Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Vegetal

Heterogeneity in *Staphylococcus aureus* response to β-lactams

Pedro Escada Cardoso Baptista Fernandes

Dissertação

Mestrado em Microbiologia Aplicada

2013

Universidade de Lisboa

Faculdade de Ciências

Departamento de Biologia Vegetal

Heterogeneity in *Staphylococcus aureus* response to β-lactams

Pedro Escada Cardoso Baptista Fernandes

Dissertação

Mestrado em Microbiologia Aplicada

Orientadores: Dr. Mariana Pinho (ITQB/UNL)

e Dr. Ricardo Dias (FCUL-BioFIG)

2013

Heterogeneity in *Staphylococcus aureus* response to β-lactams

Pedro Escada Cardoso Baptista Fernandes

MASTER THESIS

2013

This thesis was fully performed at the Bacterial Cell Biology lab in the ITQB/UNL institute under the direct supervision of Dr. Mariana Pinho.

Dr. Ricardo Dias was the internal designated supervisor in the scope of the *Master in Applied Microbiology* of the Faculty of Sciences of the University of Lisbon.

Acknowledgments

First, I would like to thank my family for the unquestionable support and for being comprehensive during this exigent year. To my supervisor, Dr Mariana Pinho for her wise guidance, for sharing her vision with me and also for teaching and helping me to develop my capacities. The constant stimulation was essential to overcome all the obstacles that appeared in our way. To Dr Sérgio Filipe for sharing his acute critical view, that helped to consolidate and improve my way of working and thinking. To Dr. Ricardo Dias for being my link between the university and ITQB and for his concern about my work. A special heartfelt thanks to all my laboratory colleagues for providing an extraordinary environment and for giving me strength every single day. I must highlight the role of Pedro Matos, João Monteiro and Raquel Pereira for being involved, since the beginning, with my research, for their incessant support and for contributing to my professional and personal development.

.

Abstract

Keywords: *Staphylococcus aureus;* cell wall; β-lactam antibiotics; cell wall stress stimulon; VraSR regulatory system; genetic noise

Multidrug resistant *Staphylococcus aureus* (MRSA) are pathogens responsible for multiple infections, even in healthy individuals, worldwide, thus requiring urgent development of new chemotherapies and a better understanding of mechanisms underlying antibiotic resistance. One of the most important cellular components is the cell wall, a structure essential for cell survival, responsible for conferring shape to bacteria, with a key role in establishing cell-environment interactions. In this way, cell wall synthesis constitutes a major target for antibiotics, such β-lactams or glycopeptides. When cell wall damage is inflicted, bacteria activate what is called the cell wall stress stimulon (CWSS), a coordinated response of several genes, in which VraSR regulatory system plays a major role. This system, composed of a histidine kinase (VraS) and cognate response regulator (VraR), is responsible for sensing cell wall threats and triggering the adequate response. Stochastic fluctuations in genetic expression, also called genetic noise, are responsible for variability in isogenic populations, a phenomenon common to all genes. In this work, we aim to investigate whether the promoters of *vraSR* and *pbp2*, a gene upregulated by VraR, which codifies for a cell wall synthesis protein, exhibit an increase in their genetic expression heterogeneity, upon cell wall stimulon stress induction. Their expression was compared to the housekeeping gene *pta*, a gene not related with the CWSS, in the presence of β-lactam oxacillin and the aminoglycoside kanamycin, a protein synthesis antibiotic that is not expected to activate the CWSS. We concluded that there was variability at the single cell level in genetic expression of CWSS promoter, but this variability did not increase upon exposure to cell wall targeting antibiotics.

Resumo

Palavras-chave: *Staphylococcus aureus;* parede celular; antibióticos β-lactâmicos; estimulão do stress da parede celular; sistema regulatório VraSR; ruído genético.

Staphylococcus aureus é uma bactéria patogénica nosocomial, bastante versátil e com grande capacidade de adaptação. A sua evolução levou ao aparecimento de estirpes resistentes resistente à meticilina (conhecidos pela sigla inglesa MRSA), que são actualmente virtualmente resistentes a todos os antibióticos disponíveis e responsáveis por diversas infecções, mesmo em indivíduos saudáveis, a nível mundial. A elevada mortalidade associada a infecções causadas por *S. aureus* e as opções limitadas de terapias eficientes, evidenciam a importância de se encontrarem novas estratégias para o seu tratamento e a necessidade da compreensão dos mecanismos subjacentes à resistência a antibióticos, adquiridos por esta bactéria patogénica. Como tal, necessita de uma resposta urgente ao nível do desenvolvimento de novas vertentes na terapia de antibióticos e um melhor conhecimento dos mecanismos subjacentes à resistência a antibióticos. Um dos principais componentes da célula bacteriana é a parede celular, uma estrutura essencial, responsável por conferir a forma às bactérias, com um papel especialmente relevante na interacção da célula com o meio envolvente. Como consequência, a síntese da parede celular constitui, desde logo, um alvo prioritário dos antibióticos desenvolvidos, como os β-lactâmicos. Esta família de antibióticos tem como alvo um dos últimos passos da síntese da parede celular, efectuado pelas proteínas de ligação à penicilina (PBPs). Uma vez aciladas pelos β-lactâmicos, estas proteínas perdem a capacidade de fazer o *crosslinking* do peptidoglicano, o que leva à lise celular. Contudo, estirpes MRSA têm a capacidade de crescer na presença de elevadas concentrações de β-lactâmicos, uma vez que adquiriram uma proteína adicional (PBP2A).

As bactérias estão constantemente em interacção com o meio externo. Para manter a integridade celular, respostas fisiológicas adaptativas foram sendo desenvolvidas pelos microorganismos, envolvendo a integração dos diversos estímulos ambientais, para desencadear a resposta adequada, sobretudo através de sistemas regulatórios. Quando é infligido algum dano à parede celular, as bactérias activam o que é conhecido como o *stimulon* do stress da parede celular (em inglês CWSS), uma resposta coordenada de vários genes. O CWSS permite que a bactéria possa lidar com o dano causado na parede celular, activando e reprimindo um conjunto vasto de genes. Entre os genes que são activados por este sistema encontram-se, nomeadamente, os responsáveis por codificar para proteínas envolvidas na síntese da parede celular. Para detectar os danos infligidos na parede celular, no caso de *S. aureus* o sistema proeminente é o sistema regulatório VraSR, homólogo do sistema LiaFSR em *Bacillus subtilis.* Este sistema regulatório desempenha um papel central no CWSS, uma vez que funciona como complexo de antena, capaz de detectar os danos na parede celular e processar a activação do CWSS. Este sistema é composto pela cinase de histidina (VraS) e pelo correspondente regulador de resposta (VraR), factor de transcrição responsável pela

activação ou silenciamento de um conjunto vasto de genes, embora recentemente se tenha revelado a presença de um terceiro elemento VraT, responsável por influenciar a fosforilação e consequente activação de VraS. Considera-se actualmente que este sistema regulatório constitui, consequentemente, um sistema de três componentes.

Todos os genes estão sujeitos a oscilações estocásticas na sua expressão. Isto significa que numa população bacteriana composta por indivíduos geneticamente iguais, cada célula será uma individualidade, considerando a variabilidade existente na expressão dos vários genes. A oscilação na expressão dos genes geradora desta variabilidade é também conhecida como ruído genético. Este ruído é o resultado de dois fenómenos: ruído extrínseco, derivado das flutuações na concentração, localização e estado de determinadas moléculas no *output* de um dado gene e o ruído intrínseco, que se refere à variação da expressão genética devida à aleatoriedade com que se processam as reacções químicas, mesmo se a população for composta por células isogénicas com concentrações e estados dos compostos celulares idênticos. Neste sentido, este ruído intrínseco é também chamado de estocasticidade inerente à expressão de determinado gene. Em determinados casos, o ruído genético pode potenciar o aparecimento de fenómenos como a bi-estabilidade, no qual uma população de células geneticamente idênticas bifurca em duas sub-populações coexistentes. Um dos exemplos mais evidentes é o aparecimento de uma sub-população de bactérias resistentes, também chamadas, neste caso, de persistentes, dentro de uma população bacteriana isogénica, quando na presença de antibióticos.

. Neste trabalho, temos como objectivo investigar se os promotores dos genes pertencentes ao CWSS, vraSR e *pbp2*, um gene regulado pelo VraR, que codifica uma proteína envolvida na síntese da parede celular, exibem um aumento da variabilidade na expressão genética, depois da activação do *stimulon* do stress da parede celular. A sua expressão foi comparada à do gene *housekeeping pta*, um gene que não está envolvido nem relacionado com o CWSS, na presença do antibiótico β-lactâmico oxacilina e do aminoglicósido canamicina, um antibiótico que não activa o *stimulon*. O controlo pelo gene *housekeeping* permitiu avaliar se o comportamento dos promotores do CWSS, em termos de variabilidade, era ou não similar ao comportamento evidenciado pelos restantes genes da bactéria. O uso dos antibióticos oxacilina e canamicina permitiu testar esse comportamento em condições de activação do CWSS e em condições em que este sistema não era activado. A expressão dos diferentes promotores foi monitorizada pelo gene repórter *gfp* em duas estirpes MRSA (MW2 e COL) e uma MSSA (Newman). Tendo em consideração a existência das variações entre células, mesmo tratando-se de populações isogénicas, foi construído um outro conjunto de estirpes, nas quais fosse possível monitorizar a expressão do promotor *housekeeping* e do promotor do CWSS simultaneamente na mesma célula. Neste caso, o promotor *housekeeping* foi monitorizado pelo gene repórter *mCherry.* Este conjunto de estirpes permitiu validar e corroborar os dados obtidos com o outro conjunto. Concluímos que existe variabilidade na expressão genética dos promotores de genes do CWSS, mas que esta variabilidade não aumenta na presença de

antibióticos que têm como alvo a síntese da parede, um comportamento registado quer em estirpes MRSA, quer na estirpe sensível MSSA. Por outro lado, colocámos de parte a possibilidade da existência do fenómeno da bi-estabilidade nos promotores do CWSS, uma vez que não houve um aumento consequente da variabilidade, nem a bifurcação em sub-populações. Continua indefinida a eventual existência de uma correlação entre os níveis de expressão dos promotores do CWSS e a rapidez com que são activados com a resistência aos antibióticos.

