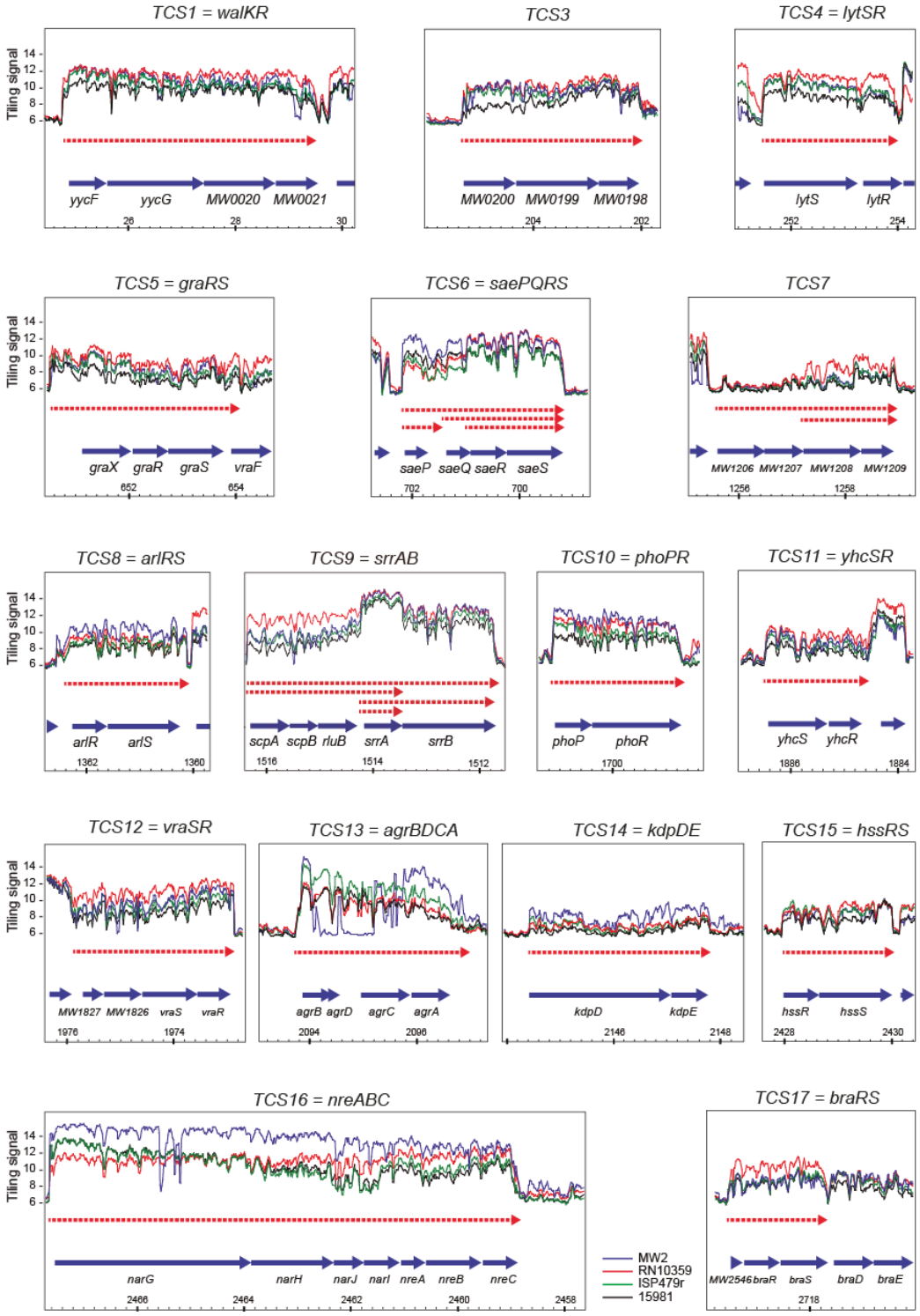


**Figure 4. Transcriptome analysis of the *S. aureus* two-component systems by tiling arrays.** Transcriptional map based on tiling data showing expression of the TCS family in four genetically unrelated *S. aureus* strains (MW2, RN10359, ISP479r and 15981). Drawings are images from IGB software showing different regions of the genome of *S. aureus* NCTC 8325. Genomic coordinates denote the position in Kb of the *S. aureus* NCTC 8325 genome. Annotated open reading frames (ORFs) are shown as blue lines. The number on the ORF indicates the gene identification.

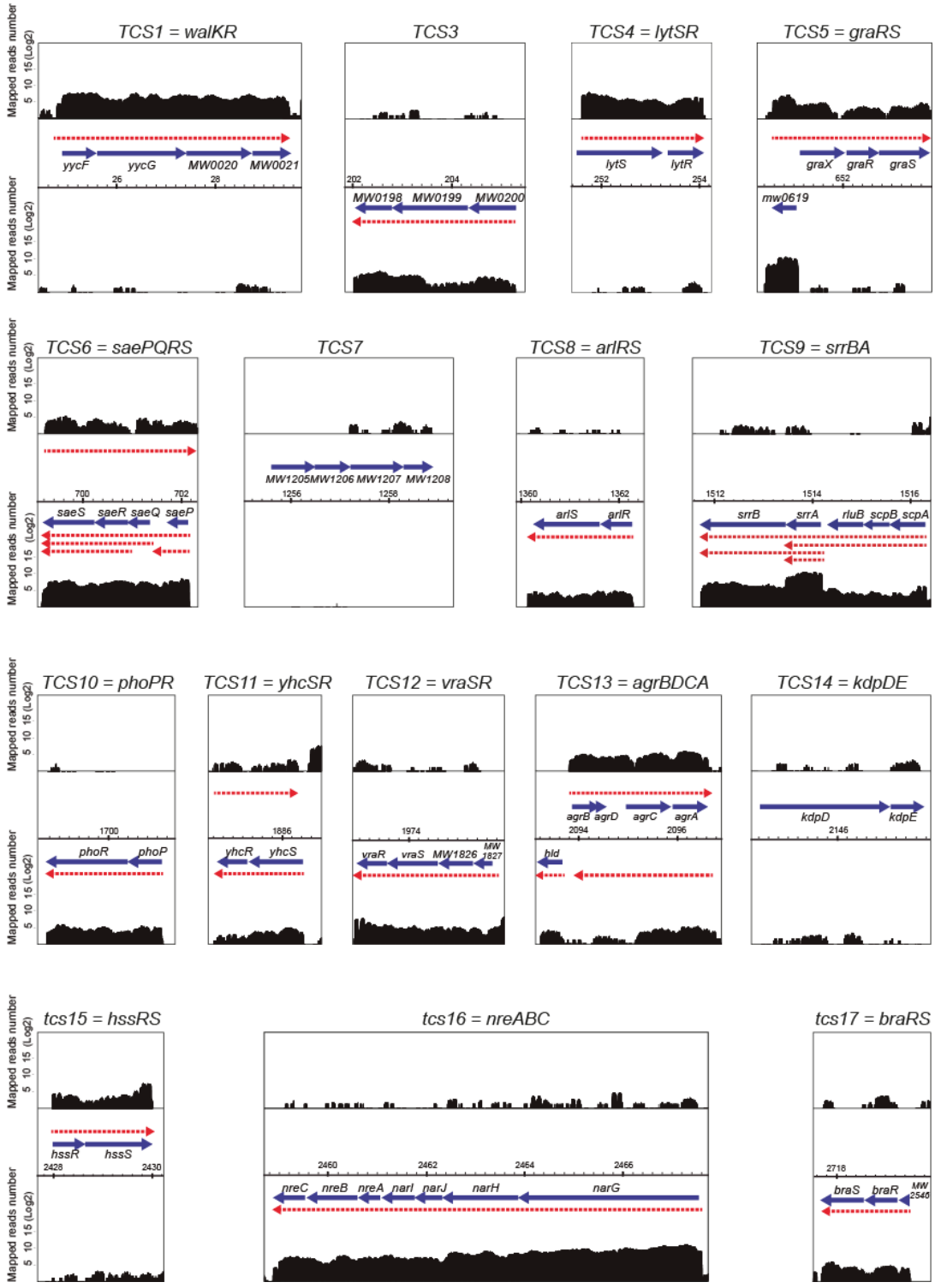


**Figure 5. Transcriptome analysis of the *S. aureus* two-component systems by RNA-seq**. Examples of mapped reads distribution of the sixteen *S. aureus* TCSs. Drawings are images from IGB software showing different regions of the genome of the *S. aureus* NCTC 8325. Annotated ORFs are shown as blue arrows. The number on the ORF indicates the gene identification. Long RNAs show the distribution of uniquely mapped reads of long RNA libraries in *S. aureus* 15981 (black). The scale ($log_2$) indicates the number of mapped reads per nucleotide position.

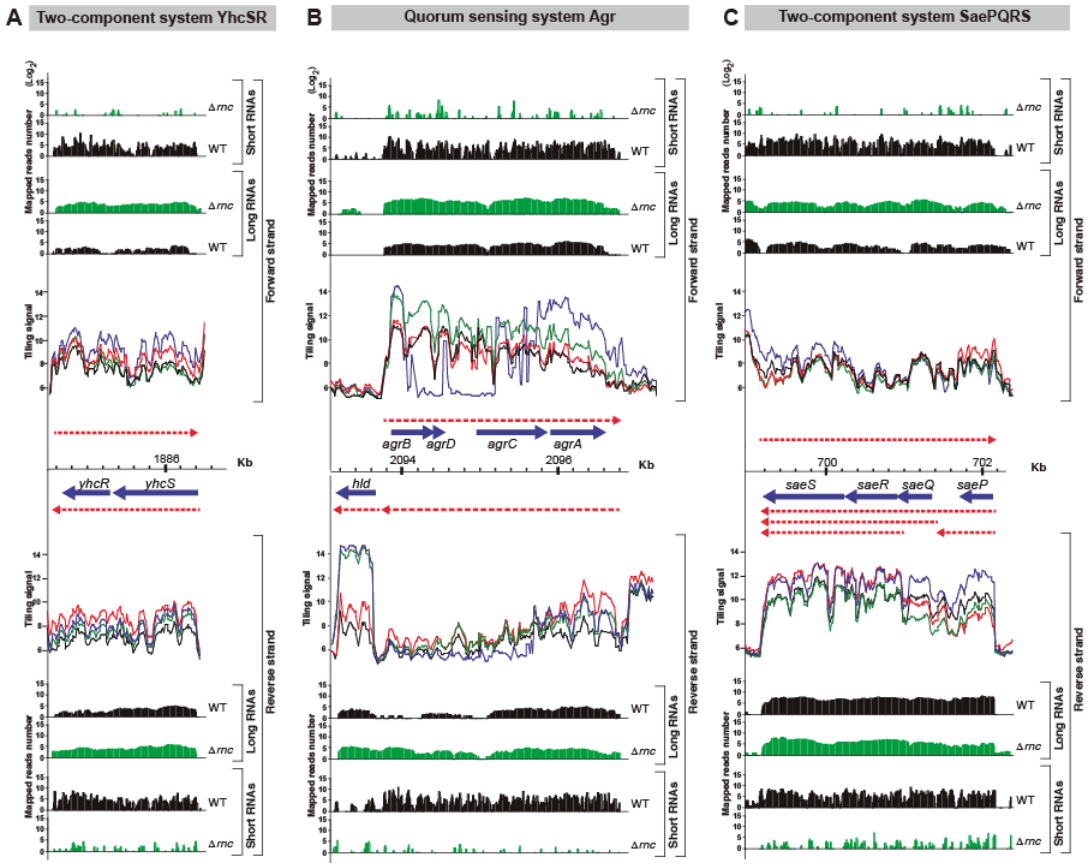


**Figure 6. Examples of mapped reads distribution of three *S. aureus* Two-component systems.** Drawings are images from IGB software showing different regions of *S. aureus* NCTC 8325. Annotated ORFs are shown as blue arrows. The number on the ORF indicates the gene identification. Long RNAs show the distribution of uniquely mapped reads of long RNA libraries in *S. aureus* 15981 (black) and *S. aureus* 15981 ∆rnc (green). Short RNAs show the distribution of uniquely mapped reads of short RNA libraries using the same pattern of colours as described for long RNAs. The scale ($log_2$) indicates the number of mapped reads per nucleotide position.

**Deletion of the fifteen non-essential TCS in a single cell**

Transcriptome analysis using RNA-seq and tilling arrays showed that most TCS were constitutively expressed in all *S. aureus* strains tested (Figs. 4 and 5). This result indicates that bacteria need to have ready the TCSs to respond to the different stimuli that migth encounter in different environmental conditions. Thus, with the aim to uncover the consequences that the absence of the complete TCS sensorial signaling pathways might have for bacteria, we sequentially deleted the fifteen non-essential TCSs in the same strain (Fig. 7). The deletions were done in two strain backgrounds, the *S. aureus* MW2 and 8325r strains. MW2 is a community-acquire MRSA strain and 8325r is a laboratory MSSA strain. The genomes of the resulting strains, *S. aureus* MW2 ΔXV and *S. aureus* 8325r ΔXV, were completely sequenced by Illumina to exclude the possibility that spontaneous mutations might have emerged during the successive deletion process. This control is important when using a reductionist approach because complementation of the final strain and restoration of the phenotypes is extremely complicated. The absence of common mutations between both strains confirmed that deletion of the fifteen TCS does not select for a particular genetic change that influenced the behaviour of the ΔXV strains. The SNPs produced spontaneously during the mutagenesis process are summarized in Tables 6 and 7. Together, these results demonstrate that a *S. aureus* strain containing a single TCS is viable at least under laboratory growth conditions.

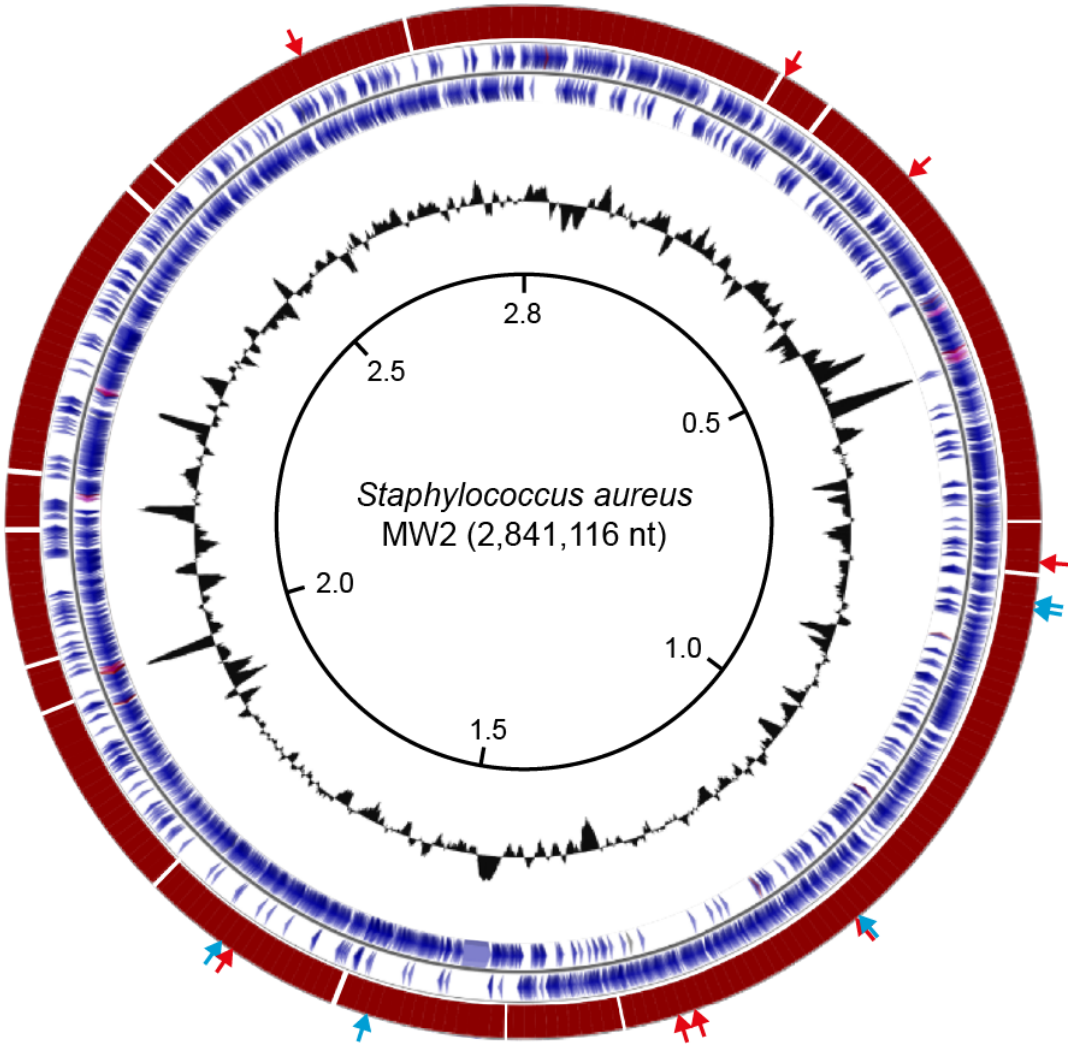


**Figure 7. Schematic circular representation of the *S. aureus* MW2 ΔXV genome, based on Illumina sequencing**. Internal two circles are the positive and negative chains. ORFs in blue, tRNAs in red and rRNAs in pink. GC content is represented in black. The red circle shows the Blastp analysis of MW2 ΔXV vs MW2 wild type. The fifteen TCS deletions are represented as 15 white lines. Red and blue arrows mark the SNPs produced spontaneously during the mutagenesis process in MW2 and 8325r strains respectively.

**Table 6.** Spontaneous changes produced during the mutagenesis on the MW2 genome

| Position | Mutation | Annotation | Gene | Description |
|---|---|---|---|---|
| 239,826 | G→A | A220T (GCA→ACA) | *pflA* → | formate acetyltransferase activating enzyme |
| 379,604 | G→T | G249V (GGA→GTA) | *MW0330* → | hypothetical protein |
| 746,147 | G→T | E123* (GAA→TAA) | *nagA* → | N-acetylglucosamine-6-phosphate deacetylase |
| 1,021,368 | G→T | D263Y (GAT→TAT) | *menB* → | naphthoate synthase |
| 1,105,909 | Ω43545 bp | Phage insertion | *rpmF* → / ← *isdB* | 50S ribosomal protein L3/hypothetical protein |
| 1,267,878 | G→T | R475L (CGT→CTT) | *pnpA* → | polynucleotide phosphorylase/polyadenylase |
| 1,283,489 | G→C | G502A (GGT→GCT) | *MW1169* → | phosphodiesterase |
| 1,691,188 | G→T | L10I (CTA→ATA) | *MW1565* ← | hypothetical protein |
| 2,639,004 | GT→TG | Y441S (TAC→TCA) | *rocA* ← | 1-pyrroline-5-carboxylate dehydrogenase |
| 2,639,012 | T→A | T439S (ACA→TCA) | *rocA* ← | 1-pyrroline-5-carboxylate dehydrogenase |

**Table 7**. Spontaneous changes produced during the mutagenesis on the 8325r genome

| Position | Mutation | Annotation | Gene | Description |
|---|---|---|---|---|
| 781,629 | C→T | Y258Y (TAC→TAT) | *eno* → | phosphopyruvate hydratase |
| 787,913 | C→T | intergenic (+907/+415) | *smpB* → / ← *SA00806* | SsrA-binding protein/hypothetical protein |
| 1,101,720 | A→G | intergenic (+149/-237) | *SA01150* → / → *SA01152* | cell division protein FtsZ/hypothetical protein |
| 1,560,503 | C→T | A190T (GCT→ACT) | *SA01646* ← | glucokinase |
| 1,703,050 | T→C | intergenic (-589/-23) | *SA01802* ← / → *SA01803* | hypothetical protein/hypothetical protein |

ID number corresponds to the 8325r strain

**Phenotypic characterization of *S. aureus* ΔXV strains**

We then determined how the removal of the complete sensorial system affects the bacterial physiology. We found that ΔXV mutants exhibited growth rates indistinguishable from the wild type under aerobic conditions at 37ºC and 44ºC, while they showed a growth defect at 28ºC (Fig. 8). Moreover, *S. aureus* ΔXV mutants showed a decreased capacity for long-term survival during desiccation in the absence of nutrients. In standard biochemical and fermentation test, both ΔXV mutant strains only exhibited a deficiency in the capacity to reduce nitrate to nitrite compared to wild type strain. Next, we examined the susceptibility of ΔXV mutants to cell lysis induced by a non-ionic detergent, such as Triton X-100, which reflects deficiencies in the regulation of autolytic activity (de Jonge *et al.*, 1991). The ΔXV mutants exhibited a much higher growth defect compared to the wild type strain in triton X-100 gradient plates (Fig 8). Since Protein A is a representative cell wall-associated exoprotein and a major determinant of virulence in *S. aureus*, we also compared the levels of Protein A between the ΔXV mutant and the wild type. The results revealed that the expression of Protein A was reduced in the ΔXV mutant compared to the wild type strain.