Contents

Introduction 1

Staphylococcus aureus, a multi-drug resistant pathogen 1

Cell wall assembly and β-lactam resistance 2

Cell wall stress stimulon and the VraSR regulatory system 5

Single cell stochasticity in genetic expression 8

Materials and Methods 10

Bacterial strains and plasmids 10

Growth conditions 11

Molecular cloning methods 11

DNA purification and manipulation 11

E. coli transformation 12

S. aureus transformation 12

S. aureus transduction 12

Construction of *gfp* promoter fusions 13

Construction of *mCherry* promoter fusions 15

Electrophoretic analysis of proteins by SDS-PAGE 16

Minimum inhibitory concentration assays 17

Fluorescence microscopy 17

Data and statistical analysis 17

Results 18

Construction of reporter *gfp* promoter fusions 18

vraSR is induced by β-lactam oxacillin and can be used as a probe of the CWSS induction 20

Cell wall stress stimulon promoters response to antibiotics 23

Analysis of genetic expression in MW2 GFP reporter strains 24

Analysis of genetic expression in Newman GFP reporter strains 27

Analysis of genetic expression in COL GFP reporter strains 30

Construction of *mCherry* promoter fusions 33

Analysis of genetic expression in MW2 mCherry/GFP reporter strains 34

Analysis of genetic expression in Newman mCherry/GFP reporter strains 36

Analysis of genetic expression in COL mCherry/GFP reporter strains 40

Discussion 43

Bibliography 48

Introduction

Staphylococcus aureus, a multi-drug resistant pathogen

Staphylococcus aureus is a gram positive coccus with a low G/C genomic content. Humans are a natural reservoir, as we can find these bacteria in the nasopharynx and on the skin. It is believed that approximately 20% of individuals are persistently nasally colonized with *S. aureus*, and 30% are intermittently colonized [1] . Colonization enables transmission among individuals in health care and community environments [1] that can be performed through skin-to-skin contact between individuals or by contaminated objects[2, 3] .Besides being commensal bacteria, *S. aureus* is also an extremely versatile pathogen, able to cause several diseases such as skin infections, pneumonia, endocarditis and pyaemia[4] . It accounts for one of the major causes of nosocomial infections worldwide [5] . Before the introduction of the antibiotics in the health system, this pathogen had been a serious concern for clinicians [5]since, for example *S. aureus* bacteremia mortality rate ranged between 75% and 83% [6]. In 2005, more than 18.000 deaths were reported in the U.S. [7], representing more deaths than AIDS and tuberculosis combined [4].

The antibiotic era began after the β-lactam penicillin was accidentally discovered in 1929 by Alexender Fleming, when he observed an inhibition halo in a *Staphylococcus* plate contaminated with a *penicillium* mould. Thereby, penicillin was one of the antibiotic pioneers in treating gram positive bacterial infections, [8]. Due to the widespread use of this antibiotic, it did not take too long until the first wave of *S. aureus* resistant strains appeared in hospitals with an incidence of 14% in 1946 which increased to 58%, in only two years [9]. The main reason behind this was the existence of strains that harbored a plasmid which encoded a penicillinase, a predominantly extracellular enzyme capable of hydrolyzing the β-lactam ring of penicillin, destroying its antimicrobial activity[10]. Penicillin resistant staphylococci subsequently spread into the community and by the late 1960s, more than 80% of both community and hospital-acquired staphylococcal isolates were resistant to penicillin[11]. Once penicillin became ineffective for staphylococcal infections, semisynthetic β-lactamase-resistant penicillins were developed, such as methicillin and oxacillin [12]. Methicillin was first introduced in Europe in 1959 but it only took a few years until the isolation of the first Methicillin Resistant *Staphylococcus aureus* (MRSA) from a patient in Colindale, UK. This strain was named COL and is has been since then one of the most studied MRSA strains [13]. This type of resistance mechanism, which did not involve antibiotic degradation, was called “intrinsic” and was effective against all β-lactam antibiotics including, besides methicillin, cephalosporines and carbapenems [14]. MRSA strains rapidly disseminated and during the early 1970´s MRSA clones were increasingly responsible for outbreaks of infections in countries around the world[15]. Since then, the situation has worsened and there are presently *S. aureus* strains virtually resistant to all known antibiotics. Consequently MRSA now more commonly stands for multidrug resistant *Staphylococcus aureus.* Although MRSA infections are particularly incident in clinical settings, it is no longer regarded as an exclusive nosocomial pathogen, since the first case of infection in an healthy individual by a community-acquired MRSA

strain (CA-MRSA) was reported in 1980 [16]. Compared to health care acquired MRSA (HA-MRSA) isolates, community-acquired MRSA isolates tend to be resistant to fewer antibiotics [17] but, more virulent [18, 19]. The current economic and clinical burden resulting from *S. aureus* infection resistant to antibiotics prompts for a better understanding of the mechanisms underlying antibiotic resistance in *S. aureus*.

Cell wall assembly and β-lactam resistance

The bacterial cell wall (CW) is an essential structure that confers shape to the cells, acts as a surface structure responsible for establishing interactions with the surrounding environment, avoids cell lysis due to internal high osmotic pressure and plays a critical role in cell division. Bacterial CW biosynthesis is, thus, one of the main antibiotic targets in the bacterial cell. The main component of the CW of most bacteria is a mesh-like structure with a scaffold composed of linear glycan chains cross-linked by short peptides, known as peptidoglycan (PGN) or murein. The structure of PGN confers the structural rigidity necessary to maintain cellular integrity, but also confers the required fluidity to adapt to modifications in bacterial cell shape during cell division, for example [20]. In *S. aureus,* as in most Gram-positive bacteria, PGN can be attached to glycopolymers, like teichoic acids (TA), and proteins [21]. The glycan strands are composed of alternating polysaccharide *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues linked by β-1,4 bonds. In each MurNac residue, the d-lactoyl group is substituted by a small peptide stem composed of different aminoacids, whose composition varies between different bacteria species [22, 23]. In *S. aureus*, the peptide moiety is sequentially composed of L-Alanine (L-Ala), D-Glutamic acid (D-Glu), L-Lysine (L-Lys), and two D-Alanine (D-Ala) amino acids. Neighboring peptides are cross-linked between the amino group of L-Lys in one peptide and the carboxyl group of D-Ala in another, via pentaglycine cross bridges, creating the three-dimensional network of PGN[24]. The biosynthesis of PGN is a complex process, involving different enzymatic reactions that take place in the cytoplasm and in the inner and outer sides of the cytoplasmic membrane, as depicted in Figure 1 [25]. This process can be divided in three stages: cytoplasmic steps; membrane-associated steps and polymerization and cross-linking of the CW on the cell surface [26]. The first step of the PGN biosynthesis occurs in the cytoplasm between sugar-linked precursor Uridine diphosphate-GlcNac (UDP-GlcNAc) and phosphoenolpyruvate, catalysed by MurA and MurB enzymes, yielding UDP-MurNAc [25, 26]. Following the production of UDP-MurNAc in the cytoplasm, serial ATP-dependent amino acid ligations occur, catalyzed by Mur ligases (Mur C-F) to synthesize and add the stem peptide chain onto UDP-MurNAc. Accordingly, moieties L-Ala, D-Glu, L-Lys and D-Ala-D-Ala are sequentially added by MurC, MurD, MurE and MurF enzymes, respectively. The second stage begins with the transfer of the MurNAc-pentapeptide from the cytoplasm to a lipid carrier commonly known as bactoprenol, on the cytoplasmic side of the membrane, catalyzed by the integral membrane protein MraY, yielding the intermediate lipid I. MurG then converts lipid I into lipid II by adding UDP-GlcNAc via a β-1,4 linkage. In *S. aureus*, five glycines are sequentially added to the L-Lys residue of the lipid II intermediate by FemXAB protein family [27]. The final step inside the

cytoplasm has the purpose to translocate the lipid II to the outer side, across the membrane, by a flippase not yet identified in *S. aureus*. The lipid anchor returns back to the cytosolic side of the membrane, where it can be reused, after being dephosphorylated to the mono-phosphate form [28]. The formation of lipid II (GlcNAc-MurNAc-pentapeptide) and its subsequent translocation across the membrane conclude the second stage of PGN biosynthesis.

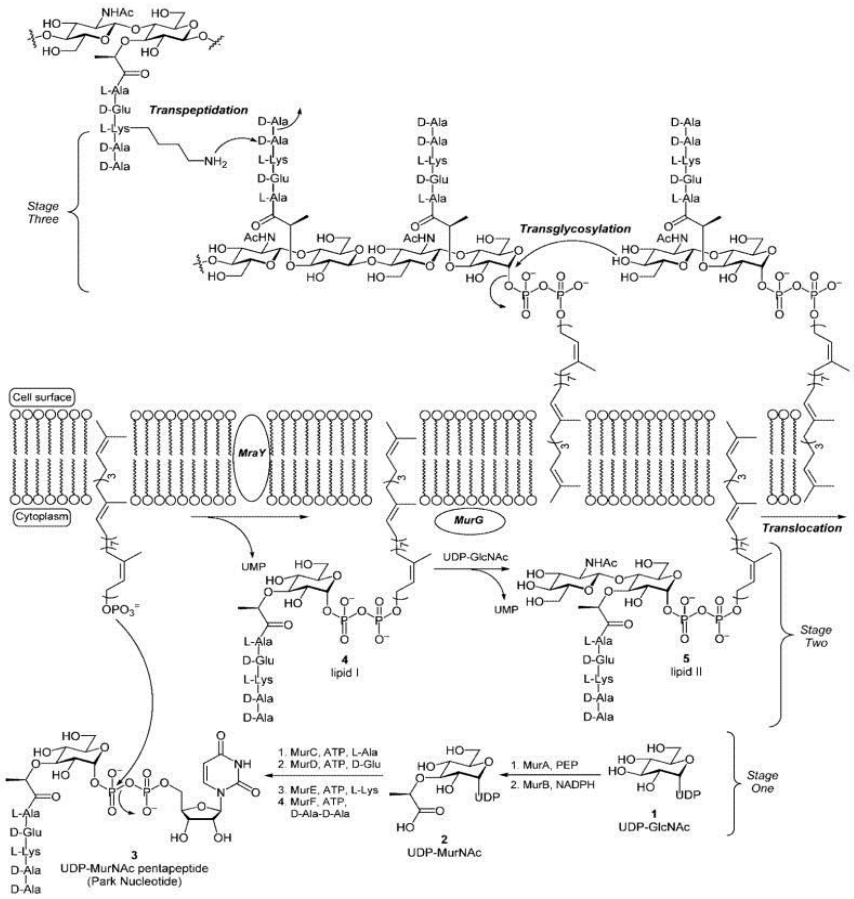


Figure 1 – Cell Wall biosynthesis in *S. aureus.* Representation of the different stages involved in the cell wall assembly: (i) cytoplasmic steps involved in the synthesis of UDP-MurNac-pentapeptide and UDP-GlcNac; (ii) membrane-bound steps implicated in the synthesis of Lipid II and (iii) outer membrane glycan chain polymerization and peptide cross-linking (Narayan, R.S. and. Vannieuwenhze, M.S., 2007)

The final stage reactions take place in the outer side of the membrane and result in the incorporation of the new PGN subunits into the growing glycan chain. This process takes place mainly, if not only, at the bacteria's division septum in *S. aureus* [29] and is catalyzed by the membrane-anchored PBPs. These enzymes can display two different roles in the last steps of PGN biosynthesis: transglycosylation (TG), responsible for glycan strands elongation and transpeptidation (TP), responsible for the peptide cross-linking between glycan chains. The TG reaction probably occurs between the reducing end of the MurNAc present in the nascent lipid-linked PG strand, and the C-4 carbon of the glucosamine residue in the lipid-linked precursor, which leads to the attachment of the new subunit to the growing glycan chain. The TP reaction, responsible for the peptide cross-linking, involves the cleavage of the D-Ala-D-Ala bond of a donor muropeptide, releasing the terminal D-Ala,

which generates the required energy to drive the subsequent reaction between the peptidyl moiety of the donor and an acceptor. In *S. aureus*, the acceptor is the last glycine of the interpeptide bridge, while in bacteria with direct cross-linking transpeptidation is performed between D-Ala of the donor muropeptide and the dibasic amino acid of the stem peptide of the muropeptide being incorporated [30]. *S. aureus* has four native PBPs (PBP1-4), all of them displaying TP activity, while PBP2, the only bifunctional PBP, is capable of both TP and TG activity[31]. Besides PBP2, *S. aureus* encodes two monofunctional glycosyl transferases with a transglycosylase (TGase) domain[32].

The transpeptidase (TPase) reaction is the target of β-lactam antibiotics, such as penicillin or methicillin[33]. These antibiotics act as substrate analogs of the D-Ala-D-Ala component of PGN and bind irreversibly to the TPase active sites of the PBPs, which blocks the binding of these enzyme to their substrate [31, 34]. Resistance to β-lactams in MRSA strains relies on the presence of the PBP2A enzyme. PBP2A is an extra PBP that contains a TPase domain with low affinity to β-lactams, which means that MRSA strains can still perform TP in the presence of high concentrations of these antibiotics [35]. Methicillin sensitive *Staphylococcus aureus* (MSSA) strains lack PBP2A and are therefore unable to grow when challenged with β-lactams. PBP2A is encoded by the *mecA* gene located in the Staphylococcal Cassette Chromosome mec (SCCmec), a mobile genetic element that integrates in the chromosome, at a unique site (*attBscc*) located near the *S. aureus* origin of replication [36]. Integration and excision of SCCmec is mediated by unique site-specific recombinases designated as cassette chromosome recombinases (*ccr*) [37]. Although the origin of the *mecA* gene remains unclear, a *mecA* homologue was found in *S. sciuri* [38] and also in a S. *haemolyticus* clinical isolate, but the later did not encode *ccr* genes, which raises the question regarding the mechanism of interspecies transfer that would have been used [39].

In the presence of β-lactams, PBP2A is assumed to take over the biosynthetic functions of the native PBPs that become irreversibly acylated [40]. In the presence of high concentrations of β-lactams, the TPase domain of PBP2 (as well as of other native PBPS) becomes inhibited and therefore unable to perform its crosslinking activity [41]. However, PBP2 also possesses a β-lactam insensitive TGase domain, which remains functional in the presence of the antibiotics. It was therefore proposed that the TGase domain of PBP2 cooperates with the TPase domain of PBP2A to synthesize the CW, in MRSA strains in the presence of β-lactams, illustrated in Figure 2. In MRSA strains, the PBP2 TGase domain becomes essential in the presence of β-lactams, probably because bacteria cannot survive if there is a simultaneous decrease in the cell wall cross-linking (as a result of PBP2A activity, which is less efficient than native PBPs) and in the length of the glycan strands (as a result of lack of PBP2 TGase activity) [42]. Accordingly, PBP2 is essential for optimal expression methicillin resistance. As demonstrated by Pinho and colleagues [40], *pbp2* inactivation in MRSA COL resulted in a strong reduction in methicillin minimum inhibitory concentration (from 1600μg/ml to 12μg/ml), even with normal levels of *mecA* expression. Therefore PBP2 was shown to be an auxiliary gene in β-lactam resistance.

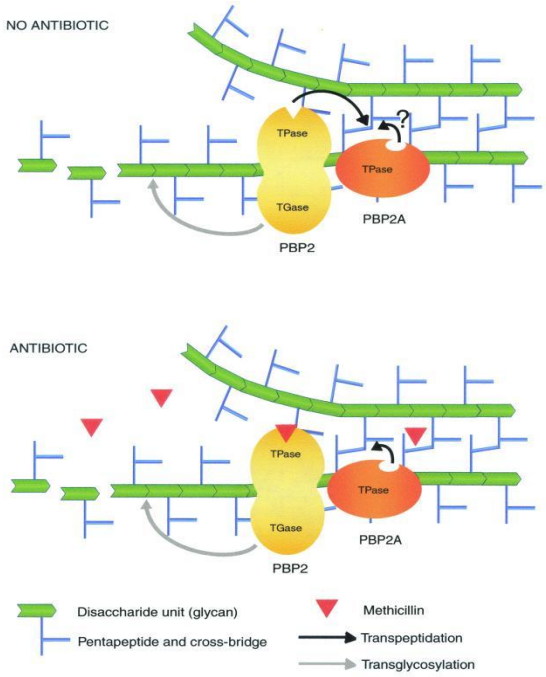


Figure 2 – Proposed model for cooperation between PBP2 transglycosilase domain (TGase) and PBP2A transpeptidation domain (TPase) in the presence of β-lactams. When in the absence of β-lactams, PBP2 is capable of both transglycosilation (TG) and transpeptidation (TP) activities. In the presence of β-lactams, PBP2 transpeptidase domain (TPase) becomes acylated, being unable to catalyze peptide cross-linking, yet in MRSA strains, which possess PBP2A, its TPase domain can substitute PBP2 TPase activity. PBP2 transglycosilation domain (TGase) remains functional in both settings (Pinho *et al*, 2001)

Cell wall stress stimulon and the VraSR regulatory system

Bacterial cells are in constant interaction with the surrounding environment, meaning that fluctuations or even abrupt physicochemical changes in the surroundings must trigger the adequate responses. In fact, bacteria rarely find natural environments where they can encounter the optimal conditions for their growth and development [43]. Adaptive physiological responses to the external stimuli are then crucial in the bacterial world in order to maintain cellular integrity, and require the sensitive integration of different environmental parameters to process the proper response, through the activity of different regulatory systems [44]. Some of the most striking examples of cellular responses to environmental challenges are the sporulation of *B. subtilis* or the development of competence in *E. coli*, processes that reveal the capacity that bacteria have to adapt to sudden external changes, allowing them to survive. One of the crucial cell structures regarding the dynamic communication between bacteria and their surroundings is precisely the CW. As stated above, the CW has an active role in establishing interactions with the exterior extracellular medium, such as signaling or molecular transport, and also displays a stress-bearing function [45]. It therefore represents the first line of defense against exterior threats. It is important to note that although many stresses like heat or osmotic shock are not referred as cell wall stress, they still affect the integrity of the CW. These stresses are sensed by signal-transducing regulatory systems that respond to the inflicted alterations, triggering the adequate reactions to repair the damage and to maintain cell integrity. This kind of response is called general stress response [43]. There are however, other regulatory systems able to

respond to stresses induced specifically in certain structures of the cell, like the cell envelope stress response [44]. For instance, in the Gram positive *B. subtilis*, the cell envelope stress response network involves at least four different alternative σ factors and a similar number of two-component systems [46]. When *S. aureus* is in the presence of CW-targeting antibiotics, CW hydrolysis or inhibition in the CW synthesis, but not of other external stresses like osmotic pressure, or the pH it triggers what is called the CW stimulon [47]. For instance, even compounds like nisin or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) which target specifically the cytoplasmatic membrane do not induce the CW stress stimulon (CWSS) [48].

The CW stimulon is an entire set of genes from different functional categories that display a coordinated response. The roles of those genes embrace CW synthesis, carbohydrate transport or even DNA repair [49]aiming to respond specifically to damage induced in the CW.

S. aureus contains several two-component systems (TCS) that act as cell-environment interfaces, consisting of a histidine kinase that senses external stimulus and a cognate response regulator that coordinates cellular response [50]. One TCS of major interest in the context of antibiotic resistance is the VraSR regulatory system [51, 52]. The Vra (standing for vancomycin resistance associated) system was first described in vancomycin-resistant strains Mu3 and Mu50, as being overexpressed when in the presence of this glycopeptide and playing a central role in vancomycin resistance [52]. Vancomycin is a CW targeting antibiotic, which binds to the D-Ala-D-Ala terminus in the PGN stem peptide, thus inhibiting TP. Besides vancomycin, the VraSR system also responds to other cell wall targeting antibiotics, including β-lactam oxacillin, D-cycloserine and bacitracin [49]. Homologues of VraSR, with a similar role in the response to CW stress, are present in other Gram-positive bacteria and include LiaRS from *Bacillus subtilis*, *Streptococcus pneumoniae* and *Streptococcus mutans,* and CesSR from *Lactococcus* species [53].

The *S. aureus* VraSR TCS is encoded in an operon [54]. VraS is a typical histidine kinase, belonging to the intramembrane sensing histidine kinase subfamily, with an N-terminal transmembrane domain and a C-terminal Histidine kinase core, while VraR belongs to the LuxR response regulators family, and is composed of an N-terminal regulatory domain and a C-terminal DNA-binding domain [55-57]. VraS, upon sensing cell wall insults, undergoes an autophosphorylation process. The phosphoryl group is subsequently transferred to the VraR response regulator, which becomes activated after conformational changes. Phosphorylated VraR binds to its own promoter and to other CW stimulon genes [55]. VraS also has phosphatase activity, which is responsible for triggering VraR inactivation [55]. VraSR therefore acts as a sentinel system, whose function is to detect conditions that represent a threat to the bacterial CW and trigger the appropriate response, in order to maintain its integrity [53, 58]. Several genes were shown, through microarray analysis, to be positively upregulated by *vraSR* system, in the presence of vancomycin. An expected, loss of induction was observed in a *vraSR* null mutant [51]. Among the genes regulated by VraSR is a set of genes involved in PGN biosynthesis, including *pbp2*, *sgtB* (monofunctional transglycosylase) and *murZ* (redundant murA isozyme) which are positively upregulated by this system, in order to increase cell wall synthesis compensating eventual damage occurred [51, 53]. Other genes overexpressed upon VraSR induction include stress response proteins, like *prsA*, as well as *vraSR* itself, due to a

positive feedback loop [51, 54]. Yin and colleagues stated that short-term induction of *pbp2* in the presence of oxacillin and vancomycin is mediate by VraSR, but longer-term *pbp2* induction might be triggered in a VraSR independent way, since only one out of the three different promoters of the *pbp2-recU* operon responds to the vraR response regulator. VraSR null mutants exhibit an increase in susceptibility to many CW targeting antibiotics, such as vancomycin, teicoplanin and oxacillin, as well as loss of induction of some PGN synthesis genes like *pbp2*. Even in the absence of antibiotic, genes involved in CW synthesis displayed a substantial decrease in their transcription, meaning that VraSR still maintains a basal expression level during regular cellular growth [51] .