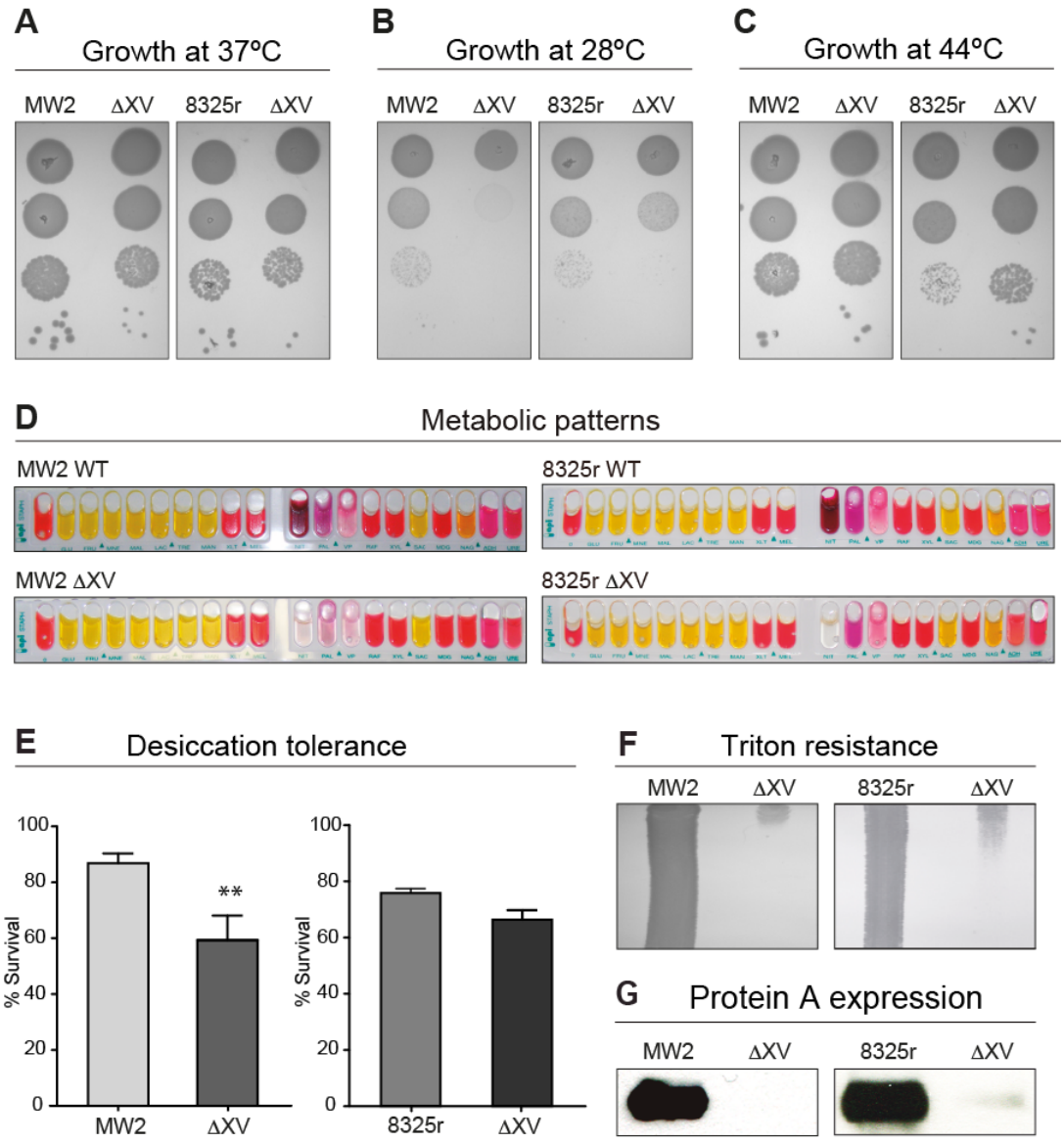


**Figure 8. Phenotypic analyses of *S. aureus* MW2, 8325r wild type strains and their corresponding ΔXV strains.** Bacterial growth on agar plates at different temperatures**.** Bacterial cultures were serially diluted ($10^{-2}$, $10^{-4}$ and $10^{-6}$) and 5 μL of each dilution were spotted onto TSA plates to determine the number of surviving CFU. The plates were then incubated overnight at (A) 37ºC, (B) 28ºC and (C) 44ºC. (D) Metabolic pattern according to API Staph galleries. (E) Desiccation tolerance after 21 days. 100 μl from overnight cultures grown in TSB-gluc medium at 37 °C were tested immediately (initial numbers) or air dried and stored in 24-well tissue culture plates at room temperature for 21 days. After rehydration of bacteria in 500 μl PBS, pH 7.4, the number of viable cells remaining in each sample was determined by serially diluting cell mixtures and plating in duplicate. The average and SD of 3 independent assays was recorded. (F) Growth phenotype on Triton X-100 concentration gradient plates. (G) Analysis of Protein A expression by Western blotting.

Finally, antimicrobial susceptibility test against the panel of antibiotics routinely used in clinics for treatment of *S. aureus* infections revealed that MW2 ΔXV mutant was more susceptible to oxacillin, levofloxacin, linezolid, nitrofurantoin and cefoxitin while the sensitivity for the rest of antibiotics tested remained similar. The 8325r ΔXV mutant showed higher susceptibility to bencilpenicillin, ampicillin, vancomycin and fosfomycin while the sensitivity for the rest of antibiotics tested remained similar (Table 8). In summary, these results revealed that the strain devoid of the fifteen non-essential TCSs showed less phenotypic deficiencies than expected, suggesting that under constant environmental conditions TCSs are not necessary for the maintenance of house-keeping functions.

**Table 8.** Antimicrobial susceptibility test against the panel of antibiotics routinely used in clinics for treatment of *S. aureus* infection

| ANTIBIOTICS | MW2 | MW2 ΔXV | 8325r | 8325r ΔXV |
|---|---|---|---|---|
| β-lactam | + | + | - | - |
| Cefoxitin detection | + | + | - | - |
| Bencilpenicillin | R | R | R (0.25) | S (0.12) |
| Ampicillin | R | R | R | S |
| Cloxacillin | R | R | S | S |
| Oxacillin | R (≥4)[a] | R (2)[a] | S | S |
| Cefalotin | R | R | S | S |
| Cefuroxime | R | R | S | S |
| Gentamicin | S | S | S | S |
| Tobramycin | S | S | S | S |
| Ciprofloxacin | S | S | S | S |
| Levofloxacin | S (≤0.25)[a] | S (≤0.12)[a] | S | S |
| Inducible resistance to Clindamycin | - | - | - | - |
| Azithromycin | S | S | S | S |
| Clarithromycin | S | S | S | S |
| Erithromycin | S | S | S | S |
| Clindamycin | S | S | S | S |
| Quinuspristin/Dalfopristin | S | S | S | S |
| Linezolid | S (2)[a] | S (1)[a] | S | S |
| Teicoplanin | S | S | S | S |
| Vancomycin | S | S | S (1)[a] | S (≤0.5)[a] |
| Tigecycline | S | S | S | S |
| Fosfomycin | S | S | S (16)[a] | S (≤8)[a] |
| Nitrofurantoin | S (32)[a] | S (≤16)[a] | S | S |
| Fusidic acid | S | S | S | S |
| Mupirocin | S | S | S | S |
| Rifampicin | S | S | S | S |
| Trimethoprim/Sulfamethoxazole | S | S | S | S |
| Cefoxitin | R | S | S | S |
| Chloramphenicol | S | S | S | S |
| MRSA | + | + | - | - |

[a]MIC, Minimal inhibitory concentration

***S. aureus* ΔXV mutant is attenuated *in vivo***

An essential step in the establishment of *S. aureus* infections is the adhesion of bacteria to surface components of epithelial cells. We analyzed the consequences of the absence of the TCS signaling on the adherence capacity of *S. aureus* to two different cell lines, a human lung epithelial (A549) and a human hepatocyte (Hep-3B) cell lines. Remarkably, ΔXV mutant strain adhered to both cell lines as efficiently as the wild type strain ($P > 0.05$) (Fig. 9A). In contrast, ΔXV mutant strain invaded both epithelial cell lines less efficiently than the wild type strain (Fig. 9B). Because *S. aureus* is a leading cause of bacteraemia and sepsis, we then investigated the contribution of TCSs to invasive staphylococcal disease using a mouse renal abscess model (Fig. 9C). The results revealed that wild type *S. aureus* MW2 formed abscesses in kidneys with a mean bacterial load of $7.59 \times 10^7$ CFU per gram of tissue. In contrast, ΔXV mutant was unable to form abscesses and displayed a >1000-fold (log 4.24) reduction in bacterial load as compared with the wild type counterpart ($P < 0.01$; Fig. 9D). We also analyzed the mice survival upon intravenous injection of $10^7$ CFU of wild type or ΔXV strains. All mice infected with the mutant strain survive after 50 days, while 50% of the animals inoculated with MW2 wild type strain died by day 21 post-infection. Taken together, these data indicated that TCS signaling system is important for the pathogenesis of *S. aureus* infections, whether measured as the ability of *S. aureus* to form abscesses and persist in host tissues or lethal bacteremia.

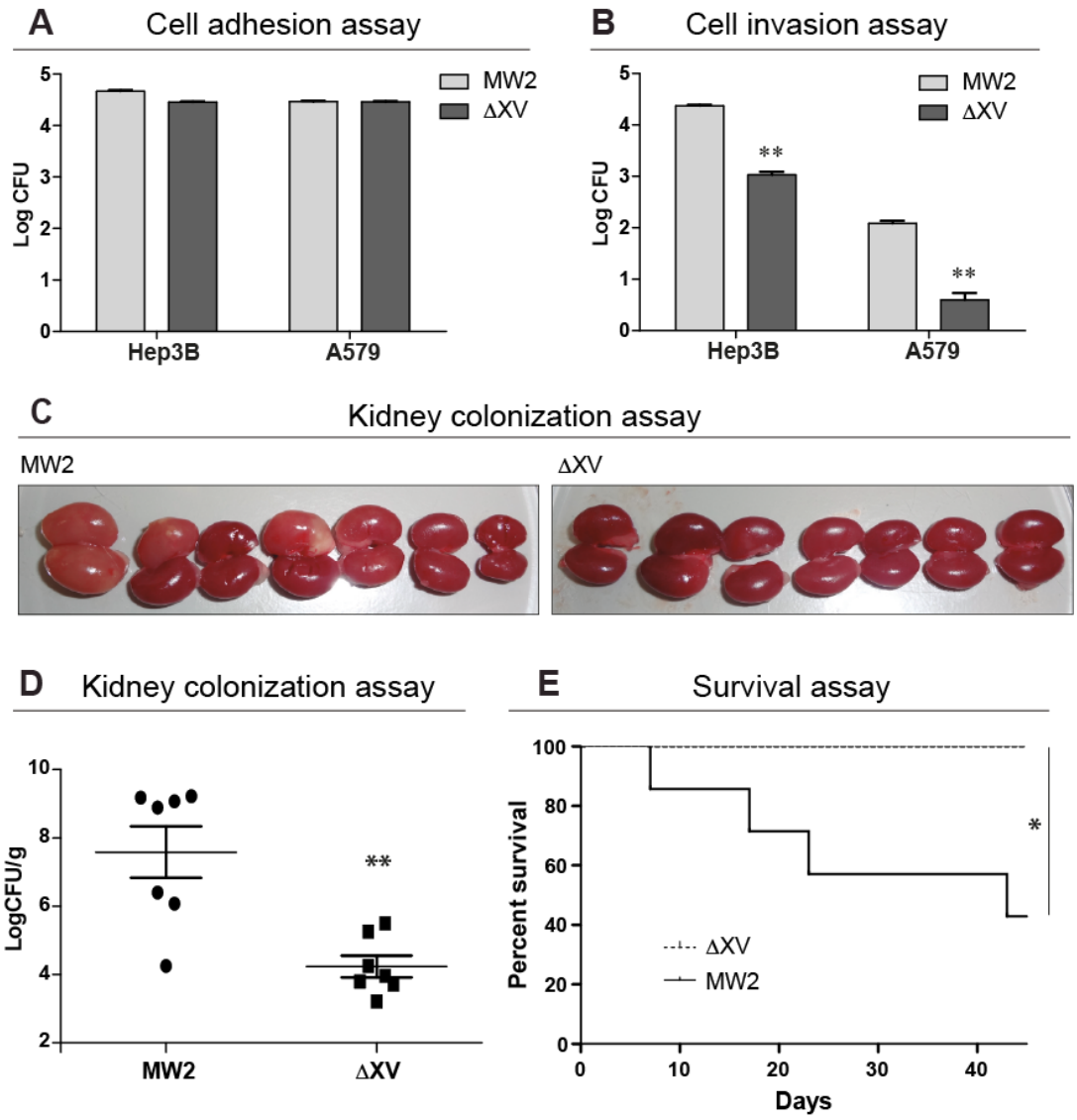


**Figure 9. Phenotypic analyses of the MW2 and MW2 ΔXV strains.** (A) Adhesion and (B) invasion of *S. aureus* MW2 wild type and ΔXV mutant to human hepathocytes (Hep3B) and human lung epithelial cells (A549). After 1 h of infection, cells were lysed and cell extracts were vigorously vortexed for 2 min. Bacterial adhesion was measured by CFU counts. Bacterial invasion was measured by CFU count after gentamicin assay. Experiments were performed in triplicate and repeated four times for each cell line (C and D) Seven mice per group were infected with $10^7$ cfu of bacteria by eye vain injection. Kidney were removed aseptically and homogenized in PBS for enumeration of viable bacteria. (E) Seven mice per group were infected with $10^7$ cfu of bacteria by eye vain injection. Mice survival was monitored every day for 7 weeks.

**Sense devoid *S. aureus* mutant**

It was well-documented that WalKR is essential for *S. aureus* viability (Dubrac and Msadek, 2004; Dubrac *et al.*, 2007), and therefore we initially excluded this TCS from our mutagenesis-based reductionist approach. However, we hypothesized that this TCS might not be essential in the absence of other TCSs. To test this hypothesis, the expression of the *walKR* operon was placed under the control of the inducible P*spac* promoter (ΔXVI* strain) (Dubrac and Msadek, 2004). In the absence of IPTG, ΔXVI* strain was unable to grow, indicating that WalKR is essential in the strain devoid of TCS (Fig. 10A). Then, we explored whether *S. aureus* could live without sensing environmental signals. To test this hypothesis, the ΔXVI* strain was complemented with a plasmid encoding the WalR gene under the control of a constitutive promoter (pEI *walR*). In the absence of IPTG, where the expression of *walKR* was inhibited, the ΔXVI* strain carrying the plasmid pEI *walR* (ΔXVI* *walR*) exhibited growth rates indistinguishable from the ΔXV strain at 37ºC, 28ºC and 44ºC whereas the strain complemented with the empty plasmid or the WalR D52A derivative protein did not grow (Fig. 10). Furthermore, ΔXVI* *walR* showed similar capacity for long-term survival during desiccation in the absence of nutrients, susceptibility to Triton X-100, deficiency in the capacity to reduce nitrate to nitrite and reduction in Protein A expression to the ΔXV strain.

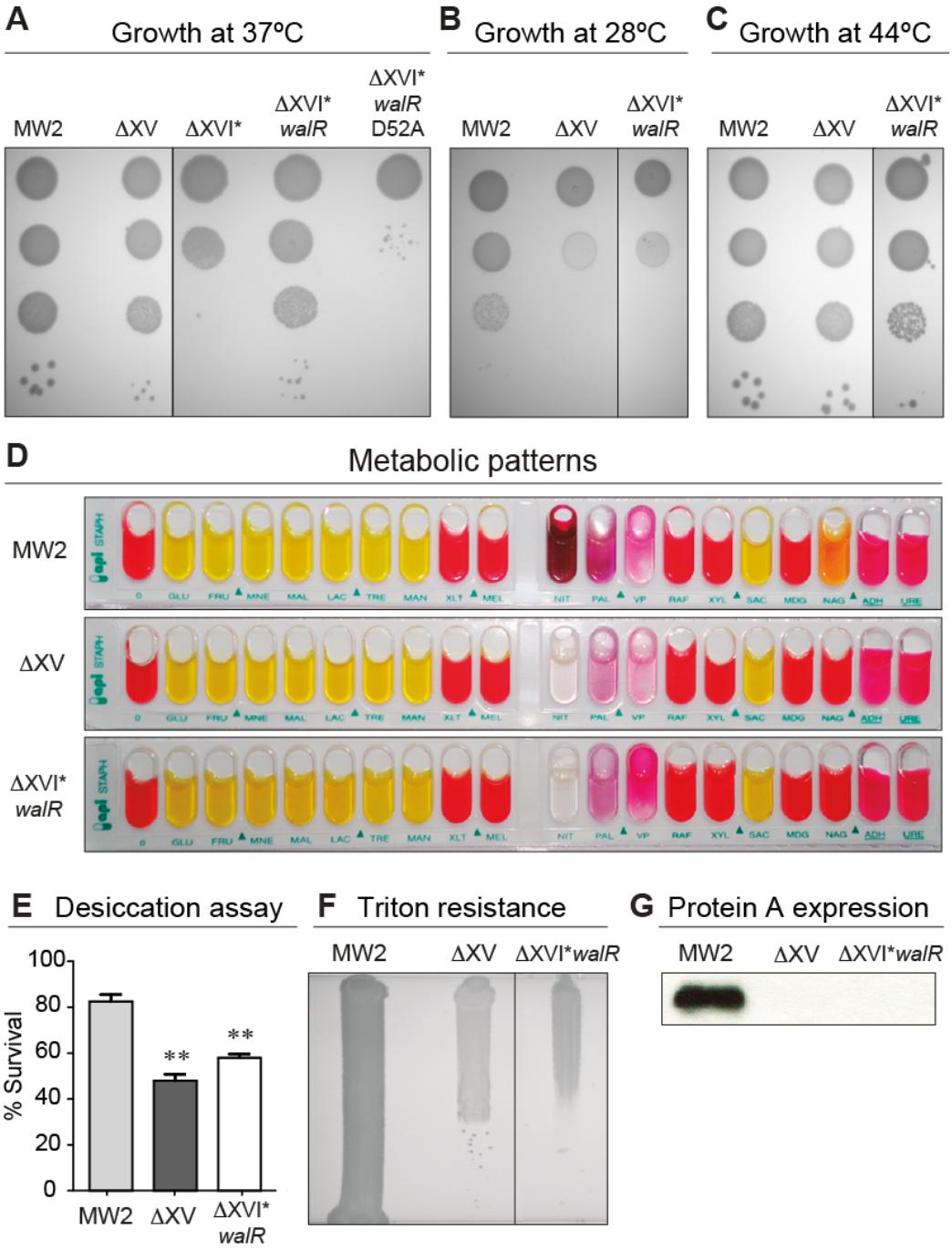


**Figure 10. Phenotypic analyses of *S. aureus* MW2 wild type, ΔXV and ΔXVI* *walR* (ΔXV Pspac-*walKR* pEI *walR*) strains.** Bacterial growth on agar plates at different temperatures: Bacterial cultures were serially diluted ($10^{-2}$, $10^{-4}$ and $10^{-6}$) and 5 μL of each dilution were spotted onto TSA plates supplemented with chloramphenicol to determine the number of surviving CFU. The plates were then incubated overnight at (A) 37ºC, (B) 28ºC and (C) 44ºC. (D) Metabolic pattern according to API Staph galleries. (E) Desiccation tolerance after 21 days. 100 μl from overnight cultures grown in TSB-gluc medium at 37ºC were tested immediately (initial numbers) or air dried and stored in 24-well tissue culture plates at room temperature for 21 days. After rehydration of bacteria in 500μl PBS, pH 7.4, the number of viable cells remaining in each sample was determined by serially diluting cell mixtures and plating in duplicate. The average and SD of 3 independent assays was recorded. (F) Growth phenotype on Triton X-100 concentration gradient plates. (G) Analysis of Protein A expression by Western blotting.