Upstream of *vraS* there is an Open Reading Frame (ORF), which was named until recently *yvqF*, as depicted in Figure 3. *yvqF* and *vraR* mutants have a similar pattern in the MIC variation for some cell wall targeting antibiotics, suggesting that this ORF might have a role as relevant as *vraR* in protecting against cell wall damage [53]. Furthermore, according to the same authors, the CW stress stimulon is not induced in ΔyvqF strains indicating that YvqF positively modulates the expression of *vraSR*. Interestingly, there is a YvqF homologous protein in *B. subtilis* and *S. mutans,* called LiaF, part of the LiaSR regulatory system. However, contrary to YvqF, LiaF is responsible for repressing LiaSR signal transduction, as *liaF* mutants resulted in constitutive expression of CW stress stimulon genes in the absence of an inducing signal [44].

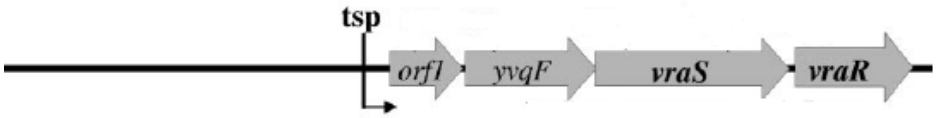


Figure 3 – Map of the *vraSR* operon. *vraSR* operon is composed of four different ORFs: ORF1, *yvqF*, *vraS* and *vraR. vraS* encodes for the VraS histidine kinase, responsible for sensing cell wall damage; *vraR* encodes for the cognate response regulator, responsible for the regulatory cascade to activate the cell wall stress stimulon (CWSS); *yvqF* encodes for a putative membrane protein with homology to LiaF from *B. subtilis*; ORF1 remains with unknown function. (Yin *et al* 2006).

Recently YvqF was renamed VraT [56]. According to Vavra and colleagues, the analyzed protein sequences of VraT revealed a conserved extracellular C-terminal domain and 4-5 transmembrane domain helices. Vavra and colleagues also showed that ΔvraS and ΔvraT showed very similar induction profiles in the presence of oxacillin, which may indicate that VraT, VraS and VraR encompass a three-component regulatory system, depicted in Figure 4. According to the new model proposed by these authors, VraT senses CW damage and, through conformational changes, promotes VraS autophosphorylation, which in turn leads to VraR activation. This data was supported by bacterial two-hybrid assays showing that VraT interacts with VraS but it does not interact directly with VraR [53].

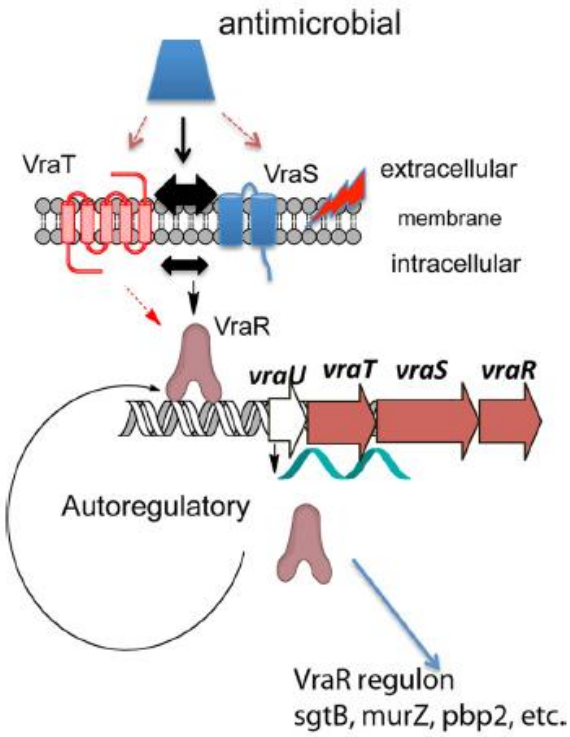


Figure 4 – VraTSR three-component regulatory system. This model proposes that the damage inflicted in the cell wall triggers a conformational change in VraT, which influences the autophosphorylation of VraS subsequently leading to the phosphorylation of VraR. VraR is responsible for activating the cell wall stimulon, through the activation and repression of several genes, including its own operon. The signal for the stimulus (lightning bolt) remains unknown, but it is likely to be a by-product of cell wall stress. Brown arrows in *vra* operon correspond to genes shown to be required for methicillin resistance; white arrow, indicates vraU which is not required for methicillin resistance; black double-headed arrows in membrane and cytoplasm, represent possible interaction between VraT and VraS following a stimulus. Dotted red and pink arrows, represent hypothetical interactions between the antimicrobial compound and VraS and VraT and between VraT and VraR. (Vavra *et al* 2013).

Single cell stochasticity in genetic expression

Every single cell in a population is a unique individual and, although many phenotypical differences can be explained by differences in genetic backgrounds, one cannot forget that environment still plays an important role in defining the different phenotypes. Yet, even genetically identical cells, subjected to the same environmental conditions can display considerable differences in their behavior. According to McAdams, variability in biological systems may be inevitable, due to the random character of physic-chemical reactions inside a single cell [59]. Once living cells possess low copy numbers of some components such as some regulatory proteins, stochasticity in genetic expression can result in substantial variability.

Jonathan and colleagues stated that there are four major sources responsible for variation among biological systems, namely the inherent stochasticity of biochemical reactions involving low abundant molecules such as during transcription; differences in gene expression due to specific cell cycle stages; differences in environmental conditions, owing to chemical gradients of a particular substance; variability due to genetic mutations. Noise of gene expression can be defined as the stochastic

fluctuation in transcription and or translation processes in isogenic cells and under identical experimental condition [60].Two kinds of noise may exist, as shown in Figure 5 [61]. Noise resulting from fluctuations in concentration, location and state of determined molecules in the output of a determined gene is called extrinsic. Intrinsic noise, which refers to variation of genetic expression due to random microscopic chemical events, even if the population is composed of isogenic cells, with identical states and concentrations of cellular components, and therefore called inherent stochasticity of a determined gene expression.

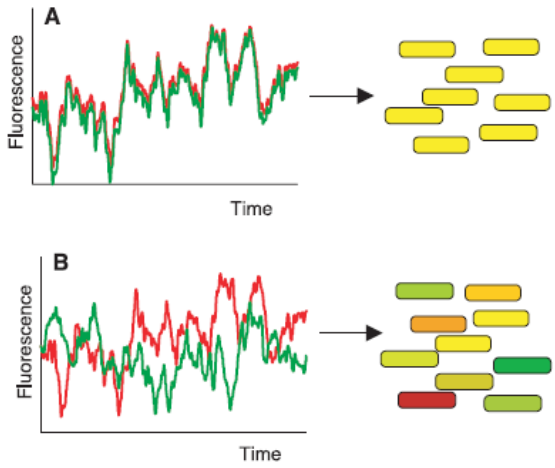


Figure 5 – Noise in genetic expression. Intrinsic and extrinsic noise (see main text) can be distinguished by expressing two genes (cfp – green, yfp - red) under the control of identical regulatory sequences. Cells with the same amount of both genes appear yellow, while cells expressing more of one fluorescent protein than the other appear red or green. (A) In the absence of intrinsic noise, the two fluorescent proteins fluctuate in a correlated way, which will render the same amount of both proteins in each cell, although that amount differs from cell to cell because of extrinsic noise. (B) The expression of the two genes, due to intrinsic noise, may become uncorrelated, generating a population where there are cells that express more of one protein than the other. (Elowitz *et al*, 2002)

There are interesting phenomena that can be associated with genetic noise that generates variability in isogenic populations. Due to genetic noise, a clonal population usually exhibits unimodal variation in the expression a determined gene. However, in some situations, genetic noise gives rise to a non-unimodal variation, in which the population bifurcates into coexisting subpopulations, known as bistable populations [62]. One important example of bistability is bacterial persistence under antibiotic treatment, which may allow survival of subpopulations in a genetically homogeneous population [63, 64]. Although this phenotype is still not yet fully understood, it was linked to the inherent heterogeneity in clonal populations, as persisters a slow or even absent cellular growth, hypothetically responsible for their tolerance to the antibiotics [65].

In this work, we want to determine if *vraSR* and *pbp2* expression is heterogeneous in isogenic populations in the presence of CW targeting antibiotics, to later determine if there are subpopulations more resistant/susceptible to antibiotic treatment.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 1.

Table 1 – Bacterial strains and Plasmids

Strain/Plasmid	Relevant Characteristics[a]	Source/Reference
Strain		
E. coli		
DC10B	Δ*dcm* in the DH10B background; Dam methylation only	[66]
S .aureus		
RN4220	MSSA strain, Restriction deficient derivative of NCTC8325-4	Rockefeller University collection
Newman	MSSA strain	[67]
MW2	Heterogeneous CA-MRSA strain	[68]
COL	Homogeneous HA-MRSA strain	Rockefeller University collection
Newman-PptafGFP	Newman expressing fast-folding GFP under the control of *pta* promoter, Erm^r	This study
Newman-Ppbp2fGFP	Newman expressing fast-folding GFP under the control of *pbp2* promoter, Erm^r	This study
Newman-PvrafGFP	Newman expressing fast-folding GFP under the control of *vraSR* operon promoter, Erm^r	This study
Newman-Ppta_mcherry-PvrafGFP	NewmanΔspa encoding mCherry under the control of *pta* promoter, from the *spa* locus, and fast-folding GFP under the control of *vraSR* promoter, Erm^r	This study
Newman-Ppta_mcherry-Ppbp2fGFP	NewmanΔspa encoding mCherry under the control of *pta* promoter from the *spa* locus and fast-folding GFP under the control of *pbp2* promoter, Erm^r	This study
MW2-PptafGFP	MW2 expressing fast-folding GFP under the control of *pta* promoter, Erm^r	This study
MW2-Ppbp2fGFP	MW2 expressing fast-folding GFP under the control of *pbp2* promoter, Erm^r	This study
MW2-PvrafgFP	MW2 expressing fast-folding GFP under the control of *vra* operon promoter, Erm^r	This study
MW2-Ppta_mcherry-PvrafGFP	MW2Δspa encoding mCherry under the control of *pta* promoter in the *spa* locus and fast-folding GFP under the control of *vra* promoter, Erm^r	This study
MW2-Ppta_mcherry-Ppbp2fGFP	MW2Δspa encoding mCherry under the control of *pta* promoter in the *spa* locus and fast-folding GFP under the control of *pbp2* promoter, Erm^r	This study
COL-PptafGFP	COL expressing fast-folding GFP under the control of *pta* promoter, Erm^r	This study
COL-Ppbp2fGFP	COL expressing fast-folding GFP under the control of *pbp2* promoter, Erm^r	This study
COL-PvrafgFP	COL expressing fast-folding GFP under the control of *vra* operon promoter, Erm^r	This study
COL-Ppta_mcherry-PvrafGFP	COLΔspa encoding mCherry under the control of *pta* promoter in the *spa* locus and fast-folding GFP under the control of *vra* promoter, Erm^r	This study
COL-Ppta_mcherry-Ppbp2fGFP	COLΔspa encoding mCherry under the control of *pta* promoter in the *spa* locus and fast-folding GFP under the control of *pbp2* promoter, Erm^r	This study
Plasmid		
pFAST	Plasmid encoding fast-folding *GFP*(P7), derivative of pSG5082;Amp^r, Erm^r	(J. Monteiro, M. G. Pinho,unpublished)
pROD17	Plasmid encoding *mcherry, Amp*^{r}	D. Sherratt

pMADspa	*E. coli – S. aureus* shuttle vector with a thermo sensitive *oriC* for Gram-positive bacteria containing the up and downstream regions of *spa*; *Amp^r, Erm^r; lacZ*	[69]
pFAST-Ppta	pFAST derivative containing *pta* promoter upstream of fGFP	This study
pFAST-Ppbp2	pFAST derivative containing *pbp2* promoter upstream of fGFP	This study
pFAST-Pvra	pFAST derivative containnig *vra* operon promoter upstream of fGFP	This study
pMADspa-Ppta_mcherry	pMADspa derivative encoding mCherry fused to pta promoter	This study

[a] abbreviations: Amp^r – Ampicillin resistant; Ery^r – Erythromycin resistant; GFP- Green Fluorescent Protein;

Growth conditions

S. aureus cells were grown at 37ºC, except where indicated, with aeration, on tryptic soy broth (TSB, Difco), or on tryptic soy agar (TSA,Difco), supplemented with erythromycin (10µg/ml) (Sigma), when required. *E. coli* strains were grown at 37ºC with aeration on Luria-Bertani broth (LB,Difco), or LB agar (Difco), supplemented with ampicillin (100µg/ml) (Sigma). Growth was followed by monitoring the optical density at 600 nm.

Molecular cloning methods

DNA purification and manipulation

Total DNA was purified from *S. aureus* cells grown overnight on TSA, at 37ºC. Cells were harvested and resuspended in in Ethylenediaminetetraacetic acid (EDTA) 50mM containing Lysostaphin 10 µg/mL (Sigma) and RNase 20 µg/mL (Sigma) and incubated at 37ºC for 30 minutes. The cells were then incubated at 80ºC with Nuclei Lysis solution (Promega) for 5 minutes. The samples were cooled to room temperature before addition of Protein Precipitation Solution (Promega) followed by 10 minutes incubation on ice. DNA was then precipitated with isopropanol, washed with ethanol 70% and resuspended in sterile water.

Plasmid DNA was purified from *E. coli* DC10B cells using the Wizard SV Plus Miniprep kit (Promega) accordingly to manufacturer's instructions. DNA fragments were digested with FastDigest restriction enzymes, purchased from Thermo Scientific (0.5-1µg DNA,1X FastDigest buffer, restriction endonuclease and water up to 20µl for 30minutes) and purified using purified using Wizard SV Plus Cleanup kit (Promega).

DNA ligations were performed using Rapid DNA ligation kit (Thermo Scientific), containing T4 DNA ligase and rapid ligation buffer. The reactions included insert and vector DNAs, 1X rapid ligation buffer and T4 DNA ligase, and were incubated during 30 minutes at room temperature.

PCR reactions were performed with GoTaq polymerase (Promega) for colony screenings and Phusion polymerase (Finnyzymes) for molecular cloning, following manufacturer's instructions.

E. coli transformation

E. coli competent cells were prepared according to the Rubidium Chloride protocol [70]. Briefly, early exponential growing cells (O.D. 0.4-0.5) were incubated on ice for 15 minutes and pelleted (6010 x g for 15 minutes at 4ºC). Cells were resuspended in 1/3 of culture volume of RF1 buffer (RbCl 100mM; MnCl2 tetrahydrate 50mM; Potassium acetate pH 7,5 35mM; Calcium chloride bihydrate 10mM; Glycerol 15%). The samples were then incubated on ice for 15 minutes, centrifuged (1320 x g for 15 minutes at 4ºC) and resuspended in 1/2 volume of cold RF2 buffer (MOPS 10 mM; $RbCl_2$ 10 mM; $CaCl_2$ bihydrate 75 mM; Glycerol 15%). Competent cells were frozen in liquid nitrogen and stored at -80ºC in 100 µL aliquots. For transformation, either plasmid or ligation mixtures were added to the thawed competent cells and kept on ice for 10 minutes. Then, cells were heat-shocked (45 seconds – 42ºC), in order to introduce incorporation of exogenous DNA, recovered (1 hour at 37ºC) in 1ml LB and plated in LA supplemented with ampicillin (100µg/ml).

S. aureus transformation

RN4220 electrocompetent cells were prepared as previously described [71]. Briefly, cells were incubated in TSB at 37ºC with aeration until an OD_{600nm} 0.4-0.5, and harvested by centrifugation (6010 x g for 15 minutes at 4ºC). The pellet was washed with an equal culture volume of ice-cold filter-sterilized 0.5M sucrose (Sigma), harvested and washed again in ½ volume of 0.5M sucrose. Cells were then incubated on ice for 15 minutes, resuspended in 1/100 of initial volume of sucrose 0.5M with 10% Glycerol (Fluka) and stored at -80ºC in 50µl aliquots. For transformation, competent cells were thawed on ice, mixed with 0.5µg of purified DNA and kept on ice for 10 minutes. The cells were then electroporated (2.5 kV; 25 µF and 100Ω) in a 0.2 cm BioRad Gene Pulser cuvette. After electroporation, the cells were recovered in 1mL TSB, incubated at 37ºC with aeration during 1 hour and plated on TSA supplemented with erythromycin (10µg/ml).

S. aureus transduction

Transduction was done using phage 80α as previously described [72]. In order to prepare phage lysates, cells from donor strains grown overnight were resuspended in TSB supplemented with $CaCl_2$ 5mM (final concentration). Phage 80α was serially diluted to 10^{-7} in phage buffer ($MgSO_4$ 1mM, $CaCl_2$ 4 mM, Tris-HCl 50 mM pH 7.8, NaCl 5.9 g/L, gelatin 1 g/L). Each of phage dilutions (10µl) were mixed to the cell suspension (10µl) in 3ml phage top agar (casamino acids 3 g/l, Difco; yeast extract 3g/L, Difco; sodium chloride 5.9 g/L, Sigma; agar 5 g/L, Difco; pH 7.8), supplemented with 5mM $CaCl_2$, stabilized for 1 hour at 50ºC. The mixtures were poured onto phage bottom agar (same composition as phage top agar but with 15g/L of agar) with 5mM $CaCl_2$ and incubated overnight at 30ºC. The plates showing confluent lysis were selected and incubated with 4mL of ice-cold phage buffer for 1 hour at 4ºC. The top agar and phage buffer were collected into a 50ml centrifuge tube and vortexed to disrupt the phage top agar. The tubes were kept 1 hour at 4ºC in order for the phage to be transferred to the phage buffer, and then centrifuged at 3500rpm for 15 minutes at 4ºC to sediment the top agar. The supernatant was collected and filtered through a 0.45 µm sterile filter.

For the transduction, the receiving strain was grown overnight in TSA at 37ºC and resuspended in 1mL TSB supplemented with 5mM $CaCl_2$. Different volumes of phage lysate (1µl, 10µl and 100µl) were mixed with 100µl of cell suspension and phage buffer to a final volume of 300µl. A control tube in which no phage lysate was added was also prepared. The transduction mixtures were incubated for 20 minutes at 37ºC and then added to 3ml 0.3GL top agar (casamino acids 3g/l, Difco; yeast extract 3g/l, Difco; NaCl, 5,9g/l Sigma; sodium l actate 60% syrup, 3,3ml/l, sigma; glycerol 50%, 2ml/l, Sigma; Tri-sodium citrate, 0,5g/l, Sigma; and agar 7,5g/l, Difco; pH 7,8) at 50ºC (pre-warmed for one hour). The samples were poured onto plates containing 10mL of 0.3GL bottom agar (identical to 0.3GL top but with 15g/l agar) with 30µg/l of Erythromycin (Sigma) and 20ml of 0.3GL bottom agar without antibiotic. The 0.3GL bottom agar plates were used within an hour after preparation.

Construction of *gfp* promoter fusions

Sequences of the primers used in this study are listed in Table 2. The restriction sites are underlined.

Table 2: Primers

Primer name	Sequence (5'-3')	Restriction Enzyme
fGFP_P2_NotI	GCGCGCGGCCGCTTAATGGTGATGATGGTGATG	NotI
mcherry_rev_nheI	GCGCGCGCTAGCTTACTTGTACAGCTCGTCCATGCCAC	NheI
mcherry-1ATG-link-15ntds	TTACAGGAGGACATTATGGCTATCATTAAAGAGTTCATGC	
pbp2_fw_new_BamHI	GCGCGCGGATCCCAACACATACTTGTACTTGCC	BamHI
Ppbp2_genome_int	CGACTACATCATTCATGAGGACGCCTCC	
Ppbp2_rev_new_EcoRI	GCGCGAATTCCGTCATACGCGGTCCTCACTTTCATC	EcoRI
pFAST3_remake_P1-GFP_ecoRI	GCGCGCGAATTCAGTAAAGGAGAAGAACTTTTCACTGG	EcoRI
pMADII	CGTCATCTACCTGCCTGGAC	
Pptafw KpnI	GCGCGGTACCGTGCTGTTGTTTCATCAAAGCGC	KpnI
pta_fw_nheI	GCGCGCGCTAGCGTGCTGTTGTTTCATCAAAGCGCATTTC	NheI
pta_genome-int_check	GAGTAGCGACTGTATAATTTCTATTGAGG	
Ppta-linker-15ntd_rev	AATGTCCTCCTGTAATATAAATCTTATTAATCATTCACGG	
Pptarev XhoI	GCGCGCCTCGAGGTAATATAAATCTTATTAATCATTCACG	XhoI
PVraSR_P1_KpnI	GCTGCGGTACCCGGTGCTATTTCTGCGCC	KpnI
PVraSR_P2_New_Xho	CGCGCTCGAGTTATAATAAGTTTTAAAATACCAAATGCGC	XhoI
Pvra_int_new	CAGGCAATGCAAGTTGGGGTACAACG	

spa-_P4_NCOI	TGCAGTCCATGGTTGAAAAAGAAAAACATTTATTC	NcoI
spaDown-p2	TGCGGTTTTAAGCCT	
spaUp-p1	AGATGTTGCTCGTGC	

In order to construct the *pta* promoter fusion, the region upstream of the gene was amplified from *S. aureus* COL genome using primers Pptafw KpnI/Pptarev XhoI, resulting in a 829bp DNA fragment. This fragment was digested and cloned into Kpn/XhoI restriction sites of pFAST, upstream of fast folding GFP, resulting in pFAST-Ppta. The *vra* promoter fusion was constructed by amplifying an 844bp region upstream of the *vra* operon, using primers PVraSR_P1_KpnI/ PVraSR_P2_New_Xho. The resulting DNA fragment was digested and cloned into Kpn/XhoI restriction sites of pFAST, upstream of fast-folding GFP, resulting in pFAST-Pvra. Both promoter fusions included the -35 and -10 consensus sequences of the respective promoter regions, but lack the Ribosome Binding Site (RBS), since fast folding *gfp* already possesses that sequence. The *pbp2* promoter region, depicted in Figure 6, was amplified using primers Ppbp2_fw_new_BamHI/ Ppbp2_rev_new_EcoRI. The 952bp region included the two promoter regions of the *recU-pbp2* operon and, in order not to disrupt *recU* gene, the RBS and the start codon of the pbp2 gene were also included in the sequence amplified. In this case, and to avoid two different ribosome binding sites and two start codons (the native and the one from GFP), these sequences were removed from *gfp* by PCR. The DNA fragment containing the *pbp2* promoter was digested and cloned into EcoRI/BamHI restriction sites of pFAST, replacing the region upstream of *gfp* and the first two codons, resulting in pFAST-Ppbp2.