Taken together, these results indicate that the constitutive expression of WalR in the absence of WalK sensor was sufficient to suppress lethality and that a *S. aureus* strain devoid of all sensor HKs is viable under laboratory conditions. On the other hand, the finding that WalR D52A is unable to restore bacterial growth suggests that WalR has to be phosphorylated to fulfill its function. However, we cannot exclude that the exchange of aspartic acid residue of position 52 by alanine could leads to an inactive protein.

**Single TCS-dependent phenotypes**

All phenotypes related with TCS previously described have been deduced from the comparison between the wild type and the corresponding isogenic mutant. This strategy allows the identification of phenotypes for which the presence of the corresponding TCS is necessary. However, identification of a TCS necessary for a phenotype does not necessarily imply that the TCS is sufficient to account for this particular phenotype in the absence of other TCSs. To tackle this question, the phenotypes previously characterized in the ΔXV strain were compared between the single and the sequential mutants (Fig. 11). We hypothesized that if a phenotype is controlled by a single TCS, then, the mutation of this TCS should affect the same phenotype both in the single and sequential mutant (Fig. 11) and the phenotype should be restored by the ectopic expression of this TCS in the ΔXV strain.

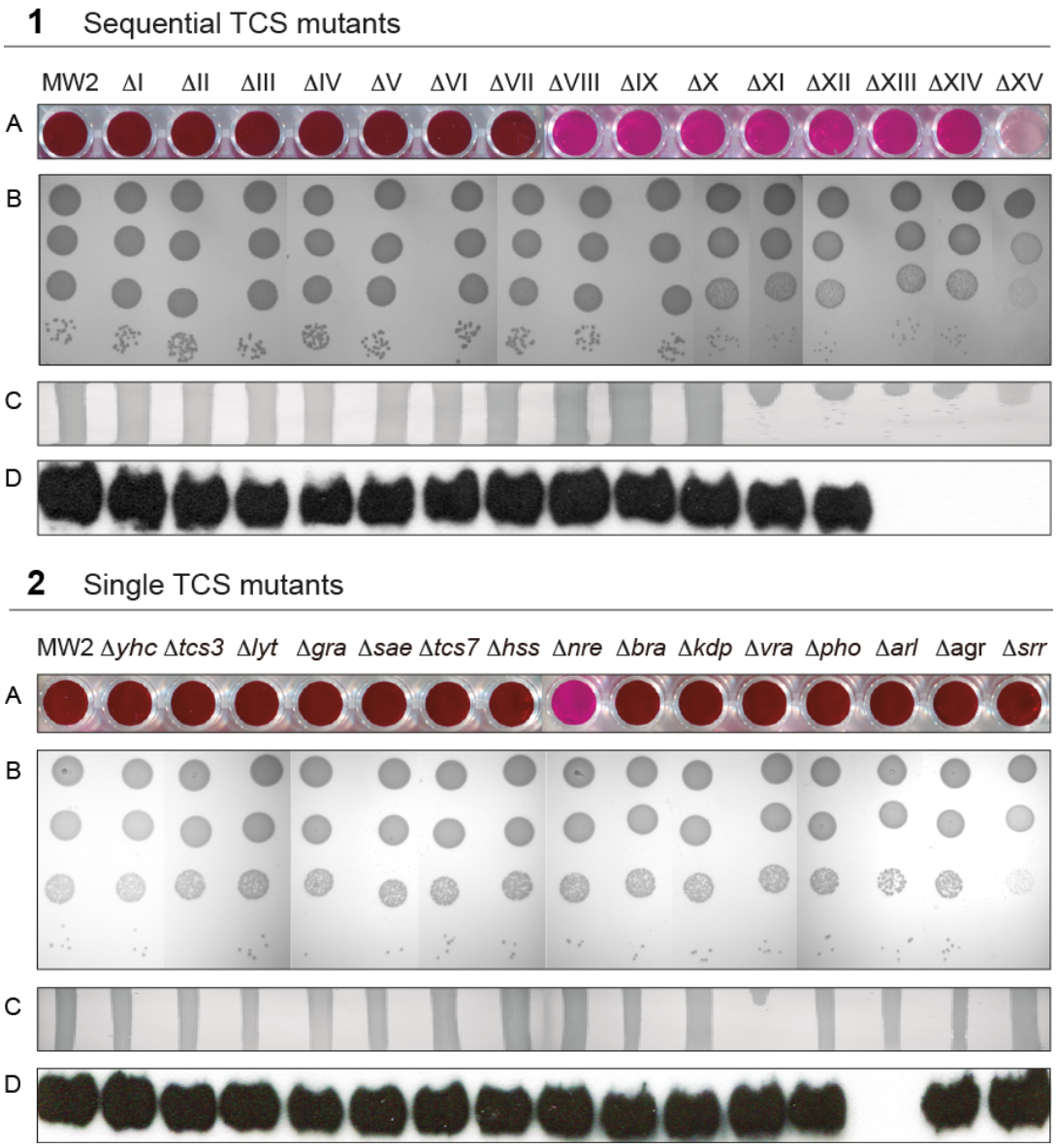


**Figure 11. Phenotypic analyses of *S. aureus* MW2 sequential TCS mutants (1) and single TCS mutants (2).** (A) Nitrate reduction capacity under low oxygen concentration. Conditions of decreased oxygen tension were created by growing the bacterial strains in 15-ml Falcon tubes that were completely filled with medium. Potassium nitrate ($KNO_3$) was added to the medium at 20 mM. Extracellular concentrations of nitrite were determined colorimetrically by the Griess Reagent System. (B) Bacterial growth on agar plates**.** Bacterial cultures were serially diluted ($10^{-2}$, $10^{-4}$ and $10^{-6}$) and 5 μL of each dilution were spotted onto TSA plates to determine the number of surviving CFU. The plates were then incubated overnight at 28ºC. (C) Growth phenotype on Triton X-100 concentration gradient plates. (D) Analysis of Protein A expression by Western blotting.

Therefore, for the complementation purposes, we engineered each HK and RR to produce individual modules of each of them that could be combined into a plasmid (Fig. 12). This strategy gave us the possibility to express each RR either alone, together with the cognate HK pair or in combination with the non-cognate HKs. The expression of HK-RR pairs could be under the control of the cadmium-inducible promoter ($P_{cad}$) or the a constitutive promoter ($P_{blaZ}$).

Phenotypic analysis of the single and sequential mutants revealed that growth defect at 28ºC depends on SrrAB, the capacity to reduce nitrate to nitrite depends on NreBC, the expression of Protein A depends on ArlRS, susceptibility to Triton X-100 depends on VraRS and WalKR is necessary to support growth at 37ºC. In all instances, ectopic expression of a functional copy of the corresponding TCS was sufficient to fully rescue each phenotype in the ΔXV strain (Fig. 13). Taken together, these results indicate that phenotypes of ΔXV strain depend on individual TCS, and ectopic expression of the corresponding TCS is sufficient to restore phenotypes associated to the ΔXV strain.

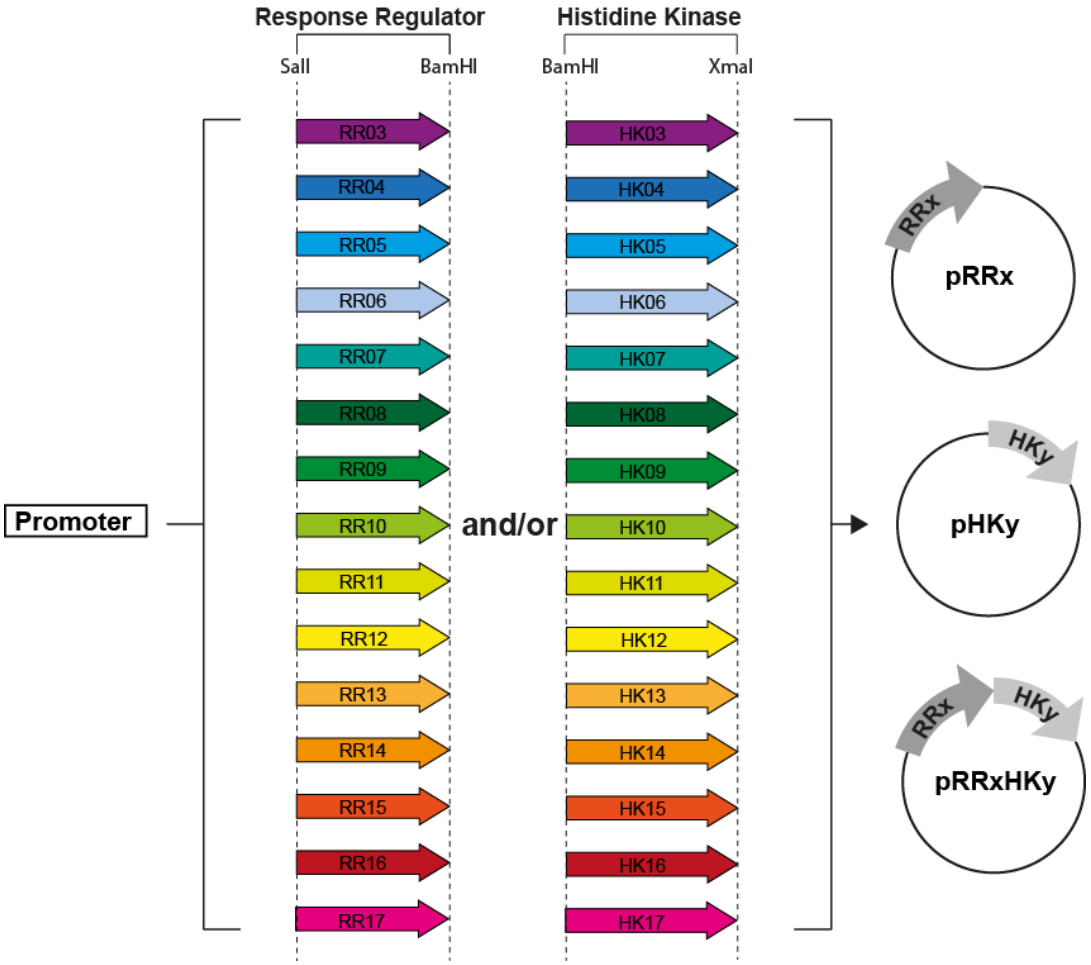


**Figure 12. Schematic representation of engineered RR and HKs modules for system-based analysis.** Response regulators are flanked by SalI and BamHI restriction sites and histidine kinases are flanked by BamHI and XmaI restriction sites to allow the easy combination of modules in a plasmid. The expression of HK-RR pairs in the plasmid is always under the control of the cadmium-inducible promoter ($P_{cad}$). This strategy provides the possibility to express each response regulator either alone, together with the cognate histidine kinase or in combination with the rest of non-cognate histidine kinases.

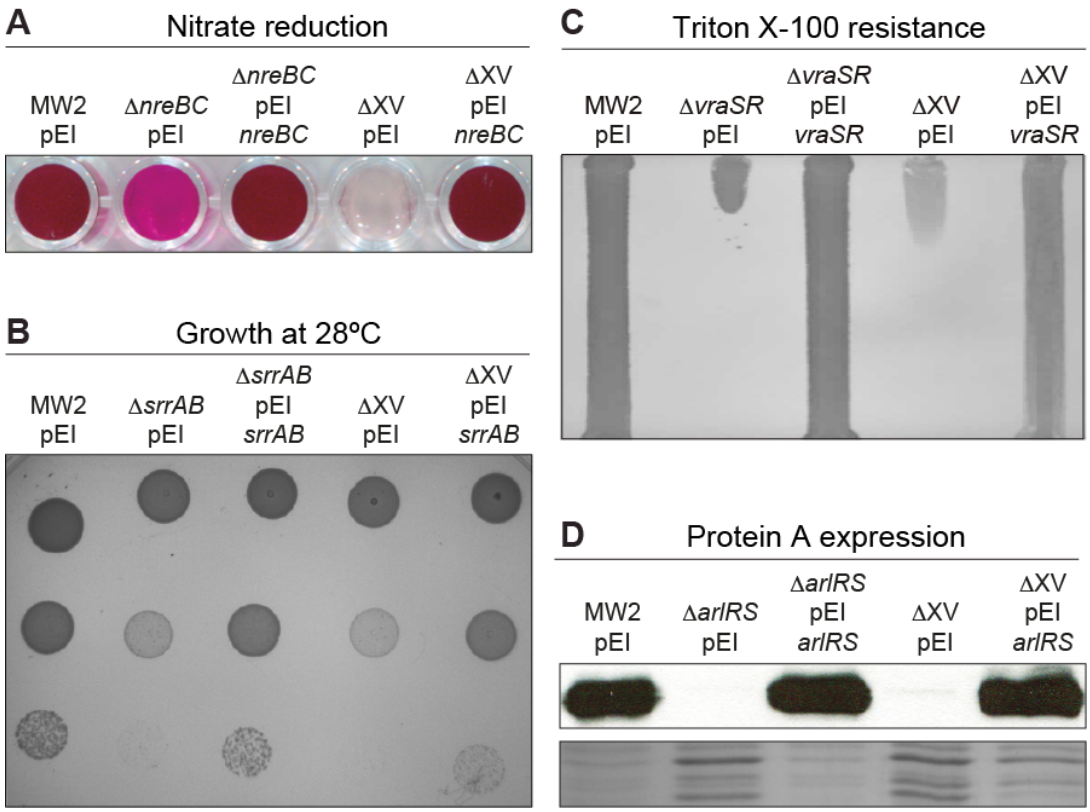


**Figure 13. Complementation of phenotypes in single and XV mutant strains by ectopic expression of a single TCS from the pEI plasmid.** (A) Nitrate reduction capacity under low oxygen concentration. Conditions of decreased oxygen tension were created by growing the bacterial strains in 15-ml Falcon tubes that were completely filled with medium. Potassium nitrate ($KNO_3$) was added to the medium at 20 mM. Extracellular concentrations of nitrite were determined colorimetrically by the Griess Reagent System. (B) Bacterial growth on agar plates. Bacterial cultures were serially diluted ($10^{-2}$, $10^{-4}$ and $10^{-6}$) and 5 μL of each dilution were spotted onto TSA plates to determine the number of surviving CFU. The plates were then incubated overnight at 28ºC. (C) Growth phenotype on Triton X-100 concentration gradient plates. (D) Analysis of Protein A expression by Western blotting.

**System-based analysis of cross-talk *in vivo***

We exploited the ΔXV strain as well as the collections of single mutants to carry on a systematic analysis of the connectivity between sensor HKs and RRs *in vivo*. Our reasoning was that if ectopic expression of a native RR is capable to activate the phenotype in the absence of its cognate sensor but it is unable to activate a specific phenotype in the ΔXV strain, it implies the existence of cross-talk from one or various non-cognate sensor HKs present in the single mutant but absent in the ΔXV strain (Fig. 14). Based on the previously observed correlation between phenotypes and single TCS, mutants in *srrAB, nreBC, arlRS, vraRS* and ΔXV were complemented with the corresponding *srrA, nreC, arlR* and *vraR* RRs.

Complementation of the single *S. aureus* Δ*arlRS* mutant with the native *arlR* RR was able to restore Protein A expression whereas complementation of ΔXV mutant with the same plasmid did not. Contrary to this, complementation with SrrA, NreC and VraR RRs were unable to activate their corresponding phenotype in the single mutants, indicating that cross-talk between non-cognate HKs and these RRs does not occur at least under laboratory growth conditions. Interestingly, VraR was able to partially restore the susceptibility to Triton X-100 of ΔXV mutant (Fig. 14). Because a similar restoration did not occur in the single mutant, it seems that activation of VraR is very likely suppressed by the phosphatase activity of a non-cognate HK.

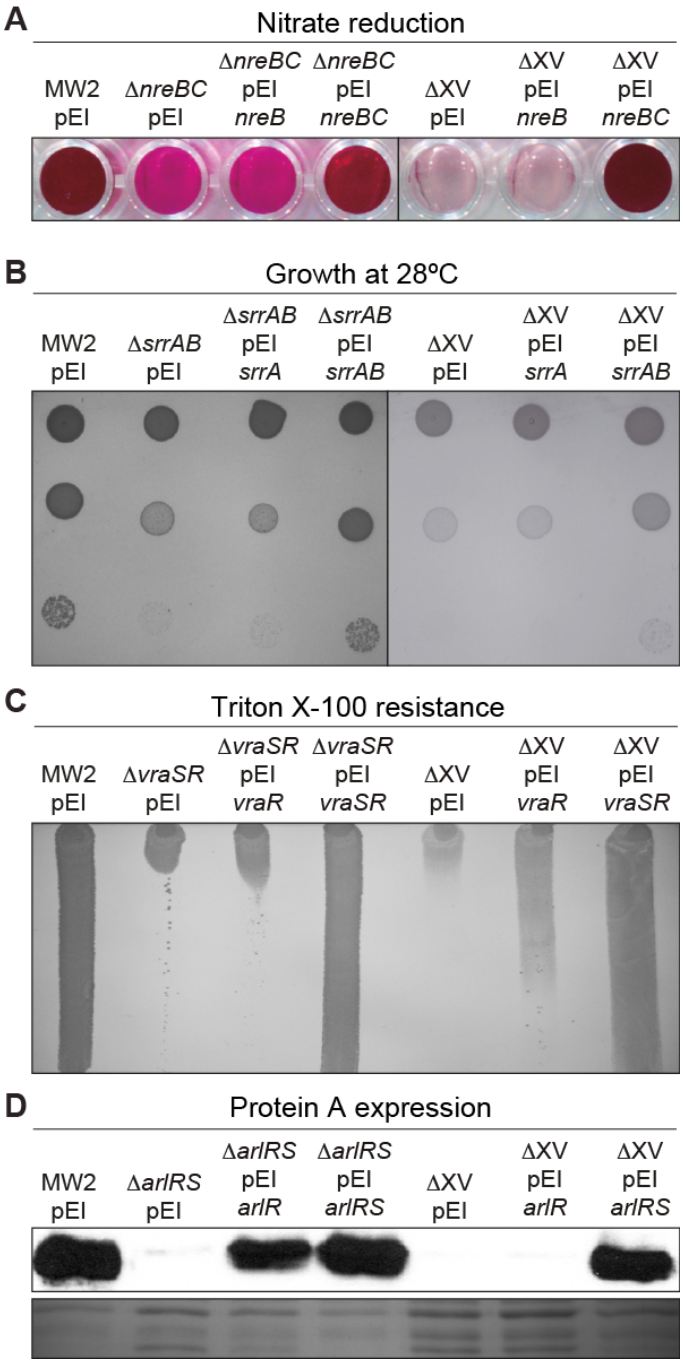


**Figure 14. Searching for cross-talk between TCSs *in vivo*. Complementation of the single and the XV mutants with the RR alone or in combination with the cognate HK.** (A) Nitrate reduction capacity under low oxygen concentration. Conditions of decreased oxygen tension were created by growing the bacterial strains in 15-ml Falcon tubes that were completely filled with medium. Potassium nitrate ($KNO_3$) was added to the medium at 20 mM. Extracellular concentrations of nitrite were determined colorimetrically by the Griess Reagent System. (B) Bacterial growth on agar plates. Bacterial cultures were serially diluted ($10^{-2}$, $10^{-4}$ and $10^{-6}$) and 5 μL of each dilution were spotted onto TSA plates to determine the number of surviving CFU. The plates were then incubated overnight at 28ºC. (C) Growth phenotype on Triton X-100 concentration gradient plates. (D) Analysis of Protein A expression by Western blotting.

Our observation that ArlR activated Protein A production in the absence of ArlS led us to hypothesize that cross-talk from another HK to ArlR occurs *in vivo*. To confirm that phosphorylation of ArlR was necessary to induce Protein A expression, the aspartic residue at position 52 in ArlR was replaced with a nonphosphorylatable alanine residue (Fig. 15A). Complementation of *arlRS* mutant with ArlR D52A allele could not activate Protein A expression, suggesting that phospho-transfer to ArlR was necessary to induce Protein A expression.

The phosphatase activity of the cognate HK provides a mechanism to reset the signal transduction to the baseline state. At the same time, it also provides a mechanisms to prevent the non-specific phosphorylation of the cognate RR. Thus, in the absence of the phosphatase catalytic activity of the cognate HK, cross-talk from non-cognate HK to a RR has been previously reported (Wanner *et al.*, 1988; Amemura *et al.*, 1990; Stock *et al.*, 2000). To test whether the cross-talk with ArlR from a non-cognate HK also occurs in the presence of the phosphatase activity of ArlS, *arlRS* single mutant was complemented with a plasmid producing ArlR together with the ArlS H242A allele, which contains an histidine-to-alanine substitution at position 242. The ArlS H242A allele retains the phosphatase activity but it loses the capacity to phosphorylate ArlR. The complemented strain showed a level of expression of Protein A similar to that of the strain complemented with ArlR alone, indicating that the phosphatase activity of ArlS cannot supress cross-talk from other HKs to ArlR (Fig. 15A).

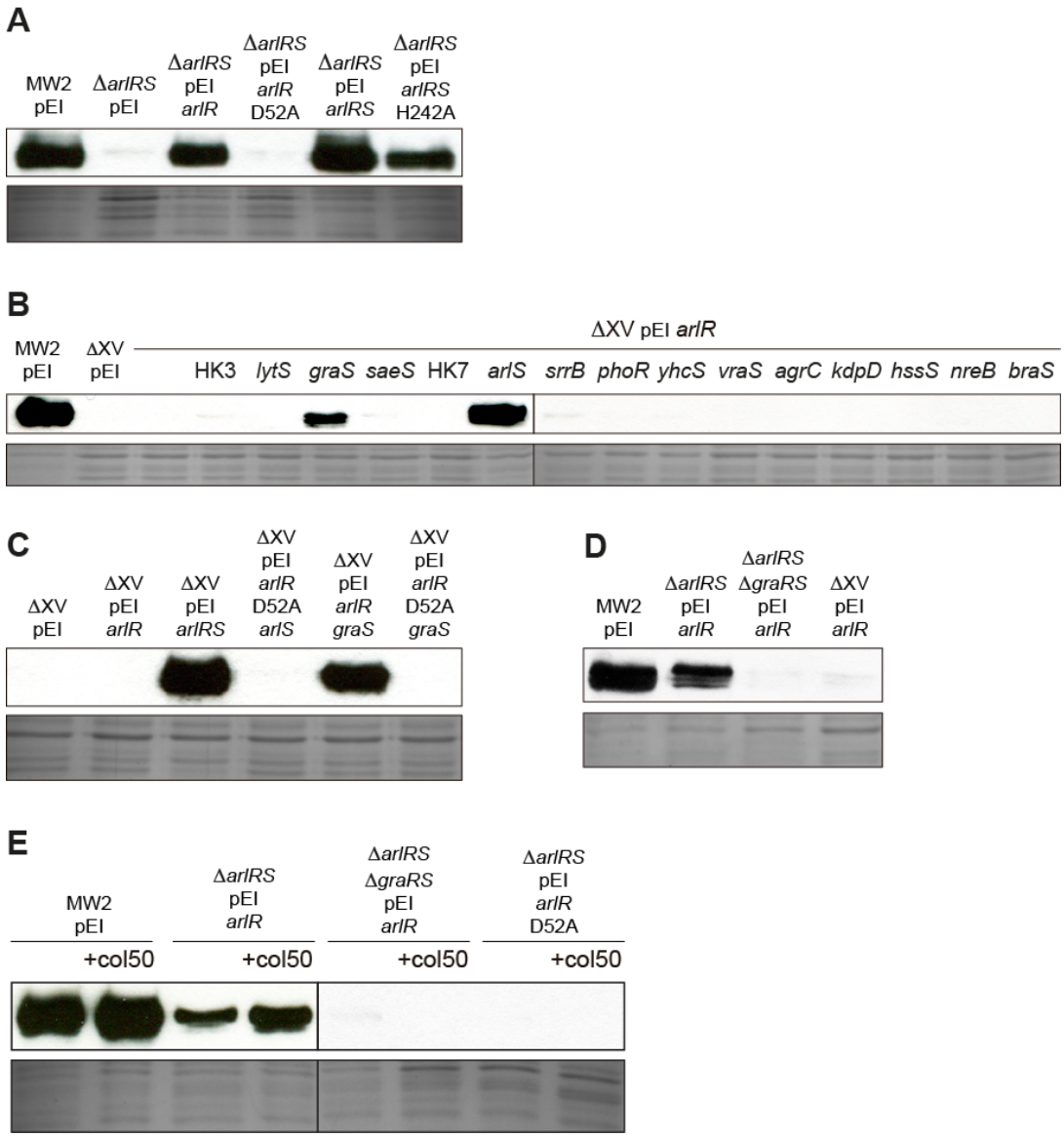


**Figure 15. System-based analysis of cross-talk *in vivo.*** Analysis of Protein A expression by Western blotting on the following strains. (A) Single *arlRS* mutant strain complemented with *arlR, arlR* D52A*, arlRS* and *arlRS* H242A. (B) ΔXV strain complemented with the set of plasmids containing *arl* RR combined with the 16 HKs. Note that GraS, apart from ArlS, is able to complement *arlR*-dependent protein A expression (C) ΔXV strain complemented with arlR or non-phosphorylatable *arlR* D52A combined with ArlS or GraS. Note that phospho-transfer is necessary for *arlR*-dependent protein A activation by GraS (D) Single Δ*arlRS*, double Δ*arlRSgraRS* and XV mutant strains complemented with *arlR*. Note that cross-talk dissappears in the absence of *graRS*. E) Single Δ*arlRS* and double Δ*arlRSgraRS* strains complemented with the *arlR* or *arlR* D52A in the presence or absence of 50 μg $ml^{-1}$ of colistin. Note that colistin activates *arlR*-dependent protein A expression.

We next developed a system-level approach to identify cross-phosphorylation between HKs and ArlR *in vivo*. A set of plasmids, each containing the *arlR* gene in combination with one of the other fifteen HKs, was used to complement ΔXV strain (Fig. 16). This strategy allows us to test the ability of non-cognate HKs to activate ArlR-dependent Protein A expression in the absence of other sources of phosphotransfer to ArlR (Fig. 15B). In addition to ArlS itself, GraS was able to activate Protein A expression in ΔXV strain. This activation was dependent on the phosphorylation of ArlR because substitution of ArlR wild type by the non-phosphorylatable ArlR D52A allele suppresses the cross-talk (Fig. 15C).

It is also notable that cross-talk with ArlR occurs in the single *arlRS* mutant, implying that GraS cross-phosphorylates ArlR even in the presence of its cognate GraR RR. In addition, mutation of the *graRS* operon eliminates cross-talk (Fig. 15D). Together, these results indicate that cross-talk between otherwise distinct TCS (ArlR and GraS) through phosphotransfer occurs *in vivo*, though it seems to be limited to few specific TCSs.

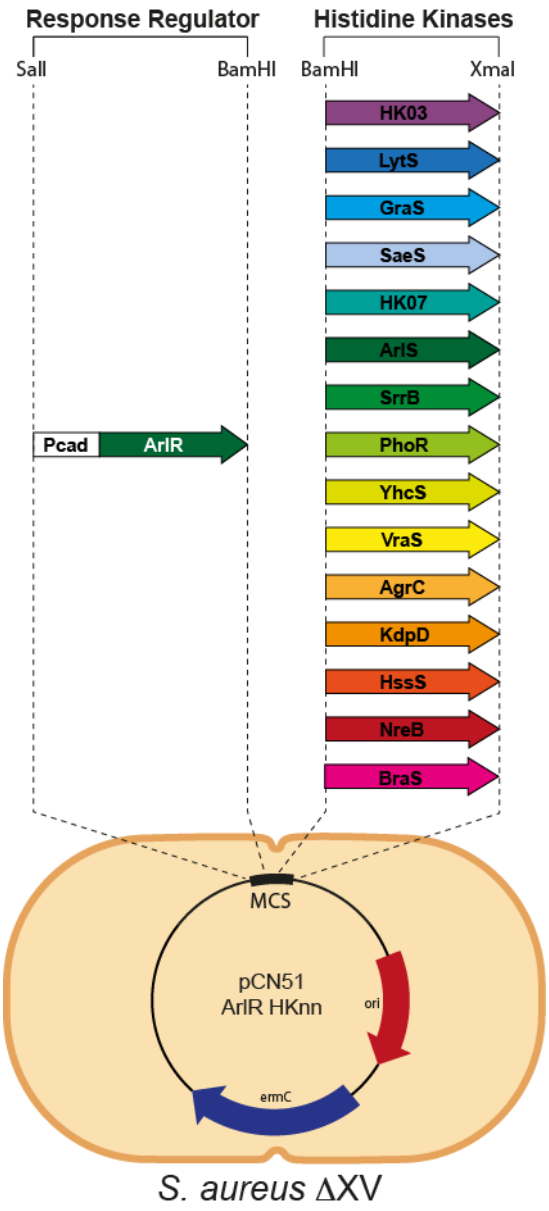


**Figure 16. Schematic representation of the plasmid platform for system-based analysis of cross-talk *in vivo*.** A set of fifteen plasmids each containing the *arlR* gene in combination with one histidine kinase is used to complement *S.aureus* MW2 ΔXV strain.

Since GraS is activated in the presence of colistin in the media, we hypothesized that cross-talk from GraS to ArlR might be induced in the presence of colistin (Fig. 15E). As expected, when GraS was stimulated with 50 μg $ml^{-1}$ of colistin, activation of the expression of Protein A was observed in the wild type and single *arlRS* mutant but not in *arlRS graSR* double mutant, where Protein A expression is null. Furthermore, activation of Protein A expression was absent when the ArlR phosphate accepting aspartate was mutated. Together these data indicate that natural activation of GraS with cationic peptides can cross-phosphorylate ArlR to activate Protein A expression *in vivo*.

**Cross-talk does not occur in NreC, SrrA and VraR RRs**

The failure of NreC, SrrA and VraR RRs to activate nitrate respiration, growth at 28ºC and resistance to Triton X-100 in the corresponding single mutant (Fig. 14) could be due to the fact that expression of HKs from the chromosome might not be sufficient to cross-activate the RRs produced from the multicopy plasmid. Therefore, we decided to continue our system-based cross-talk analysis by complementing the ΔXV strain with the set of plasmids producing each of these RRs (NreC, SrrA and VraR) combined with all the HKs. While the plasmids producing the NreBC and SrrAB genes were able to restore completely the nitrate respiration and growth rates at 28ºC, none of the non-cognate HKs were able to restore these phenotypes in ΔXV strain (Fig. 17A and 17B). In contrast, the YhcS HK was able to activate VraR and restore resistance of Triton X-100 in ΔXV strain (Fig.

17C). Since complementation of the single *vraSR* mutant with VraR was unable to restore the phenotype, we hypothesized that YhcS cross-talk might only occur in the absence of its cognate RR (YhcR). To test this idea, we complement ΔXV mutant strain with a plasmid harboring VraR or VraSR and a second compatible plasmid harboring either YhcS or YhcSR (Fig. 17D). The results showed that YhcS was unable to activate VraR in the presence of its cognate RR partner, YhcR, or in the presence of the phosphatase activity of VraS (VraS H156A) (Fig. 17D). Althogether, these results confirm that cross-talk between HKs and non-cognate RRs occurs *in vivo* but it is rather exceptional. Otherwise, internal mechanisms such as phosphatase activity and phosphorylation preference for cognate RR prevent undesired cross-talk *in vivo* as we have shown for YhcS-VraR cross-talk (Fig. 18).

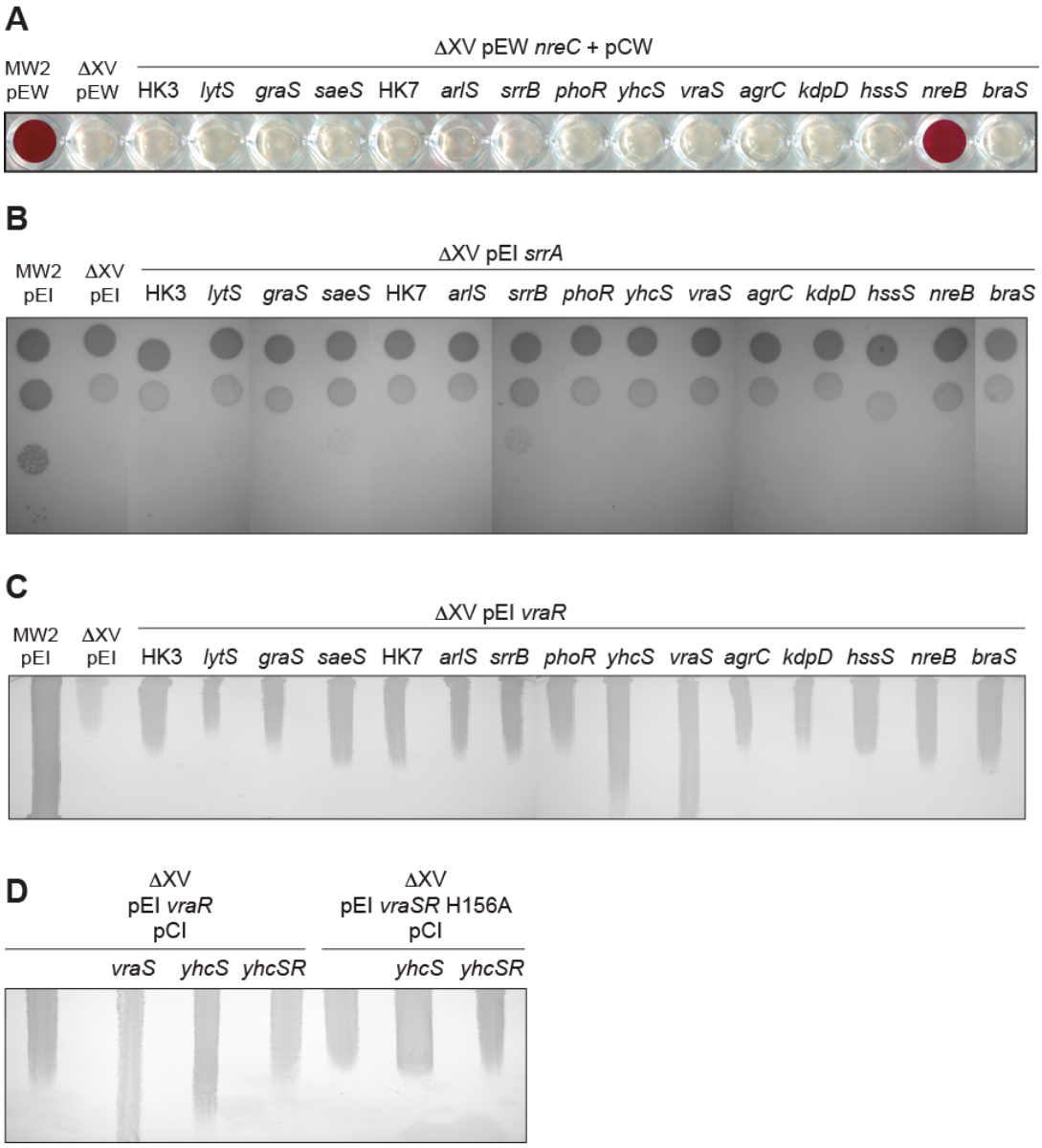


**Figure 17. Cross-talk does not occur in NreC, SrrA and VraR RRs.** (A) Nitrate reduction capacity under low oxygen concentration of ΔXV strain complemented with the set of plasmids containing *nreC* RR and all the 16 HKs. Conditions of decreased oxygen tension were created by growing the bacterial strains in 15-ml Falcon tubes that were completely filled with medium. Potassium nitrate ($KNO_3$) was added to the medium at 20 mM. Extracellular concentrations of nitrite were determined colorimetrically by the Griess Reagent System. (B) Bacterial growth on agar plates at 28ºC of ΔXV strain complemented with the set of plasmids containing *srrA* RR and all the 16 HKs. Bacterial cultures were serially diluted ($10^{-2}$, $10^{-4}$ and $10^{-6}$) and 5 μL of each dilution were spotted onto TSA plates to determine the number of surviving CFU. The plates were then incubated overnight at 28ºC. (C) Bacterial growth on Triton X-100 gradient plates of ΔXV strain complemented with the set of plasmids containing *vraR* RR and all the 16 HKs. Note that YhcS is able to restore the phenotype. (D) Bacterial growth on Triton X-100 gradient plates of ΔXV strain with a plasmid harboring VraR or VraSR H156A and a second compatible plasmid harbouring either YhcS or YhcSR. Note that YhcS is unable to restore the phenotype in the presence of the proper partners, YhcR and VraS.

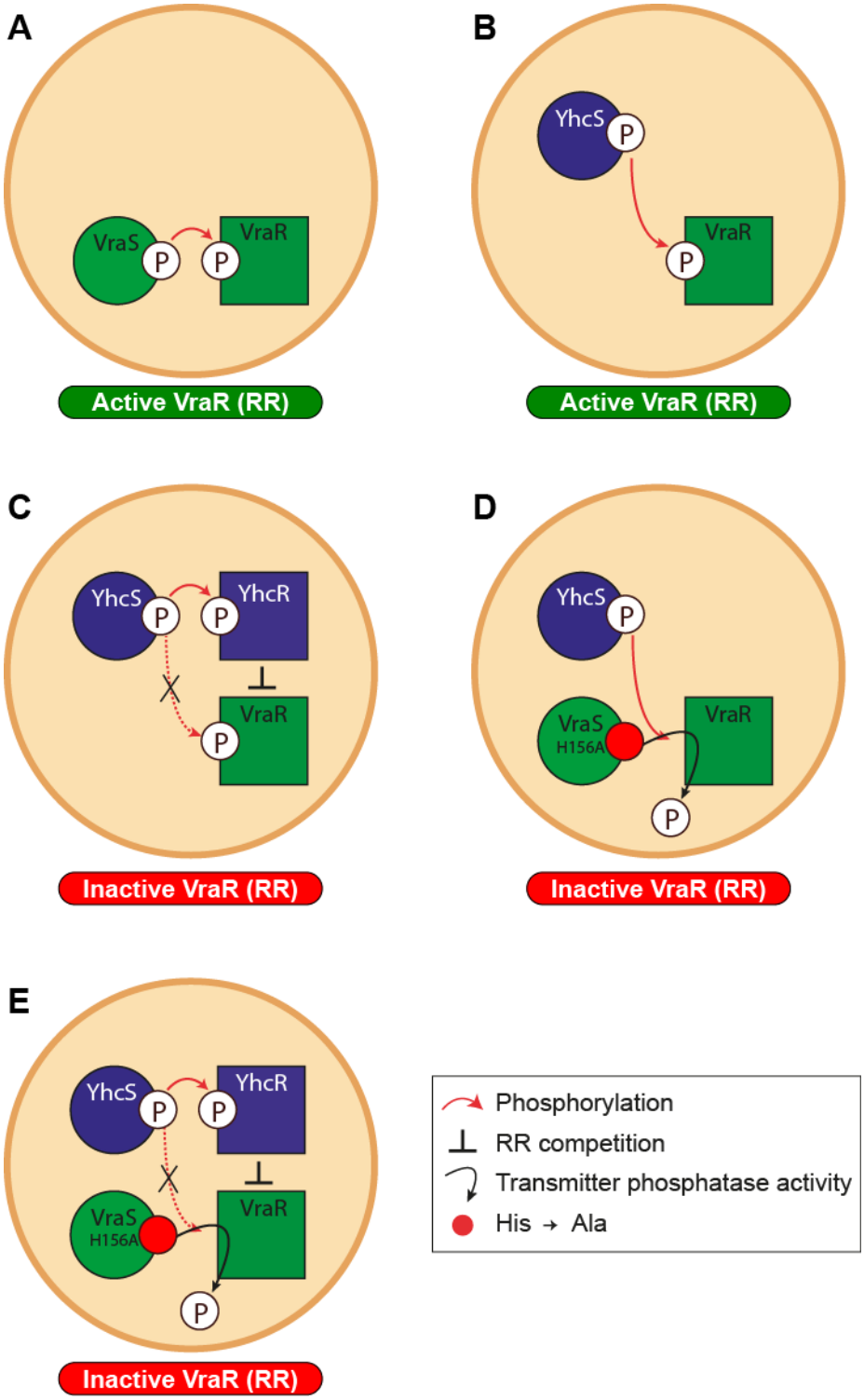


**Figure 18. Summary of cross-talk between YhcS and VraR in the presence or absence of the cognate YhcR and VraS pairs.** (A) VraS phosphorylates VraR. (B) YhcS phosphorylates VraR in the absence of cognate pairs. (C) YhcS is unable to activate VraR in the presence of YhcR. (D) YhcS is unable to activate VraR in the presence of the phosphatase activity of VraS (VraS H156A). (E) YhcS is unable to activate VraR in the presence of YhcR and the phosphatase activity of VraS (VraS H156A).