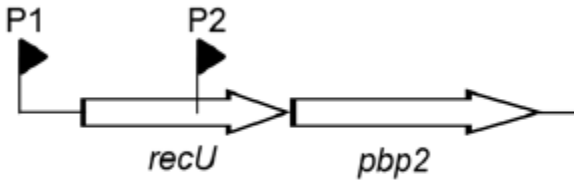


Figure 6 – The *recU*/*pbp2* operon. *pbp2* can be transcribed from different promoter regions (black flags). While P1 promoter is common to both *recU* and *pbp2*, the P2 promoter overlaps with the final fragment of *recU* coding sequence. (Pereira *et al*, 2013)

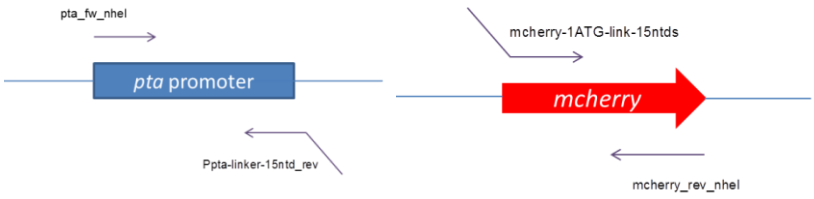
After extraction of the plasmids from *E. coli* DC10B, restriction digest confirmation was performed with the same enzymes used for cloning and the insert DNAs sequenced.

The constructed plasmids were electroporated into RN4220 competent cells and transduced into three different backgrounds: *S. aureus* Newman, MW2 and COL. Transformants were selected on erythromycin 10µg/ml plates at 37ºC and genomic integration of the three different plasmids was confirmed by PCR using the primer pairs pta_genome-int_check/ fGFP_P2_NotI for *pta* promoter fusion, Pvra_int_new/ fGFP_P2_NotI for *vra* promoter fusion and Ppbp2_genome_int/fGFP_P2_NotI for *pbp2* promoter fusion.

Construction of *mCherry* promoter fusions

The *pta-mcherry* promoter fusion was made using the overlap PCR method as previously described, depicted in Figure 7 [73]. In the first PCR, the same *pta* promoter region selected for the GFP promoter fusions was amplified from *S. aureus* COL genome using the primers pta_fw_nheI/Ppta-linker-15ntd_rev and *mCherry* was amplified from pROD17 with primers mcherry-1ATG-link-15ntds/mcherry_rev_nheI. These PCRs were done using Phusion polymerase (Finnzymes) and 20 cycles of denaturation at 98ºC for 10s, annealing at 55ºC for 30s, extension at 72ºC for 30s. In the second PCR reaction, equal amounts of both DNA fragments were mixed and primers Ppta-linker-15ntd_rev and mcherry-1ATG-link-15ntds, containing an overlapping sequence of 15 nucleotides, including the *pta* native RBS, were used. The primer pair pta_fw_nheI/ mcherry_rev_nheI was used in order to amplify the promoter fusion DNA sequence with the Phusion polymerase for 20 cycles of denaturation at 98ºC for 10s, annealing at 58ºC for 30s, extension at 72ºC for 1m. The final PCR product was digested with NheI and cloned into thermosensitive plasmid pMADspa, resulting in pMADspa-Ppta_mcherry. Correct orientation of the insert was confirmed by PCR using primers pMADII/mcherry_rev_nheI. The plasmid was extracted from E. coli DC10B, digested with NheI to confirm the presence of the insert and sequenced.

First PCR:



Second PCR:

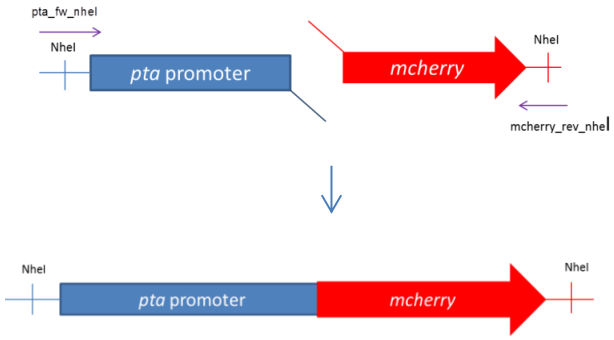


Figure 7 – Stategy for the construction of *mcherry* fusion with *pta* promoter. *pta* promoter and mCherry sequences were amplified using primer pairs pta_fw_nheI/Ppta-linker-15ntd_rev and mcherry-1ATG-link-15ntds/mcherry_rev_nheI respectively. In the second PCR, the fusion was amplified using primers pta_fw_nheI/mcherry_rev_nheI. Primers Ppta-linker-15ntds_rev and mcherry-1ATG-link-15ntds contain a complementary sequence required for the overlapping reaction. The overlapping sequence included *pta* native RBS.

The pMADspa-Ppta_mcherry plasmid was introduced in RN4220 by electroporation and then transduced to Newman, MW2 and COL strains. Erythromycin resistant colonies were inoculated at 30ºC in TSB supplemented with erythromycin 10µg/ml for 16h. The overnight culture was diluted 1:1000 into the same medium and incubated for 8 hours at the same temperature, in order to allow plasmid replication and the consequent increase of the number of plasmid copies per cell. The culture was diluted 1:1000 again in the same medium and incubated overnight at 43ºC, a temperature that prevents replication of the plasmid, since it harbors a thermosensitive origin of replication. The overnight culture was serially diluted and 100 µL of 10^{-4},10^{-5} and 10^{-6} dilutions were plated in TSA containing Ery and bromo-chloro-indolyl-galactopyranoside (X-GAL) 100µg/mL at 43ºC. Several light blue colonies (candidates for integrated plasmid) were selected and restreaked in the same conditions. The integration of pMADspa-Ppta_mcherry plasmid into the chromosome was confirmed by PCR using primers spa-_P4_NCOI and pMADII. Clones with the plasmid successfully integrated into the chromosome were inoculated in TSB at 30ºC, overnight. The overnight culture was diluted 1:500 in the same conditions at 30ºC for 8 hours and serial dilutions (10^{-3}, 10^{-4}, 10^{-5}) were plated at TSA containing X-GAL 100 µg/mL at 43ºC. White colonies, representing candidates for the double crossover event and loss of the plasmid were restreaked on TSA. Colonies which retained the *mCherry* promoter fusion in the chromosome were first selected by susceptibility to erythromycin and then by PCR screening using primers mcherry_rev_nheI and spaUp-p1. Successful exchange of the *spa* gene by the *mCherry* fusion was confirmed using primers spaUp-p1/spaDown-P2.

Electrophoretic analysis of proteins by SDS-PAGE

S. aureus cells were grown to exponential phase (40ml, $O.D_{600nm}$ 0.8) and harvested by centrifugation (845 x g, 15 minutes at 4ºC). The pellet was then washed once with equal volume of PBS (NaCl 137mM, KCl 2,7mM, Na_2HPO_4 10mM, KH_2PO_4 1,8mM) and resuspended in 1/20 of the original volume in PBS with 1 mM of phenylmethanesulfonylfluoride PMSF (Sigma). Cells were disrupted with 200 µl of glass beads in a FastPrep FP120 (Thermo Electro Corporation). Glass beads were removed by centrifugation (9400 x g, 5 minutes at 4ºC) and total protein content of the extracts was quantified by the Bradford method, using bovine serum albumin as a standard (BCA protein assay kit, Pierce). Equal amounts of protein from each sample were mixed with SDS-PAGE sample buffer and samples were applied to a 10% polyacrylamide (30%acrylamide/bis solution, 37.5:1) protein gel and separated at 120V during 2 hours. The molecular weight marker used was the SDSPAGE Molecular Weight Standards, low range (Biorad).

Minimum inhibitory concentration assays

Population analysis profiles were performed in order to determine the minimum inhibitory concentration (MIC) of the different strains. Strains were grown overnight in TSB supplemented with erythromycin 10µg/ml, when needed. Overnight cultures were serially diluted (10^0 to 10^{-6}) and 25µl were plated on TSA containing increasing antibiotic concentrations. Inocula were spread with 10µl loops, plates were incubated at 37ºC for 24 or 48 hours and the colony-forming units (CFUs) were counted. The logarithmic base ten of the number of colony forming units per mL (CFU/ml) was plotted against the antibiotic concentration, and the MIC was defined as the antibiotic concentration required to inhibit the growth of 99.9 % of cells.

Fluorescence microscopy

Strains were grown in TSB supplemented with erythromycin 10µg/ml at 37ºC, diluted 1:200 in TSB and allowed to grow until mid-exponential phase ($O.D_{600nm}$ 0.5). When needed, antibiotic was added to the medium and the cultures incubated in the same conditions. 1ml of culture was pelleted (16000 x g 1minute at room temperature) and resuspended in 20µl PBS. 1µl of culture was placed on a thin layer of 1.2% agarose in PBS. Fluorescence microscopy was performed using a Zeiss Axio Observer.Z1 microscope equipped with a Photometrics CoolSNAP HQ2 camera (Roper Scientific), using phase contrast objective Pln Apo 100 x/1.4 oil Ph3, with 0,24µm resolution and 0,55 numerical aperture. The software used was Metamorph (Molecular devices). All fluorescence microscopy images were acquired using 1000msec exposition time for GFP and 5000msec exposition time for mCherry.

Data and statistical analysis

Fluorescence quantification was performed with the cell image analysis software Cell Profiler™, using the Otsu per object thresholding method. The threshold correction factor ranged between 1.08 and 2, depending on the background intensity of the images analysed. Statistical analysis comprised either non parametric Wilcoxon MannWhitney Test for mean comparison between treated and non-treated settings or F-tests for variance comparison between the same settings. Fluorescence data was subjected to variance-stabilizing transformation, taking into consideration that variance may increase with mean increase. Statistical tests were performed with a 95% confidence interval.