**Phosphatase activity based cross-talk between TCSs**

Most of the studies dedicated to analyze cross-talk between TCS have focused their efforts in the kinase activity of the TCS sensor proteins, and highlight that loss of the sensor leads to an inactive RR that can no longer modulate gene expression. In contrast, significantly less studies have focused their attention to the significance of sensor phosphatase activity, despite the fact that RR dephosphorylation is required to reset the signal transduction baseline state upon signal depletion and to prevent aberrant RR activity in the absence of signal (Gao and Stock, 2009). We used the set of plasmids containing individual HK and two derivatives of ΔXV, harboring a chromosomal copy of either *agrBDCA* or *srrAB* (Fig. 19), to investigate the relevance of the phosphatase activity of non-cognate HK to decrease the activation of a non-cognate RR (AgrA and SrrA). Phenotypic characterization of the ΔXIV strains complemented with single HK revealed that none of the HKs was able to alter the capacity of the strain to grow at 28ºC (regulated by SrrA) and the ability of the strain to produce hemolysins (regulated through AgrA) (Fig. 20). Overall, these results suggest that phosphatase activity of HK is specific for its cognate RR and it does not interfere with the activation of non-cognate RRs.

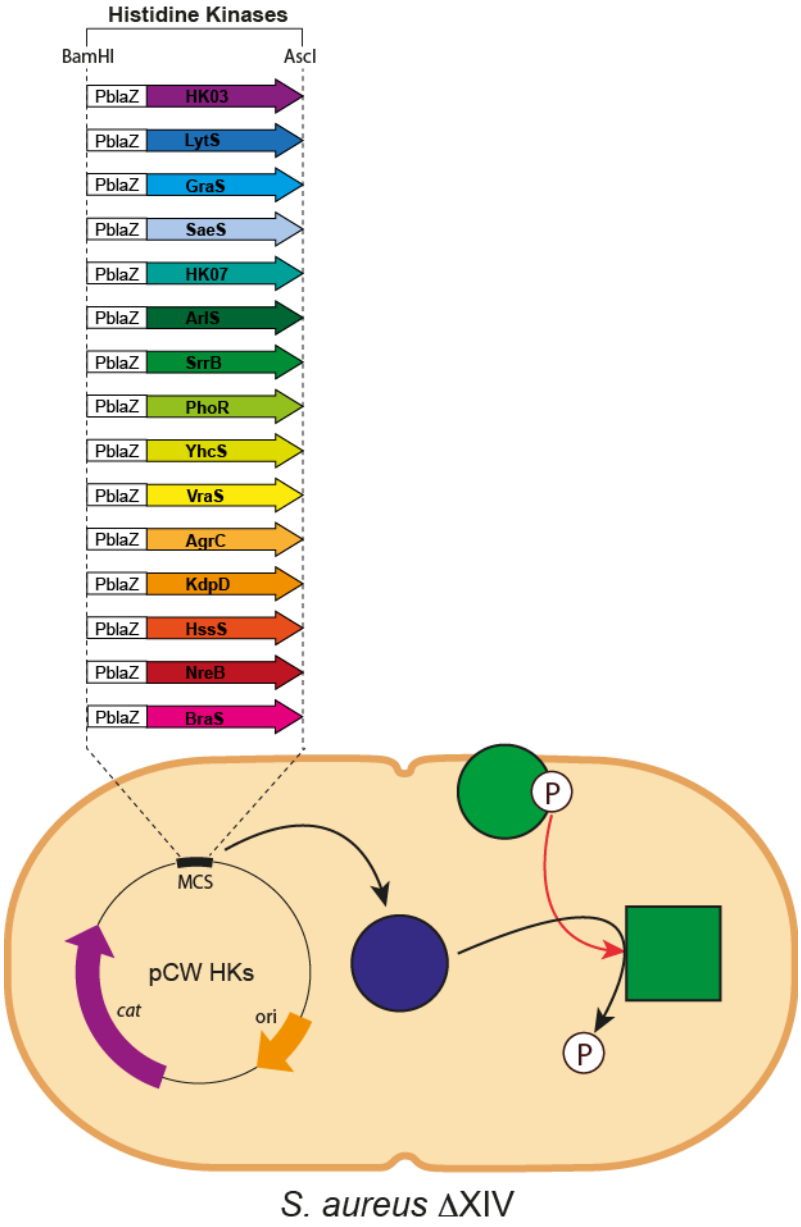


**Figure 19. Schematic representation of the strategy used to evaluate HKs phosphatase activity and cross-talk.** Two derivative strains of ΔXV, harbouring a chromosomal copy of either *agrBDCA* or *srrAB* were complemented with a set of plasmids producing single histidine kinases. Phenotypes associated to *agrA* or *srrA* were analyzed.

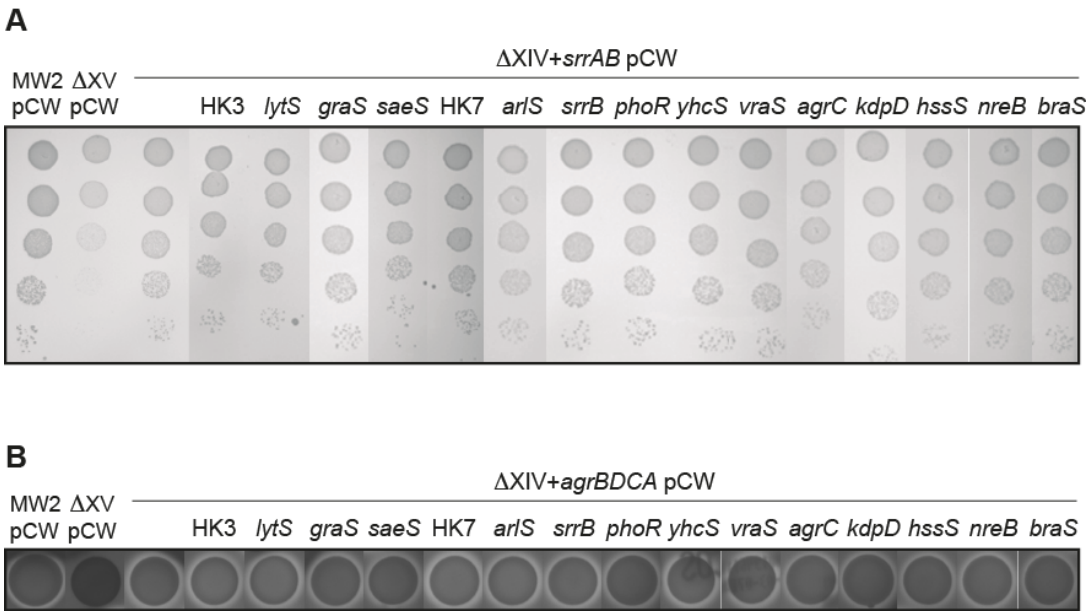


**Figure 20. HKs phosphatase activity and cross-talk.** (A) Bacterial growth on agar plates at 28ºC of ΔXIV mutant strain, harbouring the chromosomal copy of *srrAB,* complemented with a set of plasmids that contain individual histidine kinases. Bacterial cultures were serially diluted ($10^{-2}$, $10^{-4}$ and $10^{-6}$) and 5 μL of each dilution were spotted onto TSA plates to determine the number of surviving CFU. The plates were then incubated overnight at 28ºC. (B) Hemolysin production of ΔXIV mutant strain, harbouring a chromosomal copy of *agrBDCA*, complemented with a set of plasmids that contain individual histidine kinases, 5 μL of each culture were spotted onto hemolysis plates to determine the erythrocyte lysis halo. The plates were incubated overnight at 37ºC.

# *DISCUSSION*

# DISCUSSION

## High-resolution transcriptome analysis of *S. aureus*

At the beginning of this thesis, the laboratory was involved in the characterization of the entire collection of RNA molecules produced by *S. aureus*, which is currently known as the transcriptome. The analysis was performed combining two independent high-throughput technologies: microarray technology (tiling arrays) and sequencing the RNA pool (RNA-seq). Both techniques offer the advantage that the identification of the RNA content is not biased by protein-based genome annotation. The combination of both methods provides complementary information for the generation of a highly accurate transcriptome map. The results of the transcriptome can be visualized at the *S. aureus* transcriptome browser (http://staph. unavarra.es/) (Ruiz de Los Mozos *et al.*, 2013). For this thesis, we focus our attention on the transcriptome data related with the TCSs. The results showed that all TCSs are encoded in operons and most importantly that most of them are expressed under the conditions used to grow the bacteria in the laboratory. From a functional perspective, it appears reasonable that the sensorial machinery is constitutively expressed because it guarantees that the sensors will be ready to sense the stimuli in every environmental condition. However, the fact that TCSs are constitutively expressed does not mean that the expression of the TCS is not regulated. Indeed, the transcriptome uncovered the existence of transcriptional and post-transcriptional regulatory mechanisms for some TCSs. For instance, some

TCSs linked their expression with the neighbor genes (WalKR, TCS3, GraRS, SaeRS, TCS7, SrrAB, VraSR, AgrCA, NreBC) whereas others have specific promoters that uncoupled the expression between the sensor and response regulator (SrrAB). Furthermore, at least three TCSs are affected by the presence of antisense transcription (YhcSR, AgrCA, SaeRS). In this respect, the current methodology to perform transcriptome studies requires the used of micrograms of RNA that obviously can only be purified from millions of bacterial cells instead of RNA purified from a single bacterium. As a consequence, the presence of overlapping sense/antisense RNAs does not necessarily mean that both sense/antisense transcripts are simultaneously present in the same bacteria (Lasa *et al.*, 2012; Lasa and Villanueva, 2014). It might be possible that a subgroup in the bacterial population synthesized the sense transcript whereas another subgroup synthesized the antisense transcript, and consequently overlapping transcripts would never be together in the same cell. For the first time, our transcriptome analysis resolves this question and shows that both sense and antisense transcripts are present simultaneously in the same cell. This conclusion is supported by two findings. First, the presence of a collection of short RNA molecules that are symmetrically distributed in both strands of every genome region where overlapping transcription is detected. Second, RNase III is responsible for the digestion of the overlapping transcripts and the generation of the short RNA fractions. Because RNase III can only digest double stranded RNA, both sense and antisense transcripts have to be present simultaneously in the same cell to be digested by RNase III

(Lasa *et al.*, 2011). These results have been recently confirmed in a report by Lybecker *et al* (Lybecker *et al.*, 2014). Using a monoclonal antibody that recognizes double-stranded RNA molecules (dsRNA) irrespectively of the nucleotide sequence, the authors perform immunoprecipitation assays to pull down dsRNA molecules (IP-dsRNA) from a total RNA sample extracted from *E. coli*, and identified the purified dsRNA by RNA-seq. This study provides clear evidences that both sense and antisense transcripts are present simultaneously within the same bacterial cell and the amount of double stranded RNA increases significantly in the absence of RNase III.

In summary, the presence of antisense transcription in some TCSs strongly suggests that the expression of TCSs is also regulated at posttranscriptional level, though the consequence of this regulatory process remains unknown. Besides, transcriptome information has had enormous significance to design the deletion strategy of the TCSs in our genetic reductionist approach.

**Life without sensing in *S. aureus***

The term “signal transduction” has been typically reserved for the TCSs, however, according to the census of signal transduction proteins encoded in bacterial genomes at the NCBI (http://www.ncbi.nlm.nih.gov/Complete_Genomes/SignalCensus.html) (Galperin, 2010), *S. aureus* contains additional signal transduction systems that included: (i) GdpS, the only GGDEF domain protein with a conserved GGDEF motif (Tu Quoc *et al.*, 2007; Holland *et al.*, 2008); (ii) a c-di-AMP cyclase (Dac) and

phosphodiesterase (GpdP) (Corrigan *et al.*, 2011) and (iii) a Ser/Thr protein-kinase (Stk1) and two Ser/Thr protein-phosphatases (Stp1 and RsbU). Among these signal transducers, only GdpS and Stk1 contain transmembrane sensor domain capable to respond to extracelular signals. Biochemical analysis showed that GdpS does not synthesize c-di-GMP (Holland *et al.*, 2008). Thus, *S. aureus* lacks the c-di-GMP signaling pathway and therefore, the role of this protein in signal transduction remains unknown. In contrast, Stk1 contains three PASTA domains in the carboxy-terminal part that are suggested to interact with the cell-wall (Maestro *et al.*, 2011). Recent studies have indicated that Stk1 phosphorylates GraR to modulate D-Ala content in the teichoic acids and the cell-wall charge (Fridman *et al.*, 2013). In the absence of GraR, Stk1 dependent remodeling of cell wall is compromised. Hence, although the presence of Stk1 might question whether the elimination of all TCSs results in a senseless bacterium, the dependency of Stk1 signaling pathway on the presence of the TCS supports this designation.

This Doctoral Thesis aimed to construct a senseless bacteria (here we used *S. aureus* as a model) to determine whether a sensorial system is necessary when bacteria is growing under constant environmental conditions and also to establish the consequence of the removal of the complete TCS signaling for the bacterial biology. We used a genetic reductionist approach and generated a strain that is completely devoid of TCS signaling machinery. The removal was done in two steps. First, the fifteen non-essential TCSs were deleted from the chromosome (ΔXV) and

then, the expression of the remnant WalKR essential system was switched off in a strain producing ectopically *walR* under the control of a constitutive promoter. Thus, the resulting strain lacks all the sensor HKs and it contains a single RR (WalR). We carried out the deletion process in two genetically unrelated strains because it allows comparing the phenotypes caused by the gradual mutation of TCSs in both ΔXV strains. This strategy is extremely time consuming and demands an enormous effort but in our opinion is irreplaceable when using reductionist approaches because complementation of the final strain to restore the phenotypes is technically very difficult. We next confirmed that the phenotypes of the ΔXV mutant were not dependent on mutations generated during the sequential mutation process. For that, the genomes of the two ΔXV mutants and their corresponding wild type strains were completely sequenced *de novo*. This analysis confirms the absence of common polymorphisms in both ΔXV strains compared with their corresponding wild type parental strains. These results exclude that the phenotypes displayed by the ΔXV strains are due to presence of compensatory mutations generated during the sequential deletion process of the TCSs.

The first surprise in relation with the ΔXV and ΔXVI HK strains is the little phenotypic consequences that the absence of the TCS signaling has for the bacteria. The *S. aureus* ΔXV strains exhibited growth defect at 28ºC, decreased capacity for long-term survival during desiccation, lost the capacity to reduce nitrate to nitrite, showed higher autolysis rate in the presence of Triton X-100 and higher susceptible to some antibiotics. The

overall conclusion of these results is that under unstressed environmental conditions, *S. aureus* does not need a sensorial system. In contrast, when the environmental conditions are perturbed (presence of antibiotics, detergents, changes in temperature), the absence of TCSs impairs the proper adaptation to the new environmental condition and this has a fitness cost for the bacteria. These results are in agreement with the observation that those bacteria inhabiting stable host environments, such as obligate intracellular parasites, usually encode for few or even none signaling systems (Luke E Ulrich and Zhulin, 2010).

Interestingly, MW2 ΔXV strain did not show any deficiency in the adherence capacity of S. *aureus* to eukaryotic cells suggesting that the expression of adhesion-involved CWA proteins is not affected by the absence of TCSs. In contrast, the ΔXV mutant showed strong deficiency in the invasion of host cells and/or survival inside the host cells and consequently its virulence potential is clearly attenuated. These results confirm that growth conditions in host tissues are different from laboratory conditions and consequently *S. aureus* needs the TCS sensorial machinery to adapt to the host tissues. In this case, we have not tackled the question of whether it is one or various the TCSs responsible for the phenotype, mainly because this analysis requires the sacrifice of a high number of animals. With the aim of reducing the number of animals, we are planning to establish an alternative method inspire in the STM mutagenesis technique that would allow us to infect the animals simultaneously with the fifteen single mutants and the ΔXV strain (Hensel *et al.*, 1995). We aware of the

relevance that these results might have because in the case that a single TCS would be responsible for the virulence deficiency, this TCS would represent an excellent target for the development of new antimicrobials. TCSs are considered excellent targets for development o new antimicrobials for two reasons: TCSs are not present in mammalian cells and consequently drugs blocking this proteins will not have toxic effects. Besides, in opposition to conventional antibiotics that usually target proteins that carry out essential functions, TCSs are not essential, reducing the probability of selecting for drug resistant variants. Thus, there is a big hope that drugs that target TCSs required for growth may serve as new antibiotics that can kill multi-drug-resistant bacteria such as methicillin-resistant bacteria such as *S. aureus* MRSA strains (Dubrac and Msadek, 2008; Watanabe *et al.*, 2008; Gotoh *et al.*, 2010).

**Correlation between phenotypes and individual TCSs**

Because the ΔXV mutant is still genetically manageable, it provides an excellent platform for the systematic analysis of the phenotypes controlled by the TCS network. We have generated a collection of ΔXV derivatives, each of them producing from a plasmid a single TCS. This collection of strains has been used to analyze the phenotypes previously described in the ΔXV strain and to identify the TCSs responsible for each phenotype. This analysis is important because the regulon affected by a TCS is currently inferred from the phenotype and transcription profile displayed by the single mutant and restoration of the phenotype after complementation.

However, this approach cannot distinguish between those members of the regulon that are direct targets of the TCS from those targets whose expression depends indirectly on a second TCS. We used ΔXV mutant complemented with the battery of TCSs to analyze whether individual TCSs were able to restore the phenotypes characteristic of ΔXV strain. Interestingly, single TCSs are sufficient to restore the phenotypes of ΔXV strain, supporting the notion that TCS regulatory pathways are insulated from one another and little interdependency exists among them. This system-level insulation might explain why phenotypes of ΔXV strains correspond to the sum of phenotypes controlled by each TCS, without noticeable pleiotropic effects on other cellular processes. Following this reasoning and in relation with the virulence deficiency, it is likely that virulence attenuation could be due exclusively to the absence of a particular TCS.

**System-based analysis of cross-talk between TCSs**

Detailed system-level analysis with purified proteins have shown that specificity between HK and RR occurs through a small number of residues in the protein-protein interaction interface that provide strong kinetic preference to the HK for transferring or removing the phosphate from the cognate RR pair depending on its activation state (Skerker *et al.*, 2005; Yamamoto *et al.*, 2005; Laub *et al.*, 2007; Procaccini *et al.*, 2011). There has been a general interest in determining whether a similar specificity in the TCS signaling exists *in vivo*. However, technical difficulties due to the

presence of dozens of TCSs in the same bacterial cell have impaired to answer this question through global system-level approaches. Instead, cross regulation studies have been focused on specific TCS partners (Wanner *et al.*, 1988; Amemura *et al.*, 1990; Wanner, 1992; Matsubara *et al.*, 2000; Stewart, 2003; Howell *et al.*, 2006; Siryaporn and Goulian, 2008; Goodman *et al.*, 2009; Guckes *et al.*, 2013; Mike *et al.*, 2014). The general conclusion of these studies has been that cross-talk between non-cognate HK-RR pairs is possible, but rare.

We have used the ΔXV strain together with the knowledge of the phenotypes activated by each TCS to perform a systematic analysis of cross-talk *in vivo*. First, we analyzed the capacity of a RR to activate the corresponding phenotype in the single mutant compared to the ΔXV strain. This simple strategy allows the rapid identification of those RRs that are activated in the presence of non-cognate HKs. We focused on those RRs for which a clear phenotype in the ΔXV strain was detected. In agreement with previous studies showing that cross-talk between TCS is rare, only *arlR*, among the response regulators analyzed, was activated by non-cognate histidine kinases. Second, we searched for the histidine kinases capable to activate *arlR* by complementing the ΔXV strain with a set of plasmids containing the combination of *arlR* and the whole family of histidine kinases. Because ArlRS regulates Protein A production (Fournier *et al.*, 2001), we used this protein as a sensitive and easily detectable target to identify HKs able to activate ArlR. The results showed that GraS was able to activate ArlR in the conditions tested. Both systems, ArlS and

GraS, have been involved in the regulation of bacterial autolysis (Fournier and Hooper, 2000; Meehl *et al.*, 2007). Thus, our results indicate that these TCSs are interconnected through a cross-phosphorylation event to modulate bacterial autolysis (Fig. 21). The fact that cross-talk between GraS and ArlR can occur in the presence of ArlS indicates that the phosphatase activity of ArlS cannot prevent the phosphorylation of ArlR by GraS. Then, we tested the biological relevance of the cross-talk between GraS and ArlR by analyzing whether a natural stimuli that activates GraS was able to activate ArlR–mediated regulation of Protein A. The results showed that activation of GraS with colistin increased the production of Protein A in the wild type and *arlRS* mutant complemented with ArlR, whereas the levels remain similar in the GraRS mutant independently of the presence of *arlR*. We know that ArlR is responsible for the inhibition of the autolysis process (Fournier and Hooper, 2000), which needs to be extremely well regulated to avoid unnecessary bacterial death, whereas GraS is an intramembrane-sensing HK that senses Cationic Antimicrobial Peptides. Sequence identity between GraS and ArlS is 30% or 29% when the whole protein or the HK domain is considered, respectively. Assigning functions to RRs based on sequence similarity is problematic. In contrast, family assignments of RRs based on their domain architectures are relatively robust and provide a good orientation of their function. Although GraS and ArlS belong to the HisKA family of HKs they do not share the 5 key residues in the $\alpha$1 helix responsible for the specificity (Fig. 22).

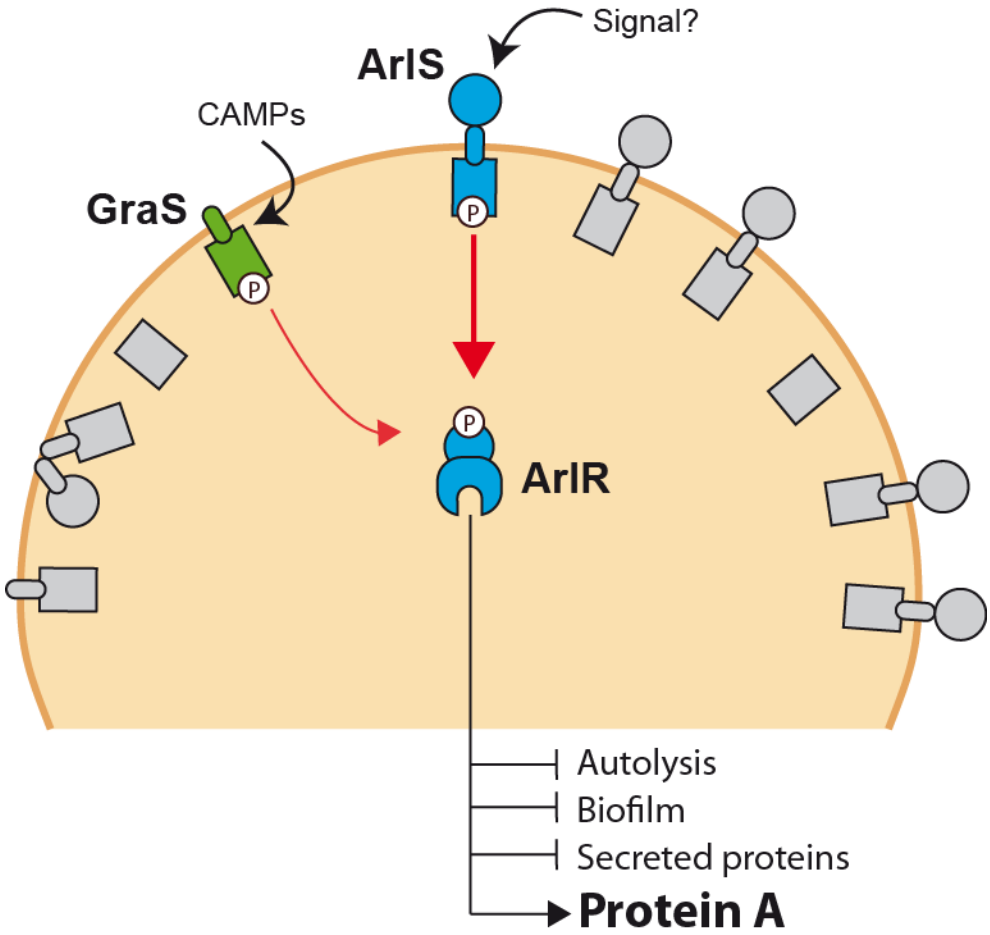


**Figure 21. Schematic representation of cross-activation of ArlR by GraS.** ArlR regulates many relevant cellular functions as autolysis, biofilm formation or protein secretion. ArlR is activated mainly by its cognate partner ArlS, but under the presence of specific environmental stimuli, such as the presence of antimicrobial cationic peptides (CAMP), it can be activated also by GraS.

One mechanism to preserve the specificity and avoid cross-activations is the phosphatase activity of the HKs. This activity is responsible for the dephosphorylation of the RR when the stimulus is over. Also, it aids to eliminate the phosphorylation from unspecific sources (Siryaporn and Goulian, 2008). Accordingly, our results indicate that cross-activation of VraR by YhcS disappeared in the presence of the VraS.

Interestingly, VraS and YhcS belong to the HisKA_3 family of HKs and share 5 of the key residues in the $\alpha$1 helix responsible for the specificity (Fig. 22) (Guckes *et al.*, 2013), it is tempting to speculate that both TCS have recently evolve from the same ancestral and the proteins have not yet been diversified (Capra and Laub, 2012; Capra *et al.*, 2012).

In summary, these studies provide the first example of a free-living bacterium in which the whole TCS signalling system has been removed. This strategy is especially useful to elucidate the connectivity between environmental signals, the sensor responsible for its perception and the set of regulated genes. Also, it may aid to carry out studies to investigate the molecular mechanisms that govern the specificity between sensors and RRs and to understand the interdependency between different TCSs. From a biotechnological point of view, the senseless *S. aureus* mutant can be an ideal bacterial chassis for synthetic biology applications (Nikel *et al.*, 2014).

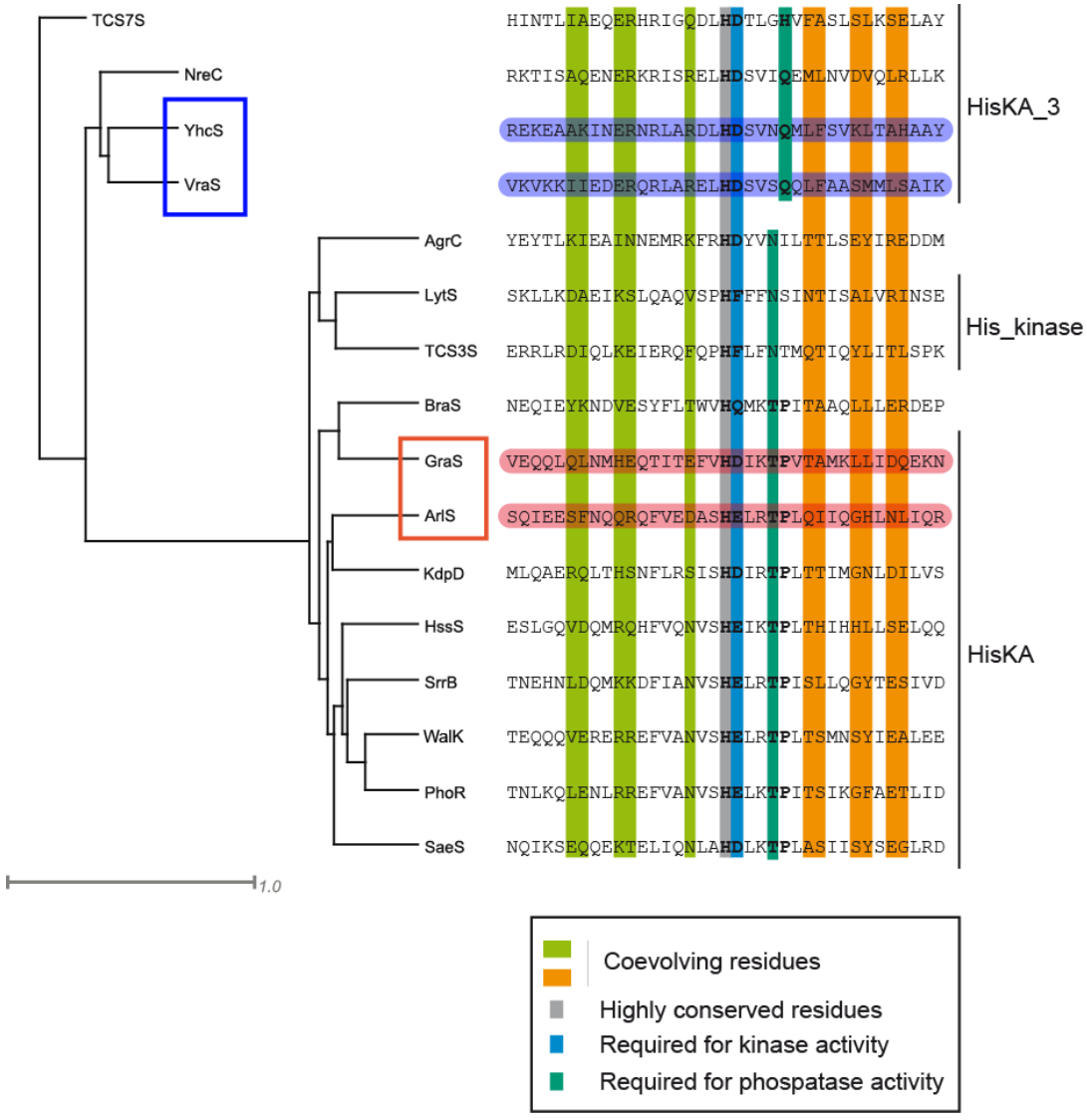


**Figure 22. Phylogenetic tree of the *S. aureus* histidine kinase DHp domains.** Both YhcS and VraS share common specificity residues. GraS and ArlS have not common specificity residues.

*S. aureus* ΔXV has a genome that encodes the basic biological functions required for self-maintenance and growth, but is deleted of signal-sensing components that divert resources. Under natural circumstances, signaling functions enable microorganisms to interact with their environment, but they may not be required in the synthetic biology laboratory or in biotechnological settings. *S. aureus* ΔXV is robust, stable (low spontaneous variability) and easily amenable to genetic manipulations. Thus, *S. aureus* ΔXV can be engineered to create novel connections between stimuli and gene expression networks to develop different types of useful engineered cells (Ninfa, 2010). For example, using inducible regulatory elements (such as riboswitches, thermosensors, or transcriptional regulators) to control the expression of WalKR would allow us to create a biosafe *S. aureus* strain able to grow only in presence of the corresponding inductor.

Reductionist approaches have been previously used to disentangle the complex c-di-GMP dependent signal transduction pathway in *Salmonella enteritidis* and *Caulobacter crescentus* (Solano *et al.*, 2009; Abel *et al.*, 2013). As tools for the genetic manipulation of bacterial chromosomes developed, this strategy will be instrumental to understand the function of complex TCS signaling networks in different bacteria.

# *CONCLUSIONS*

# CONCLUSIONS

1. The transcriptomic analysis has shown that *S. aureus* TCSs are often transcribed constitutively and together with the neighbour genes.

2. The complete deletion of the *S. aureus* TCS sensorial system neither affects bacterial viability, nor the capacity of the bacteria to grow under constant environmental conditions. The strains devoid of TCS sensorial system show growth rates at 37ºC and 44ºC similar to the wild type strain and their capacity to metabolize different carbon sources remains intact.

3. The two-component sensorial system is necessary when *S. aureus* grows in environmental conditions distinct from those at the laboratory. The strains devoid of TCSs have lower growth rates at 28ºC, higher sensitivity to detergents, show reduced capacity to survive in the environment and lose the capacity to reduce nitrate to nitrite.

4. Related to virulence, *S. aureus* needs the TCS sensorial system to survive inside the host and cause the infection. In the absence of TCSs bacteria is unable to invade and/or survive within eukaryotic cells and colonize organs, rendering the bacteria avirulent.

5. *S. aureus* strains devoid of TCS sensorial system provide a platform to study the biological processes regulated by each two-component

system and to study the specificity on the signal transmission (cross-talk) between different two-component systems.

6. Phenotypic analysis of a collection of *S. aureus* strains harbouring a single TCS revealed that each two-component system is sufficient for the activation of its regulon and the corresponding phenotypes. This situation explains why deficiencies in the strain devoid of TCS sensorial system are the sum of phenotypes regulated by individual TCS.

7. We have used the strain devoid of TCS to develope a simple methodology to carry out systematic studies on the cross-activation or cross-talk between TCSs *in vivo*. The results have shown that under the environmental conditions used in this study and for the phenotypes analyzed, only GraS is able to cross-activate ArlR RR. The activation of ArlR by GraS in the presence of a natural stimulus occurs even in the presence of the cognate ArlS phosphatase activity and GraR phosphorylation substrate.

# *CONCLUSIONES*

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1. El análisis transcriptómico ha mostrado que los sistemas de dos-componentes forman con frecuencia unidades de transcripción con los genes adyacentes y su expresión es constitutiva.

2. La deleción completa del sistema sensorial de *S. aureus*, mediante mutación de los 16 TCSs, no afecta a la viabilidad de la bacteria y a su capacidad de crecimiento bajo condiciones ambientales constantes. Las cepas que carecen del sistema sensorial tienen una velocidad de crecimiento similar a los de la cepa salvaje a 37ºC y 44ºC y su capacidad para metabolizar distintas fuentes de carbono se mantiene intacta.

3. El sistema sensorial es necesario cuando *S. aureus* crece en condiciones ambientales distintas a las de laboratorio. Las cepas que carecen de TCSs tienen ralentizado el crecimiento a 28ºC, muestran menor capacidad de supervivencia en ausencia de nutrientes, son más sensibles a la presencia de detergentes y pierden la capacidad de reducir los nitratos a nitritos.

4. En relación con la virulencia, los TCSs son necesarios para que *S. aureus* pueda sobrevivir en el huesped y causar infección. En ausencia del sistema sensorial, la bacteria no puede invadir o sobrevivir en el interior de las células eucariotas, colonizar órganos y en consecuencia las bacteria es avirulenta.

5. Las cepas de *S. aureus* deficientes en el sistema sensorial de dos-componentes proporcionan una plataforma para el estudio de los procesos biológicos que regula cada TCS y para el estudio de la especificidad en la transmisión de la señal (cross-talk) entre distintos TCS.

6. El análisis fenotípico de una colección de cepas de *S. aureus* que contiene un único TCS nos ha permitido analizar que cada TCS es suficiente para activar de forma autónoma su regulón, sin que exista interdependencia entre los mismos. Esta situación explica porque los fenotipos que muestra el mutante deficiente en el sistema sensorial de dos-componentes sea la suma de fenotipos que regulan cada uno de los TCSs.

7. Hemos desarrollado una metodología sencilla para realizar estudios sistematicos de la existencia de activación cruzada o *cross-talk in vivo* utilizando la cepa deficiente en el sistema sensorial de dos-componentes. Los resultados han mostrado que en las condiciones ambientales estudiadas y para los fenotipos analizados mayoritariamente existe activación cruzada entre el sensor GraS y el regulador de respuesta ArlR. La activación de GraS por un estímulo natural es capaz de activar ArlR en presencia de la actividad fosfatasa de AlrS y de su sustrato de fosforilación directo, GraR.

# *REFERENCES*

# REFERENCES

Abel, S., Bucher, T., Nicollier, M., Hug, I., Kaever, V., Abel Zur Wiesch, P., and Jenal, U. (2013) Bi-modal distribution of the second messenger c-di-GMP controls cell fate and asymmetry during the *caulobacter* cell cycle. *PLoS Genet* **9**: e1003744.

Amemura, M., Makino, K., Shinagawa, H., and Nakata, A. (1990) Cross talk to the phosphate regulon of *Escherichia coli* by PhoM protein: PhoM is a histidine protein kinase and catalyzes phosphorylation of PhoB and PhoM-open reading frame 2. *J Bacteriol* **172**: 6300–6307.

Arnaud, M., Chastanet, A., and Débarbouillé, M. (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Appl Environ Microbiol* **70**: 6887–6891.

Augustin, J., Rosenstein, R., Wieland, B., Schneider, U., Schnell, N., Engelke, G., *et al.* (1992) Genetic analysis of epidermin biosynthetic genes and epidermin-negative mutants of *Staphylococcus epidermidis. Eur J Biochem* **204**: 1149–1154.

Ausmees, N., Mayer, R., Weinhouse, H., Volman, G., Amikam, D., Benziman, M., and Lindberg, M. (2001) Genetic data indicate that proteins containing the GGDEF domain possess diguanylate cyclase activity. *FEMS Microbiol Lett* **204**: 163–167.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K.-I., Oguchi, A., *et al.* (2002) Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* **359**: 1819–1827.

Barrick, J.E., Yu, D.S., Yoon, S.H., Jeong, H., Oh, T.K., Schneider, D., *et al.* (2009) Genome evolution and adaptation in a long-term experiment with Escherichia coli. *Nature* **461**: 1243–1247.

Batchelor, J.D., Doucleff, M., Lee, C.-J., Matsubara, K., De Carlo, S., Heideker, J., *et al.* (2008) Structure and regulatory mechanism of Aquifex aeolicus NtrC4: variability and evolution in bacterial transcriptional regulation. *J Mol Biol* **384**: 1058–1075.

Beenken, K.E., Dunman, P.M., McAleese, F., Macapagal, D., Murphy, E., Projan, S.J., *et al.* (2004) Global gene expression in *Staphylococcus aureus* biofilms. *J Bacteriol* **186**: 4665–4684.

Bilwes, A.M., Alex, L.A., Crane, B.R., and Simon, M.I. (1999) Structure of CheA, a signal-transducing histidine kinase. *Cell* **96**: 131–141.

Blake, K.L., Randall, C.P., and O'Neill, A.J. (2011) In vitro studies indicate a high resistance potential for the lantibiotic nisin in *Staphylococcus aureus* and define a genetic basis for nisin resistance. *Antimicrob Agents Ch* **55**: 2362–2368.

Boisset, S., Geissmann, T., Huntzinger, E., Fechter, P., Bendridi, N., Possedko, M., *et al.* (2007) *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev* **21**: 1353–1366.

Brunskill, E.W., and Bayles, K.W. (1996a) Identification and molecular characterization of a putative regulatory locus that affects autolysis in *Staphylococcus aureus. J Bacteriol* **178**: 611–618.

Brunskill, E.W., and Bayles, K.W. (1996b) Identification of LytSR-regulated genes from *Staphylococcus aureus. J Bacteriol* **178**: 5810–5812.

Capra, E.J., and Laub, M.T. (2012) Evolution of two-component signal transduction systems. *Annu Rev Microbiol* **66**: 325–347.

Capra, E.J., Perchuk, B.S., Skerker, J.M., and Laub, M.T. (2012) Adaptive mutations that prevent crosstalk enable the expansion of paralogous signaling protein families. *Cell* **150**: 222–232.

Casino, P., Rubio, V., and Marina, A. (2009) Structural Insight into Partner Specificity and Phosphoryl Transfer in Two-Component Signal Transduction. *Cell* **139**: 325–336.

Casino, P., Rubio, V., and Marina, A. (2010) The mechanism of signal transduction by two-component systems. *Curr Opin Struc Biol* **20**: 763–771.

Charpentier, E., Anton, A.I., Barry, P., Alfonso, B., Fang, Y., and Novick, R.P. (2004) Novel cassette-based shuttle vector system for gram-positive bacteria. *Appl Environ Microbiol* **70**: 6076–6085.

Chaudhuri, R.R., Allen, A.G., Owen, P.J., Shalom, G., Stone, K., Harrison, M., *et al.* (2009) Comprehensive identification of essential *Staphylococcus aureus* genes using Transposon-Mediated Differential Hybridisation (TMDH). *BMC Genomics* **10**: 291.

Chavakis, T., Hussain, M., Kanse, S.M., Peters, G., Bretzel, R.G., Flock, J.-I., *et al.* (2002) *Staphylococcus aureus* extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes. *Nat Med* **8**: 687–693.

Cluzel, M.-E., Zanella-Cléon, I., Cozzone, A.J., Fütterer, K., Duclos, B., and Molle, V. (2010) The *Staphylococcus aureus* autoinducer-2 synthase LuxS is regulated by Ser/Thr phosphorylation. *J Bacteriol* **192**: 6295–6301.

Corrigan, R.M., Abbott, J.C., Burhenne, H., Kaever, V., and Gründling, A. (2011) c-di-AMP is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress. *PLoS Pathog* **7**: e1002217.