Results

Construction of reporter *gfp* promoter fusions

In order to study the promoter activity of the *vraSR* operon and *pbp2* gene, their promoter sequences were fused to a gene encoding the fluorescent reporter fast-folding green fluorescent protein GFP [74]. These constructs were inserted into their native *S. aureus* chromosomal loci, which resulted in the duplication of the promoter regions, as depicted in Figure 8. This strategy allows the native operons to remain intact, while we monitor the expression of the promoters. The constructs were inserted into three different *S. aureus* backgrounds, HA-MRSA MW2, CA-MRSA COL and MSSA Newman. Hence, we can find if there are different responses to the presence of β-lactams, depending on the strain background. The *vraSR* and *pbp2* genes belong to the cell wall stimulon [75] , and are upregulated by VraR response regulator [51, 54, 76]. Consequently these genes respond, by increasing their expression, to cell wall targeting antibiotics like the β-lactam oxacillin or the glycopeptide vancomycin.

We have also made a *gfp* promoter fusion using the *pta* promoter. This construct was used as a control since *pta* is a housekeeping gene that has a role in acetyl-CoA metabolism [77-79] and its expression is not expected to vary in the absence or presence of different antibiotics. The *pta* promoter was fused to the gene encoding fast-folding GFP, similarly to the promoters from the cell wall stress stimulon, and inserted in its native locus in the *S. aureus* genome. The set of the three constructed strains (Figure 8) therefore allows us to study expression from the *vraSR*, *pbp2* and *pta* promoters, using GFP as a reporter.

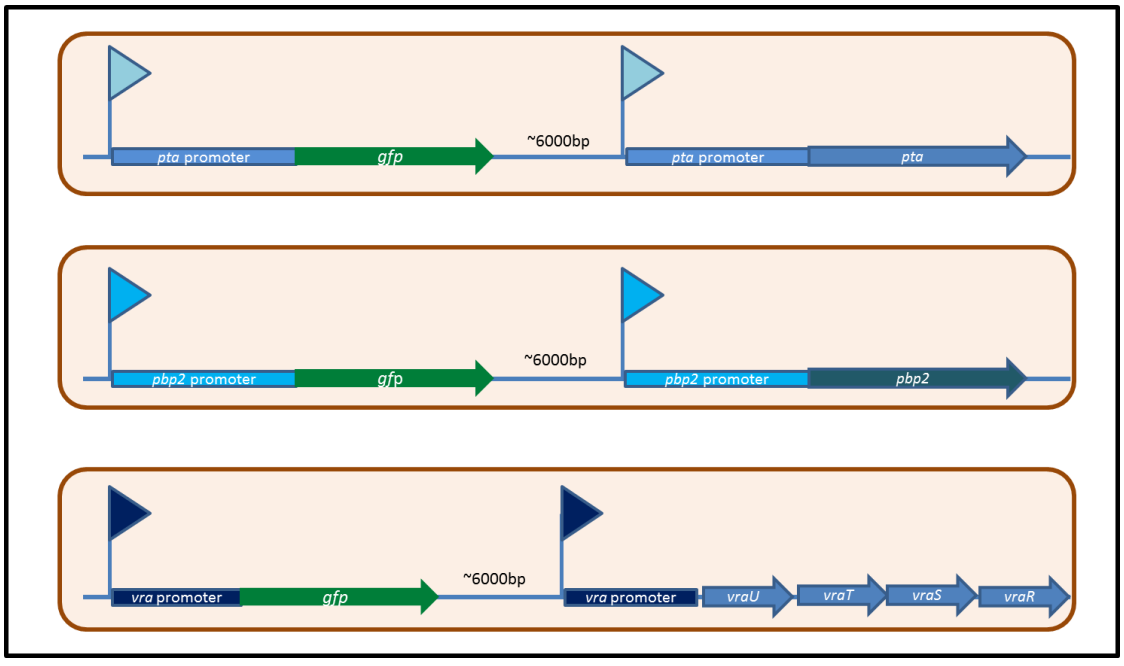


Figure 8 – Schematic representation of the chromosomal organization of strains expressing *vraSR*, *pbp2* and *pta* promoter fusions to *gfp***.** The integration of the different constructs into the genome resulted in the duplication of the promoter sequences. Therefore, we can follow the activity of the different promoters while maintaining the integrity of the native operons.

As referred in the Materials and Methods section, the RBS from *gfp* was maintained in all promoter fusions, except for the case of *pbp2*, in which the *pbp2* RBS was used, in order not to disrupt the *recU* gene coding sequence. The *recU* gene is immediately upstream of the *pbp2* gene and its last two codons overlap the ATG codon of *pbp2.* Therefore *pbp2* RBS sequence is in the coding sequence of *recU.* Furthermore *recU* coding sequence contains one of the *pbp2* promoters. Therefore, excluding *pbp2* RBS from the sequence cloned in the plasmid result in the presence of a truncated *recU* gene and could result in unwanted phenotypes [80].

Given that the aim of our work was to study the response of *S. aureus* to the presence of antibiotics, we wanted to ascertain that the genetic manipulations that were required to obtain the reporter strains did not have an effect on their antibiotic resistance profiles. For that purpose we determined the population analysis profiles of strains constructed in Newman, MW2 and COL backgrounds for oxacillin and vancomycin. We also used kanamycin, a protein synthesis inhibitor, as we wanted to use it as a control antibiotic to determine if phenotypes observed in subsequent experiments were specific for cell wall targeting antibiotics.

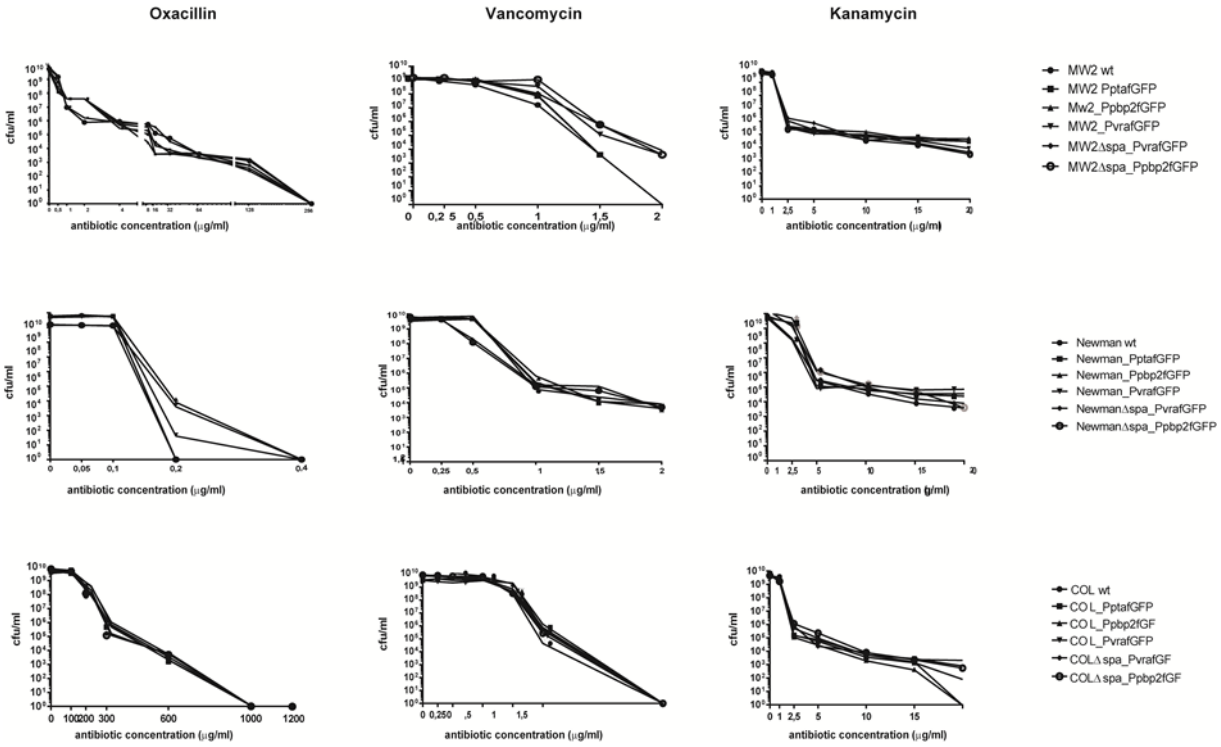


Figure 9 – Population analysis profiles of GFP reporter strains for oxacillin, kanamycin and vancomycin. First row: MW2 strains; **Second row**: Newman strains; **Third row**: COL strains. All strains exhibit the same MIC as their respective *wt* parental strains The MICs obtained were the following: MW2-Oxacillin: 256μg/ml (see main text); MW2-Kanamycin: 2,5μg/ml; MW2-Vancomycin:1,5μg/ml; COL-Oxacillin: 300μg/ml; COL-Kanamycin:2,5μg/ml; COL-Vancomycin: 2μg/ml; Newman-Oxacillin:0,2μg/ml; Newman-Kanamycin:5μg/ml and Newman-Vancomycin:1μg/ml

Figure 9 shows that the GFP reporter strains have the same MIC for all the antibiotics tested as the respective wild-type parental strains, which indicates that introduction of the promoter fusions in the genome did not change antibiotic susceptibility. As expected, there is a large difference between the oxacillin MICs of MRSA and MSSA backgrounds, while vancomycin and kanamycin MICs are similar. Regarding oxacillin, MSSA Newman exhibited a typical susceptible behavior, in which almost all the cells die when the MIC is reached. As for heterogeneously-resistant MRSA MW2, we could see that, in an isogenic population, different cells displayed different oxacillin susceptibilities and only when we reach 256µg /ml of antibiotic concentration, all cells are killed. Considering the MIC to be a 3 log decrease in cfu/ml, the MIC for MW2 would be around 2-4µg/ml, but since it is a heterogeneous-resistant strain, this concentration would not kill the resistant subpopulations. In order to embrace all subpopulations, for the purpose of this work we considered 1x MIC that of the resistant subpopulation (256µg/ml). Homogeneously-resistant MRSA COL cells had an MIC of 300µg/ml for oxacillin. Contrary to the results obtained with oxacillin, the susceptibility to vancomycin was uniform in all backgrounds and all strains were susceptible to this antibiotic. All strains showed an uniform susceptibility to the aminoglycoside kanamycin although resistant subpopulations were present. As kanamycin targets 30S subunit of the ribosome, point mutations in the target, that unable the binding of the antibiotic molecule, might be one plausible explanation.

vraSR is induced by β-lactam oxacillin and can be used as a probe of the CWSS induction

VraSR constitutes a two-component regulatory system, which is responsible for sensing cell wall damage through VraS and triggering the physiological response through the cognate VraR response regulator. The *vraSR* promoter has a basal expression in the absence of cell wall targeting antibiotics and it is activated in their presence [51, 58]

As an initial test to confirm that the strains constructed to study *vraSR* expression were indeed responding to the presence of cell wall targeting antibiotics, a SDS-PAGE gel was performed. As controls, we tested the activity of *pta* promoter in the presence of oxacillin, as well as the activity of both promoter fusions in the presence of kanamycin. Conditions expected to result in strong *vraSR* induction were used (1x MIC during 1 hour). This assay was performed using MRSA and MSSA strains to check if there were differences between those backgrounds in the promoter responses to the different conditions. Figure 10 shows that the *vraSR* promoter exhibited, as expected, a very low basal expression in the absence of antibiotics or in the presence of kanamycin. However, when the cell wall targeting antibiotic oxacillin was added to the media, *vraS* was induced. This was observed both in MRSA and MSSA backgrounds. Regarding the *pta* promoter, its expression was essentially constant in the different conditions tested. In MW2, addition of kanamycin seemed to have slightly lowered the level of genetic expression driven by *pta.* It is possible that this variation was due to sample loading error, despite the fact that we have measure the total protein content in each sample

and loaded the same amount in each lane. In Newman, the *pta* promoter expression was constant in the conditions tested.