Corrigan, R.M., Campeotto, I., Jeganathan, T., Roelofs, K.G., Lee, V.T., and Gründling, A. (2013) Systematic identification of conserved bacterial c-di-AMP receptor proteins. *Proc Natl Acad Sci USA* **110**: 9084–9089.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M. (1995) Microbial biofilms. *Annu Rev Microbiol* **49**: 711–745.

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., and Penadés, J.R. (2001) Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol* **183**: 2888–2896.

D'Argenio, D.A., and Miller, S.I. (2004) Cyclic di-GMP as a bacterial second messenger. *Microbiology* **150**: 2497–2502.

de Haas, C.J.C., Veldkamp, K.E., Peschel, A., Weerkamp, F., Van Wamel, W.J.B., Heezius, E.C.J.M., *et al.* (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J Exp Med* **199**: 687–695.

de Jonge, B.L., de Lencastre, H., and Tomasz, A. (1991) Suppression of autolysis and cell wall turnover in heterogeneous Tn551 mutants of a methicillin-resistant *Staphylococcus aureus* strain. *J Bacteriol* **173**: 1105–1110.

Delaune, A., Dubrac, S., Blanchet, C., Poupel, O., Mäder, U., Hiron, A., *et al.* (2012) The WalKR system controls major staphylococcal virulence genes and is involved in triggering the host inflammatory response. *Infec Immun* **80**: 3438–3453.

Delaune, A., Poupel, O., Mallet, A., Coic, Y.-M., Msadek, T., and Dubrac, S. (2011) Peptidoglycan Crosslinking Relaxation Plays an Important Role in *Staphylococcus aureus* WalKR-Dependent Cell Viability. *PLoS ONE* **6**: e17054.

Débarbouillé, M., Dramsi, S., Dussurget, O., Nahori, M.-A., Vaganay, E., Jouvion, G., *et al.* (2009) Characterization of a serine/threonine kinase involved in virulence of *Staphylococcus aureus*. *J Bacteriol* **191**: 4070–4081.

Donat, S., Streker, K., Schirmeister, T., Rakette, S., Stehle, T., Liebeke, M., *et al.* (2009) Transcriptome and functional analysis of the eukaryotic-type serine/threonine kinase PknB in *Staphylococcus aureus*. *J Bacteriol* **191**: 4056–4069.

Dubrac, S., and Msadek, T. (2004) Identification of genes controlled by the essential YycG/YycF two-component system of *Staphylococcus aureus*. *J Bacteriol* **186**: 1175–1181.

Dubrac, S., and Msadek, T. (2008) Tearing down the wall: Peptidoglycan metabolism and the WalK/WalR (YycG/YycF) essential two-component system. *Adv Exp Med Biol* **631**: 214–228.

Dubrac, S., Bisicchia, P., Devine, K.M., and Msadek, T. (2008) A matter of life and death: cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. *Mol Microbiol* **70**: 1307–1322.

Dubrac, S., Boneca, I.G., Poupel, O., and Msadek, T. (2007) New Insights into the WalK/WalR (YycG/YycF) Essential Signal Transduction Pathway Reveal a Major Role in Controlling Cell Wall Metabolism and Biofilm Formation in *Staphylococcus aureus*. *J Bacteriol* **189**: 8257–8269.

Dunman, P.M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S., *et al.* (2001) Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. *J Bacteriol* **183**: 7341–7353.

Dutta, R., Qin, L., and Inouye, M. (1999) Histidine kinases: diversity of domain organization. *Mol Microbiol* **34**: 633–640.

Eiff, von, C., Heilmann, C., Proctor, R.A., Woltz, C., Peters, G., and Gotz, F. (1997) A site-directed *Staphylococcus aureus* hemB mutant is a small-colony variant which persists intracellularly. *J Bacteriol* **179**: 4706–4712.

Falord, M., Karimova, G., Hiron, A., and Msadek, T. (2012) GraXSR proteins interact with the VraFG ABC transporter to form a five-component system required for cationic antimicrobial peptide sensing and resistance in *Staphylococcus aureus*. *Antimicrob Agents Ch* **56**: 1047–1058.

Falord, M., Mäder, U., Hiron, A., Dbarbouillé, M., and Msadek, T. (2011) Investigation of the *Staphylococcus aureus* GraSR Regulon Reveals Novel Links to Virulence, Stress Response and Cell Wall Signal Transduction Pathways. *PLoS ONE* **6**: e21323.

Foster, T.J., and Höök, M. (1998) Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol* **6**: 484–488.

Foster, T.J., Geoghegan, J.A., Ganesh, V.K., and Höök, M. (2014) Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. *Nat Rev Microbiol* **12**: 49–62.

Fournier, B., and Hooper, D.C. (2000) A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. *J Bacteriol* **182**: 3955–3964.

Fournier, B., Klier, A., and Rapoport, G. (2001) The two-component system ArlS-ArlR is a regulator of virulence gene expression in *Staphylococcus aureus*. *Mol Microbiol* **41**: 247–261.

Freeman, Z.N., Dorus, S., and Waterfield, N.R. (2013) The KdpD/KdpE Two-Component System: Integrating K+ Homeostasis and Virulence. *PLoS Pathog* **9**: e1003201.

Fridman, M., Williams, G.D., Muzamal, U., Hunter, H., Siu, K.W.M., and Golemi-Kotra, D. (2013) Two unique phosphorylation-driven signaling pathways crosstalk in *Staphylococcus aureus* to modulate the cell-wall charge: Stk1/Stp1 meets GraSR. *Biochemistry* **52**: 7975–7986.

Friedman, D.B., Stauff, D.L., Pishchany, G., Whitwell, C.W., Torres, V.J., and Skaar, E.P. (2006) *Staphylococcus aureus* redirects central metabolism to increase iron availability. *PLoS Pathog* **2**: e87.

Fukushima, T., Szurmant, H., Kim, E.-J., Perego, M., and Hoch, J.A. (2008) A sensor histidine kinase co-ordinates cell wall architecture with cell division in *Bacillus subtilis*. *Mol Microbiol* **69**: 621–632.

Galperin, M.Y. (2010) Diversity of structure and function of response regulator output domains. *Curr Opin Microbiol* **13**: 150–159.

Galperin, M.Y., Nikolskaya, A.N., and Koonin, E.V. (2001) Novel domains of the prokaryotic two-component signal transduction systems. *FEMS Microbiol Lett* **203**: 11–21.

Gao, R., and Stock, A.M. (2009) Biological insights from structures of two-component proteins. *Annu Rev Microbiol* **63**: 133–154.

Gardete, S., Wu, S.W., Gill, S., and Tomasz, A. (2006) Role of VraSR in Antibiotic Resistance and Antibiotic-Induced Stress Response in *Staphylococcus aureus*. *Antimicrob Agents Ch* **50**: 3424–3434.

Geiger, T., Goerke, C., Mainiero, M., Kraus, D., and Wolz, C. (2008) The virulence regulator Sae of Staphylococcus aureus: promoter activities and response to phagocytosis-related signals. *J Bacteriol* **190**: 3419–3428.

Giraudo, A.T., Raspanti, C.G., Calzolari, A., and Nagel, R. (1994) Characterization of a Tn551-mutant of *Staphylococcus aureus* defective in the production of several exoproteins. *Can J Microbiol* **40**: 677–681.

Goodman, A.L., Merighi, M., Hyodo, M., Ventre, I., Filloux, A., and Lory, S. (2009) Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. *Genes Dev* **23**: 249–259.

Gordon, R.J., and Lowy, F.D. (2008) Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin Infect Dis* **46 Suppl 5**: S350–9.

Gotoh, Y., Eguchi, Y., Watanabe, T., Okamoto, S., Doi, A., and Utsumi, R. (2010) Two-component signal transduction as potential drug targets in pathogenic bacteria. *Curr Opin Microbiol* **13**: 232–239.

Gresham, H.D., Lowrance, J.H., Caver, T.E., Wilson, B.S., Cheung, A.L., and Lindberg, F.P. (2000) Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *J Immunol* **164**: 3713–3722.

Groban, E.S., Clarke, E.J., Salis, H.M., Miller, S.M., and Voigt, C.A. (2009) Kinetic Buffering of Cross Talk between Bacterial Two-Component Sensors. *J Mol Biol* **390**: 380–393.

Groicher, K.H., Firek, B.A., Fujimoto, D.F., and Bayles, K.W. (2000) The *Staphylococcus aureus lrgAB* operon modulates murein hydrolase activity and penicillin tolerance. *J Bacteriol* **182**: 1794–1801.

Guckes, K.R., Kostakioti, M., Breland, E.J., Gu, A.P., Shaffer, C.L., Martinez, C.R., *et al.* (2013) Strong cross-system interactions drive the activation of the QseB response regulator in the absence of its cognate sensor. *Proc Natl Acad Sci USA*.

Hanssen, A.-M., and Ericson Sollid, J.U. (2006) SCCmec in staphylococci: genes on the move. *FEMS Immunol Med Microbiol* **46**: 8–20.

Helmann, J.D. (2002) The extracytoplasmic function (ECF) sigma factors. *Adv Microb Physiol* **46**: 47–110.

Hensel, M., Shea, J.E., Gleeson, C., Jones, M.D., Dalton, E., and Holden, D.W. (1995) Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**: 400–403.

Herbert, S., Bera, A., Nerz, C., Kraus, D., Peschel, A., Goerke, C., *et al.* (2007) Molecular Basis of Resistance to Muramidase and Cationic Antimicrobial Peptide Activity of Lysozyme in Staphylococci. *PLoS Pathog* **3**: e102.

Hiron, A., Falord, M., Valle, J., Débarbouillé, M., and Msadek, T. (2011) Bacitracin and nisin resistance in *Staphylococcus aureus*: a novel pathway involving the BraS/BraR two-component system (SA2417/SA2418) and both the BraD/BraE and VraD/VraE ABC transporters. *Mol Microbiol* **81**: 602–622.

Holland, L.M., O'Donnell, S.T., Ryjenkov, D.A., Gomelsky, L., Slater, S.R., Fey, P.D., *et al.* (2008) A staphylococcal GGDEF domain protein regulates biofilm formation independently of cyclic dimeric GMP. *J Bacteriol* **190**: 5178–5189.

Howell, A., Dubrac, S., Noone, D., Varughese, K.I., and Devine, K. (2006) Interactions between the YycFG and PhoPR two-component systems in *Bacillus subtilis*: the PhoR kinase phosphorylates the non-cognate YycF response regulator upon phosphate limitation. *Mol Microbiol* **59**: 1199–1215.

Hsing, W., and Silhavy, T.J. (1997) Function of conserved histidine-243 in phosphatase activity of EnvZ, the sensor for porin osmoregulation in Escherichia coli. *J Bacteriol* **179**: 3729–3735.

Huynh, T.N., Noriega, C.E., and Stewart, V. (2010) Conserved mechanism for sensor phosphatase control of two-component signaling revealed in the nitrate sensor NarX. *Proc Natl Acad Sci USA* **107**: 21140–21145.

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S., and Ciofu, O. (2010) Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Ag* **35**: 322–332.

Janzon, L., Löfdahl, S., and Arvidson, S. (1989) Identification and nucleotide sequence of the delta-lysin gene, hld, adjacent to the accessory gene regulator (*agr*) of *Staphylococcus aureus*. *Mol Gen Genet* **219**: 480–485.

Jenal, U. (2004) Cyclic di-guanosine-monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria? *Curr Opin Microbiol* **7**: 185–191.

Jenal, U., and Malone, J. (2006) Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu Rev Genet* **40**: 385–407.

Jeong, D.-W., Cho, H., Jones, M.B., Shatzkes, K., Sun, F., Ji, Q., *et al.* (2012) The auxiliary protein complex SaePQ activates the phosphatase activity of sensor kinase SaeS in the SaeRS two-component system of Staphylococcus aureus. *Mol Microbiol* **86**: 331-348.

Jones, R.N. (2008) Key considerations in the treatment of complicated staphylococcal infections. *Clin Microbiol Infect* **14 Suppl 2**: 3–9.

Jung, K., Fried, L., Behr, S., and Heermann, R. (2012) Histidine kinases and response regulators in networks. *Curr Opin Microbiol* **15**: 118–124.

Kamps, A., Achebach, S., Fedtke, I., Unden, G., and Götz, F. (2004) Staphylococcal NreB: an O(2)-sensing histidine protein kinase with an O(2)-labile iron-sulphur cluster of the FNR type. *Mol Microbiol* **52**: 713–723.

Kavanaugh, J.S., Thoendel, M., and Horswill, A.R. (2007) A role for type I signal peptidase in *Staphylococcus aureus* quorum sensing. *Mol Microbiol* **65**: 780–798.

Kennedy, A.D., Otto, M., Braughton, K.R., Whitney, A.R., Chen, L., Mathema, B., *et al.* (2008) Epidemic community-associated methicillin-resistant *Staphylococcus aureus*: recent clonal expansion and diversification. *Proc Natl Acad Sci USA* **105**: 1327–1332.

Keshav, S., Chung, P., Milon, G., and Gordon, S. (1991) Lysozyme is an inducible marker of macrophage activation in murine tissues as demonstrated by in situ hybridization. *J Exp Med* **174**: 1049–1058.

Kinkel, T.L., Roux, C.M., Dunman, P.M., and Fang, F.C. (2013) The *Staphylococcus aureus* SrrAB Two-Component System Promotes Resistance to Nitrosative Stress and Hypoxia. *mBio* **4**.

Klevens, R.M., Morrison, M.A., Nadle, J., Petit, S., Gershman, K., Ray, S., *et al.* (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *Jama* **298**: 1763–1771.

Kluytmans, J., van Belkum, A., and Verbrugh, H. (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* **10**: 505–520.

Koenig, R.L., Ray, J.L., Maleki, S.J., Smeltzer, M.S., and Hurlburt, B.K. (2004) *Staphylococcus aureus* AgrA binding to the RNAIII-agr regulatory region. *J Bacteriol* **186**: 7549–7555.

Kolar, S.L., Nagarajan, V., Oszmiana, A., Rivera, F.E., Miller, H.K., Davenport, J.E., *et al.* (2011) NsaRS is a cell-envelope-stress-sensing two-component system of *Staphylococcus aureus*. *Microbiology* **157**: 2206–2219.

Kuroda, H., Kuroda, M., Cui, L., and Hiramatsu, K. (2007) Subinhibitory concentrations of beta-lactam induce haemolytic activity in *Staphylococcus aureus* through the SaeRS two-component system. *FEMS Microbiol Lett* **268**: 98–105.

Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H., and Hiramatsu, K. (2003) Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Mol Microbiol* **49**: 807–821.

Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., *et al.* (2001) Whole genome sequencing of meticillin-resistant *Staphylococcus aureus. Lancet* **357**: 1225–1240.

Labandeira-Rey, M., Couzon, F., Boisset, S., Brown, E.L., Bes, M., Benito, Y., *et al.* (2007) *Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia. *Science* **315**: 1130–1133.

Lang, S., Livesley, M.A., Lambert, P.A., Littler, W.A., and Elliott, T.S. (2000) Identification of a novel antigen from *Staphylococcus epidermidis. FEMS Immunol Med Microbiol* **29**: 213–220.

Lasa, I., and Villanueva, M. (2014) Overlapping transcription and bacterial RNA removal. *Proc Natl Acad Sci USA* **111**: 2868–2869.

Lasa, I., Toledo-Arana, A., and Gingeras, T.R. (2012) An effort to make sense of antisense transcription in bacteria. *RNA Biol* **9**: 1039–1044.

Lasa, I., Toledo-Arana, A., Dobin, A., Villanueva, M., de los Mozos, I.R., Vergara-Irigaray, M., *et al.* (2011) Genome-wide antisense transcription drives mRNA processing in bacteria. *Proc Natl Acad Sci USA* **108**: 20172–20177.

Laub, M.T., and Goulian, M. (2007) Specificity in Two-Component Signal Transduction Pathways. *Annu Rev Genet* **41**: 121–145.

Laub, M.T., Biondi, E.G., and Skerker, J.M. (2007) Phosphotransfer profiling: systematic mapping of two-component signal transduction pathways and phosphorelays. *Meth Enzymol* **423**: 531–548.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., *et al.* (2009) The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**: 2078–2079.

Li, M., Cha, D.J., Lai, Y., Villaruz, A.E., Sturdevant, D.E., and Otto, M. (2007) The antimicrobial peptide-sensing system aps of *Staphylococcus aureus*. *Mol Microbiol* **66**: 1136–1147.

Li, M., Lai, Y., Villaruz, A.E., Cha, D.J., Sturdevant, D.E., and Otto, M. (2007) Gram-positive three-component antimicrobial peptide-sensing system. *Proc Natl Acad Sci USA* **104**: 9469–9474.

Liang, X., Yu, C., Sun, J., Liu, H., Landwehr, C., Holmes, D., and Ji, Y. (2006) Inactivation of a two-component signal transduction system, SaeRS, eliminates adherence and attenuates virulence of *Staphylococcus aureus*. *Infec Immun* **74**: 4655–4665.

Liebeke, M., Meyer, H., Donat, S., Ohlsen, K., and Lalk, M. (2010) A metabolomic view of *Staphylococcus aureus* and its ser/thr kinase and phosphatase deletion mutants: involvement in cell wall biosynthesis. *Chem Biol* **17**: 820–830.

Lowy, F.D. (1998) *Staphylococcus aureus* infections. *N Engl J Med* **339**: 520–532.

Löffler, B., Tuchscherr, L., Niemann, S., and Peters, G. (2014) *Staphylococcus aureus* persistence in non-professional phagocytes. *Int J Med Microbiol* **304**: 170–176.

Luo, R., Liu, B., Xie, Y., Li, Z., Huang, W., Yuan, J., *et al.* (2012) SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. *Gigascience* **1**: 18.

Luo, Y., and Helmann, J.D. (2012) Analysis of the role of *Bacillus subtilis* σ(M) in β-lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. *Mol Microbiol* **83**: 623–639.

Lybecker, M., Zimmermann, B., Bilusic, I., Tukhtubaeva, N., and Schroeder, R. (2014) The double-stranded transcriptome of *Escherichia coli*. *Proc Natl Acad Sci USA* **111**: 3134–3139.

Maestro, B., Novaková, L., Hesek, D., Lee, M., Leyva, E., Mobashery, S., *et al.* (2011) Recognition of peptidoglycan and β-lactam antibiotics by the extracellular domain of the Ser/Thr protein kinase StkP from *Streptococcus pneumoniae*. *FEBS Lett* **585**: 357–363.

Martin, P.K., Li, T., Sun, D., Biek, D.P., and Schmid, M.B. (1999) Role in cell permeability of an essential two-component system in *Staphylococcus aureus*. *J Bacteriol* **181**: 3666–3673.

Martinez, S.E., Beavo, J.A., and Hol, W.G.J. (2002) GAF domains: two-billion-year-old molecular switches that bind cyclic nucleotides. *Mol Interv* **2**: 317–323.

Martínez-Hackert, E., and Stock, A.M. (1997) Structural relationships in the OmpR family of winged-helix transcription factors. *J Mol Biol* **269**: 301–312.

Mascher, T. (2006) Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in Firmicutes bacteria. *FEMS Microbiol Lett* **264**: 133–144.

Mascher, T., Helmann, J.D., and Unden, G. (2006) Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev* **70**: 910–938.

Matsubara, M., Kitaoka, S.I., Takeda, S.I., and Mizuno, T. (2000) Tuning of the porin expression under anaerobic growth conditions by His-to-Asp cross-phosphorelay through both the EnvZ-osmosensor and ArcB-anaerosensor in *Escherichia coli*. *Genes Cells* **5**: 555–569.

McCallum, N., Meier, P.S., Heusser, R., and Berger-Bachi, B. (2011) Mutational Analyses of Open Reading Frames within the vraSR Operon and Their Roles in the Cell Wall Stress Response of *Staphylococcus aureus*. *Antimicrob Agents Ch* **55**: 1391–1402.

McCarthy, A.J., and Lindsay, J.A. (2010) Genetic variation in *Staphylococcus aureus* surface and immune evasion genes is lineage associated: implications for vaccine design and host-pathogen interactions. *BMC Microbiol* **10**: 173.

McDougal, L.K., Steward, C.D., Killgore, G.E., Chaitram, J.M., McAllister, S.K., and Tenover, F.C. (2003) Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. *J Clin Microbiol* **41**: 5113–5120.

Meehl, M., Herbert, S., Götz, F., and Cheung, A. (2007) Interaction of the GraRS two-component system with the VraFG ABC transporter to support vancomycin-intermediate resistance in *Staphylococcus aureus*. *Antimicrob Agents Ch* **51**: 2679–2689.

Memmi, G., Nair, D.R., and Cheung, A. (2012) Role of ArlRS in autolysis in methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* strains. *J Bacteriol* **194**: 759–767.

Mike, L.A., Choby, J.E., Brinkman, P.R., Olive, L.Q., Dutter, B.F., Ivan, S.J., *et al.* (2014) Two-component system cross-regulation integrates *Bacillus anthracis* response to heme and cell envelope stress. *PLoS Pathog* **10**: e1004044.

Milani, M., Leoni, L., Rampioni, G., Zennaro, E., Ascenzi, P., and Bolognesi, M. (2005) An active-like structure in the unphosphorylated StyR response regulator suggests a phosphorylation- dependent allosteric activation mechanism. *Structure* **13**: 1289–1297.

Mills, E., Pultz, I.S., Kulasekara, H.D., and Miller, S.I. (2011) The bacterial second messenger c-di-GMP: mechanisms of signalling. *Cell Microbiol* **13**: 1122–1129.

Müllner, M., Hammel, O., Mienert, B., Schlag, S., Bill, E., and Unden, G. (2008) A PAS domain with an oxygen labile [4Fe-4S](2+) cluster in the oxygen sensor kinase NreB of *Staphylococcus carnosus*. *Biochemistry* **47**: 13921–13932.

Nikel, P.I., Martínez-García, E., and de Lorenzo, V. (2014) Biotechnological domestication of pseudomonads using synthetic biology. *Nat Rev Microbiol* **12**: 368–379.

Ninfa, A.J. (2010) Use of two-component signal transduction systems in the construction of synthetic genetic networks. *Curr Opin Microbiol* **13**: 240–245.

Novick, R.P. (2003) The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing. *Microbiology* **149**: 2709–2717.

Novick, R.P., and Muir, T.W. (1999) Virulence gene regulation by peptides in staphylococci and other Gram-positive bacteria. *Curr Opin Microbiol* **2**: 40–45.

O'Hara, B.P., Norman, R.A., Wan, P.T., Roe, S.M., Barrett, T.E., Drew, R.E., and Pearl, L.H. (1999) Crystal structure and induction mechanism of AmiC-AmiR: a ligand-regulated transcription antitermination complex. *EMBO J* **18**: 5175–5186.

Ohlsen, K., and Donat, S. (2010) The impact of serine/threonine phosphorylation in *Staphylococcus aureus*. *Int J Med Microbiol* **300**: 137–141.

Otto, M. (2009) Bacterial sensing of antimicrobial peptides. *Contrib Microbiol* **16**: 136–149.

Otto, M. (2013) Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. *Annu Rev Med* **64**: 175–188.

Otto, M. (2014) *Staphylococcus aureus* toxins. *Curr Opin Microbiol* **17**: 32–37.

Palazzolo-Ballance, A.M., Reniere, M.L., Braughton, K.R., Sturdevant, D.E., Otto, M., Kreiswirth, B.N., *et al.* (2008) Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant *Staphylococcus aureus*. *J Immunol* **180**: 500–509.

Palmqvist, N., Patti, J.M., Tarkowski, A., and Josefsson, E. (2004) Expression of staphylococcal clumping factor A impedes macrophage phagocytosis. *Microbes Infect* **6**: 188–195.

Patton, T.G., Yang, S.-J., and Bayles, K.W. (2006) The role of proton motive force in expression of the Staphylococcus aureus cid and lrg operons. *Mol Microbiol* **59**: 1395–1404.

Peng, H.L., Novick, R.P., Kreiswirth, B., Kornblum, J., and Schlievert, P. (1988) Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *J Bacteriol* **170**: 4365–4372.

Perocchi, F., Xu, Z., Clauder-Münster, S., and Steinmetz, L.M. (2007) Antisense artifacts in transcriptome microarray experiments are resolved by actinomycin D. *Nucleic Acids Res* **35**: e128.

Peterson, P.K., Verhoef, J., Sabath, L.D., and Quie, P.G. (1977) Effect of protein A on staphylococcal opsonization. *Infec Immun* **15**: 760–764.

Podgornaia, A.I., and Laub, M.T. (2013) Determinants of specificity in two-component signal transduction. *Curr Opin Microbiol*.

Polarek, J.W., Williams, G., and Epstein, W. (1992) The products of the kdpDE operon are required for expression of the Kdp ATPase of Escherichia coli. *J Bacteriol* **174**: 2145–2151.

Pragman, A.A., Yarwood, J.M., Tripp, T.J., and Schlievert, P.M. (2004) Characterization of virulence factor regulation by SrrAB, a two-component system in *Staphylococcus aureus*. *J Bacteriol* **186**: 2430–2438.

Prévost, G., Cribier, B., Couppié, P., Petiau, P., Supersac, G., Finck-Barbançon, V., *et al.* (1995) Panton-Valentine leucocidin and gamma-hemolysin from *Staphylococcus aureus* ATCC 49775 are encoded by distinct genetic loci and have different biological activities. *Infec Immun* **63**: 4121–4129.

Procaccini, A., Lunt, B., Szurmant, H., Hwa, T., and Weigt, M. (2011) Dissecting the specificity of protein-protein interaction in bacterial two-component signaling: orphans and crosstalks. *PLoS ONE* **6**: e19729.

Proctor, R.A., Eiff, von, C., Kahl, B.C., Becker, K., McNamara, P., Herrmann, M., and Peters, G. (2006) Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol* **4**: 295–305.

Proctor, R.A., van Langevelde, P., Kristjansson, M., Maslow, J.N., and Arbeit, R.D. (1995) Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. *Clin Infect Dis* **20**: 95–102.

Qiu, R., Pei, W., Zhang, L., Lin, J., and Ji, G. (2005) Identification of the putative staphylococcal AgrB catalytic residues involving the proteolytic cleavage of AgrD to generate autoinducing peptide. *J Biol Chem* **280**: 16695–16704.

Queck, S.Y., Jameson-Lee, M., Villaruz, A.E., Bach, T.-H.L., Khan, B.A., Sturdevant, D.E., *et al.* (2008) RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus. Mol Cell* **32**: 150–158.

Ramadurai, L., Lockwood, K.J., Nadakavukaren, M.J., and Jayaswal, R.K. (1999) Characterization of a chromosomally encoded glycylglycine endopeptidase of Staphylococcus aureus. *Microbiology* **145 ( Pt 4)**: 801–808.

Rice, K.C., Mann, E.E., Endres, J.L., Weiss, E.C., Cassat, J.E., Smeltzer, M.S., and Bayles, K.W. (2007) The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus. Proc Natl Acad Sci USA* **104**: 8113–8118.

Rissman, A.I., Mau, B., Biehl, B.S., Darling, A.E., Glasner, J.D., and Perna, N.T. (2009) Reordering contigs of draft genomes using the Mauve aligner. *Bioinformatics* **25**: 2071–2073.

Rooijakkers, S.H.M., Ruyken, M., Roos, A., Daha, M.R., Presanis, J.S., Sim, R.B., *et al.* (2005) Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nat Immunol* **6**: 920–927.

Roux, A., Todd, D.A., Velázquez, J.V., Cech, N.B., and Sonenshein, A.L. (2014) CodY-Mediated Regulation of the *Staphylococcus aureus* Agr System Integrates Nutritional and Population Density Signals. *J Bacteriol* **196**: 1184–1196.

Römling, U., and Amikam, D. (2006) Cyclic di-GMP as a second messenger. *Curr Opin Microbiol* **9**: 218–228.

Römling, U., Gomelsky, M., and Galperin, M.Y. (2005) C-di-GMP: the dawning of a novel bacterial signalling system. *Mol Microbiol* **57**: 629–639.

Ruiz de Los Mozos, I., Vergara-Irigaray, M., Segura, V., Villanueva, M., Bitarte, N., Saramago, M., *et al.* (2013) Base Pairing Interaction between 5“- and 3-”UTRs Controls icaR mRNA Translation in *Staphylococcus aureus*. *PLoS Genet* **9**: e1004001.

Schenk, S., and Laddaga, R.A. (1992) Improved method for electroporation of Staphylococcus aureus. *FEMS Microbiol Lett* **73**: 133–138.

Schlag, S., Fuchs, S., Nerz, C., Gaupp, R., Engelmann, S., Liebeke, M., *et al.* (2008) Characterization of the oxygen-responsive NreABC regulon of *Staphylococcus aureus*. *J Bacteriol* **190**: 7847–7858.

Segura, V., Toledo-Arana, A., Uzqueda, M., Lasa, I., and Muñoz-Barrutia, A. (2012) Wavelet-based detection of transcriptional activity on a novel *Staphylococcus aureus* tiling microarray. *BMC Bioinformatics* **13**: 222.

Sharma-Kuinkel, B.K., Mann, E.E., Ahn, J.-S., Kuechenmeister, L.J., Dunman, P.M., and Bayles, K.W. (2009) The *Staphylococcus aureus* LytSR two-component regulatory system affects biofilm formation. *J Bacteriol* **191**: 4767–4775.

Sidote, D.J., Barbieri, C.M., Wu, T., and Stock, A.M. (2008) Structure of the Staphylococcus aureus AgrA LytTR domain bound to DNA reveals a beta fold with an unusual mode of binding. *Structure* **16**: 727–735.

Siryaporn, A., and Goulian, M. (2008) Cross-talk suppression between the CpxA-CpxR and EnvZ-OmpR two-component systems in *E. coli*. *Mol Microbiol* **70**: 494–506.

Skerker, J.M., Perchuk, B.S., Siryaporn, A., Lubin, E.A., Ashenberg, O., Goulian, M., and Laub, M.T. (2008) Rewiring the Specificity of Two-Component Signal Transduction Systems. *Cell* **133**: 1043–1054.

Skerker, J.M., Prasol, M.S., Perchuk, B.S., Biondi, E.G., and Laub, M.T. (2005) Two-component signal transduction pathways regulating growth and cell cycle progression in a bacterium: a system-level analysis. *PLoS Biol* **3**: e334.

Sobral, R.G., Jones, A.E., Etages, Des, S.G., Dougherty, T.J., Peitzsch, R.M., Gaasterland, T., *et al.* (2007) Extensive and Genome-Wide Changes in the Transcription Profile of *Staphylococcus aureus* Induced by Modulating the Transcription of the Cell Wall Synthesis Gene *murF*. *J Bacteriol* **189**: 2376–2391.

Solano, C., García, B., Latasa, C., Toledo-Arana, A., Zorraquino, V., Valle, J., *et al.* (2009) Genetic reductionist approach for dissecting individual roles of GGDEF proteins within the c-di-GMP signaling network in *Salmonella*. *Proc Natl Acad Sci USA* **106**: 7997–8002.

Stauff, D.L., and Skaar, E.P. (2009) The heme sensor system of *Staphylococcus aureus*. *Contrib Microbiol* **16**: 120–135.

Stauff, D.L., Torres, V.J., and Skaar, E.P. (2007) Signaling and DNA-binding activities of the *Staphylococcus aureus* HssR-HssS two-component system required for heme sensing. *J Biol Chem* **282**: 26111–26121.

Steinhuber, A., Goerke, C., Bayer, M.G., Döring, G., and Wolz, C. (2003) Molecular architecture of the regulatory Locus *sae* of *Staphylococcus aureus* and its impact on expression of virulence factors. *J Bacteriol* **185**: 6278–6286.

Stewart, V. (2003) Nitrate-and nitrite-responsive sensors NarX and NarQ of proteobacteria. *Bioc Soc T* **31**: 1–10.

Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000) Two-component signal transduction. *Annu Rev Biochem* **69**: 183–215.

Sun, F., Ji, Q., Jones, M.B., Deng, X., Liang, H., Frank, B., *et al.* (2012) AirSR, a [2Fe-2S] Cluster-Containing Two-Component System, Mediates Global Oxygen Sensing and Redox Signaling in *Staphylococcus aureus*. *J Am Chem Soc* **134**: 305–314.

Sun, F., Liang, H., Kong, X., Xie, S., Cho, H., Deng, X., *et al.* (2012) Quorum-sensing agr mediates bacterial oxidation response via an intramolecular disulfide redox switch in the response regulator AgrA. *Proc Natl Acad Sci USA* **109**: 9095–9100.

Sun, H., Yang, Y., Xue, T., and Sun, B. (2013) Modulation of cell wall synthesis and susceptibility to vancomycin by the two-component system AirSR in *Staphylococcus aureus* NCTC8325. *BMC Microbiol* **13**: 286.

Sun, J., Zheng, L., Landwehr, C., Yang, J., and Ji, Y. (2005) Identification of a novel essential two-component signal transduction system, YhcSR, in *Staphylococcus aureus*. *J Bacteriol* **187**: 7876–7880.

Tamayo, R., Pratt, J.T., and Camilli, A. (2007) Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annu Rev Microbiol* **61**: 131–148.

Tamayo, R., Tischler, A.D., and Camilli, A. (2005) The EAL domain protein VieA is a cyclic diguanylate phosphodiesterase. *J Biol Chem* **280**: 33324–33330.

Thakker, M., Park, J.S., Carey, V., and Lee, J.C. (1998) *Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. *Infec Immun* **66**: 5183–5189.

Toledo-Arana, A., Dussurget, O., Nikitas, G., Sesto, N., Guet-Revillet, H., Balestrino, D., *et al.* (2009) The Listeria transcriptional landscape from saprophytism to virulence. *Nature* **459**: 950–956.

Toledo-Arana, A., Merino, N., Vergara-Irigaray, M., Débarbouillé, M., Penadés, J.R., and Lasa, I. (2005) *Staphylococcus aureus* develops an alternative, ica-independent biofilm in the absence of the *arlRS* two-component system. *J Bacteriol* **187**: 5318–5329.

Torres, V.J., Stauff, D.L., Pishchany, G., Bezbradica, J.S., Gordy, L.E., Iturregui, J., *et al.* (2007) A *Staphylococcus aureus* regulatory system that responds to host heme and modulates virulence. *Cell Host Microbe* **1**: 109–119.

Tu Quoc, P.H., Genevaux, P., Pajunen, M., Savilahti, H., Georgopoulos, C., Schrenzel, J., and Kelley, W.L. (2007) Isolation and characterization of biofilm formation-defective mutants of *Staphylococcus aureus. Infec Immun* **75**: 1079–1088.

Ulrich, L.E., and Zhulin, I.B. (2010) The MiST2 database: a comprehensive genomics resource on microbial signal transduction. *Nucleic Acids Res* **38**: D401–7.

Ulrich, M., Bastian, M., Cramton, S.E., Ziegler, K., Pragman, A.A., Bragonzi, A., *et al.* (2007) The staphylococcal respiratory response regulator SrrAB induces *ica* gene transcription and polysaccharide intercellular adhesin expression, protecting *Staphylococcus aureus* from neutrophil killing under anaerobic growth conditions. *Mol Microbiol* **65**: 1276–1287.

Utaida, S. (2003) Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. *Microbiology* **149**: 2719–2732.

Úbeda, C., Barry, P., Penadés, J.R., and Novick, R.P. (2007) A pathogenicity island replicon in *Staphylococcus aureus* replicates as an unstable plasmid. *Proc Natl Acad Sci USA* **104**: 14182–14188.

Valle, J., Latasa, C., Gil, C., Toledo-Arana, A., Solano, C., Penadés, J.R., and Lasa, I. (2012) Bap, a biofilm matrix protein of *Staphylococcus aureus* prevents cellular internalization through binding to GP96 host receptor. *PLoS Pathog* **8**: e1002843.

Valle, J., Toledo-Arana, A., Berasain, C., Ghigo, J.-M., Amorena, B., Penadés, J.R., and Lasa, I. (2003) SarA and not sigmaB is essential for biofilm development by *Staphylococcus aureus. Mol Microbiol* **48**: 1075–1087.

Vollmer, W., Joris, B., Charlier, P., and Foster, S. (2008) Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiology Reviews* **32**: 259–286.

Voyich, J.M., Vuong, C., DeWald, M., Nygaard, T.K., Kocianova, S., Griffith, S., *et al.* (2009) The SaeR/S gene regulatory system is essential for innate immune evasion by *Staphylococcus aureus*. *J Infect Dis* **199**: 1698–1706.

Walker, J.N., Crosby, H.A., Spaulding, A.R., Salgado-Pabón, W., Malone, C.L., Rosenthal, C.B., *et al.* (2013) The *Staphylococcus aureus* ArlRS two-component system is a novel regulator of agglutination and pathogenesis. *PLoS Pathog* **9**: e1003819.

Wanner, B.L. (1992) Is cross regulation by phosphorylation of two-component response regulator proteins important in bacteria? *J Bacteriol* **174**: 2053–2058.

Wanner, B.L., WILMES, M.R., and YOUNG, D.C. (1988) Control of bacterial alkaline phosphatase synthesis and variation in an *Escherichia coli* K-12 phoR mutant by adenyl cyclase, the cyclic AMP receptor protein, and the phoM operon. *J Bacteriol* **170**: 1092–1102.

Watanabe, T., Okada, A., Gotoh, Y., and Utsumi, R. (2008) Inhibitors targeting two-component signal transduction. *Adv Exp Med Biol* **631**: 229–236.

Wertheim, H.F.L., Melles, D.C., Vos, M.C., van Leeuwen, W., van Belkum, A., Verbrugh, H.A., and Nouwen, J.L. (2005) The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect Dis* **5**: 751–762.

White, A.P., Gibson, D.L., Kim, W., Kay, W.W., and Surette, M.G. (2006) Thin aggregative fimbriae and cellulose enhance long-term survival and persistence of Salmonella. *J Bacteriol* **188**: 3219–3227.

Willett, J.W., and Kirby, J.R. (2012) Genetic and biochemical dissection of a HisKA domain identifies residues required exclusively for kinase and phosphatase activities. *PLoS Genet* **8**: e1003084.

Willett, J.W., Tiwari, N., Müller, S., Hummels, K.R., Houtman, J.C.D., Fuentes, E.J., and Kirby, J.R. (2013) Specificity residues determine binding affinity for two-component signal transduction systems. *mBio* **4**: e00420–13.

Winkler, M.E., and Hoch, J.A. (2008) Essentiality, Bypass, and Targeting of the YycFG (VicRK) Two-Component Regulatory System in Gram-Positive Bacteria. *J Bacteriol* **190**: 2645–2648.

Woodward, J.J., Iavarone, A.T., and Portnoy, D.A. (2010) c-di-AMP secreted by intracellular *Listeria monocytogenes* activates a host type I interferon response. *Science* **328**: 1703–1705.

Wu, J.A., Kusuma, C., Mond, J.J., and Kokai-Kun, J.F. (2003) Lysostaphin disrupts *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms on artificial surfaces. *Antimicrob Agents Ch* **47**: 3407–3414.

Xue, T., You, Y., Hong, D., Sun, H., and Sun, B. (2011) The *Staphylococcus aureus* KdpDE Two-Component System Couples Extracellular $K^+$ Sensing and Agr Signaling to Infection Programming. *Infec Immun* **79**: 2154–2167.

Yamamoto, K., Hirao, K., Oshima, T., Aiba, H., Utsumi, R., and Ishihama, A. (2005) Functional characterization in vitro of all two-component signal transduction systems from *Escherichia coli. J Biol Chem* **280**: 1448–1456.

Yan, M., Yu, C., Yang, J., and Ji, Y. (2011) The essential two-component system YhcSR is involved in regulation of the nitrate respiratory pathway of *Staphylococcus aureus*. *J Bacteriol* **193**: 1799–1805.

Yarwood, J.M., Bartels, D.J., Volper, E.M., and Greenberg, E.P. (2004) Quorum sensing in *Staphylococcus aureus* biofilms. *J Bacteriol* **186**: 1838–1850.

Yarwood, J.M., McCormick, J.K., and Schlievert, P.M. (2001) Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus*. *J Bacteriol* **183**: 1113–1123.

Yoshida, Y., Matsuo, M., Oogai, Y., Kato, F., Nakamura, N., Sugai, M., and Komatsuzawa, H. (2011) Bacitracin sensing and resistance in *Staphylococcus aureus*. *FEMS Microbiol Lett* **320**: 33–39.

Zhang, L., and Ji, G. (2004) Identification of a staphylococcal AgrB segment(s) responsible for group-specific processing of AgrD by gene swapping. *J Bacteriol* **186**: 6706–6713.

Zhao, L., Xue, T., Shang, F., Sun, H., and Sun, B. (2010) *Staphylococcus aureus* AI-2 quorum sensing associates with the KdpDE two-component system to regulate capsular polysaccharide synthesis and virulence. *Infec Immun* **78**: 3506–3515.