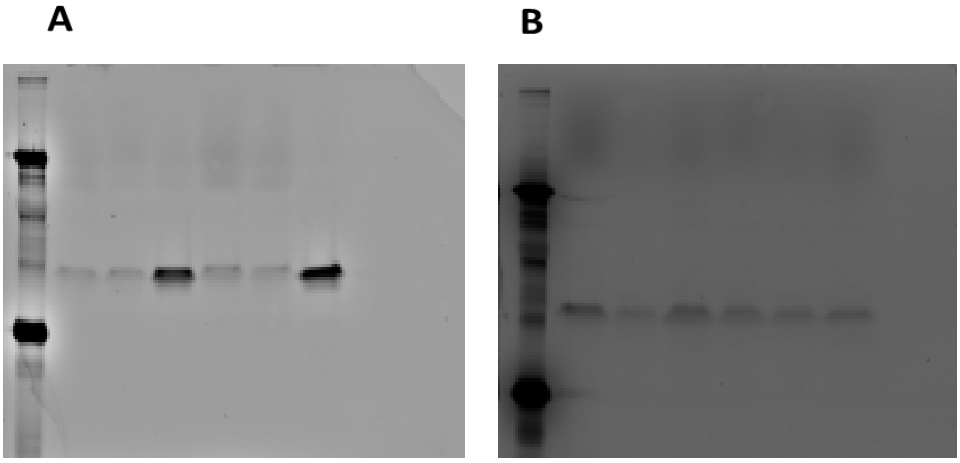


Figure 10 – *vraSR* but not *pta* promoter is induced by β-lactams. Blue laser light scanning (to detect GFP fluorescence) of the protein extracts from the Pvra (A) and Ppta (B) GFP reporter strains in MW2 and Newman after SDS-PAGE separation**. (A)** Lane 1 – MW2_PvrafGFP in the absence of antibiotics; Lane 2 – MW2_PvrafGFP + Kanamycin; Lane 3 – MW2_PvrafGFP + Oxacillin; Lane 4 – Newman_PvrafGFP in the absence of antibiotics; Lane 5 – Newman_PvrafGFP + Kanamycin; Lane 6 – Newman_PvrafGFP + Oxacillin. **(B)** Lane 1 – MW2_PptafGFP in the absence of antibiotics; Lane 2 – MW2_PptafGFP + Kanamycin; Lane 3 – MW2_PptafGFP + Oxacillin; Lane 4 – Newman_PptafGFP in the absence of antibiotics; Lane 5 – Newman_PptafGFP + Kanamycin; Lane 6 – Newman_PptafGFP + Oxacillin. Cells were exposed to antibiotics at 1X MIC during one hour.

In order to choose an experimental condition that would allow the comparison of the promoter activity of both MRSA strains (COL and MW2) and the MSSA strain (Newman), we first determined which antibiotics concentrations would activate the *vraSR* promoter in all strains to easily detectable levels. Given that we are working with strains with very different oxacillin MICs (MRSA and MSSA backgrounds) we could not select one oxacillin concentration that would induce the *vraSR* system to high levels in the three backgrounds, but did not kill any of the strains. We therefore decided to work with constant fraction of each strains MIC, rather that constant antibiotic concentrations. For the preliminary tests, we chose the *pta* and *vraSR* promoter GFP fusions and defined three different standard concentrations: ¼ x MIC, ½ x MIC, 1 x MIC and two time points, 30 and 60 min. To analyze the results, mean fluorescence values were compared between antibiotic treated/ non-treated conditions.

Quantification of the fluorescence signal in the microscopy images was performed by delimitating cell borders, using the contrast between the signal of image background and fluorescence intensity within the cytoplasm as illustrated in Figure 11. The fluorescence signal of each pixel inside the cytoplasm was automatically summed and the value subsequently divided by the area occupied by the cell, rendering the integrated fluorescence. Background signal was then subtracted to all images, which allowed standardizing of fluorescence values obtained in the different conditions, showed in Tables 3 and 4.

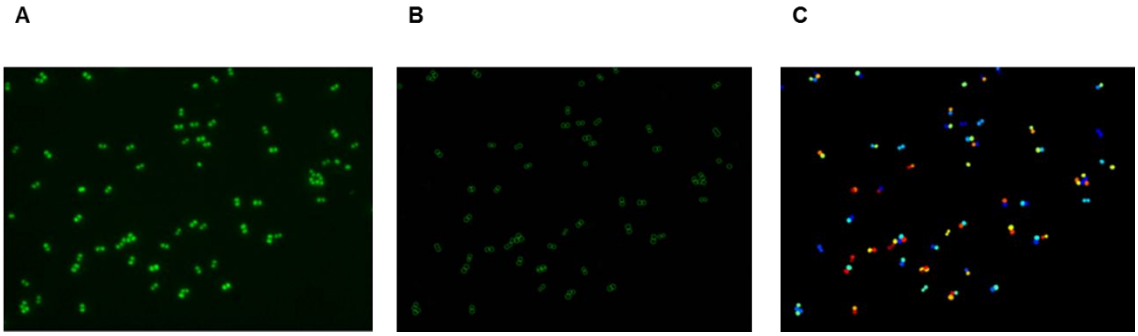


Figure 11 - Images illustrating the quantification process by Cell Profiler software: (A) Fluorescence microscopy image of *S. aureus* expressing GFP **(B)** Automatic delimitation of cellular borders, according to the fluorescence intensity displayed **(C)** Calculation of the area occupied by each cell.

	no antibiotic	1/4 x MIC	1/2 x MIC	1x MIC
MW2_Pvra	4,897	13,368	17,148	18,383
MW2_Ppta	4,569	7,348	5,182	6,421
Newman_Pvra	6,783	6,934	9,222	13,827
Newman_Ppta	8,594	8,47 *	10,923	5,773
COL_Pvra	6,048	11,553	17,388	9,957
COL_Ppta	6,148	6,64	7,709	6,123 *

Table 3: *vraSR* and *pta* promoters activity in the presence of oxacillin for 30 min: Average values of fluorescence intensity displayed by cells encoding promoter reporters for *vraSR* and *pta* promoters, in the presence of different oxacillin concentrations. Fluorescence intensity data was obtained after 30 minutes of antibiotic exposure. * represent values not statistically different relatively to the non-treated condition.

	no antibiotic	1/4 x MIC	1/2 x MIC	1x MIC
MW2_Pvra	5,245	56,801	51,693	47,791
MW2_Ppta	4,662	8,549	8,513	7,918
Newman_Pvra	7,504	10,871	8,319	48,787
Newman_Ppta	7,76	11,297	7,849	10,793
COL_Pvra	3,7	8,457	11,377	16,838
COL_Ppta	6,373	7,311	9,277	8,572

Tabela 4: *vraSR* and *pta* promoters activity in the presence of oxacillin for 60 min: Average values of fluorescence intensity displayed by cells encoding promoter reporters for *vraSR* and *pta* promoters in the presence of different oxacillin concentrations. Fluorescence intensity data was obtained after 1 hour of antibiotic exposure. * represent values not statistically different relatively to the non-treated condition.

In the absence of antibiotic, the *vraSR* promoter exhibited a basal expression. When cells were challenged with ¼ x MIC during 30 minutes, *vraSR* induction was evident only in MW2 background. After 1 hour of antibiotic exposure, the promoter response could be observed in both MRSA backgrounds, while *pta* expression increased only slightly. However, this antibiotic concentration, at both time points, was not sufficient to induce vraSR response in MSSA strain Newman, due to the low

antibiotic concentration used (0,05µg/ml), most likely insufficient to acylate PBPs and result in cell wall damage. When we used ½ x MIC, both MRSA backgrounds but not Newman showed over two fold *vraSR* induction as soon as 30 minutes after antibiotic addition. Treating Newman strains with 1x oxacillin MIC (0,2µg/ml) induced a clear *vraSR* response in this background, which was even more pronounced after 1 hour of antibiotic exposure. In this particular experiment COL did not exhibit a strong *vraSR* response after 30 minutes of antibiotic exposure at 1X MIC, which is not in line with the remaining data obtained for this strain, namely given that 30 min exposure to ½ X oxacillin MIC was sufficient to induce *vraSR* expression almost three fold.

Taking together the data collected in these preliminary experiments on *vraSR* activation, the conditions chosen to test cell wall stimulon promoters were ½ x MIC (30min and 1h), conditions that induce *vraSR* but do not result in considerable cell death, as judged through staining with cell death dye propidium iodide (data not shown). Additionally, we decided to use 1 x MIC for strains in Newman background, because ½ X MIC corresponds to very low oxacillin concentration, which does not activate the *vraSR* system.

Cell wall stress stimulon promoters response to antibiotics

The aim of this work is to determine if there genetic expression variability in the expression of CWSS promoters in response to the presence of cell wall targeting antibiotics. We have used the β-lactam oxacillin, which targets peptidoglycan synthesis and which we have shown above induces *vraSR,* to test the system. To ensure that our system was not promiscuous, but instead responded specifically to cell wall targeting antibiotics, we used the aminoglycoside kanamycin as a control. Kanamycin inhibits protein synthesis by disrupting the ribosome translational complex. As it does not target any of the cell wall components, it is a good control to verify the specificity of the induction of CWSS promoters by different antibiotics. We chose to treat the cells with 1 X kanamycin MIC and analyze them after 1 hour of antibiotic exposure. These conditions will provide a proper control, since we want to study heterogeneity in cell wall stimulon promoters. As such, we chose the treatment that is supposed to generate more variability within a population derived from cellular stress.

Cells were challenged in the conditions selected above and imaged by fluorescence microscopy. The integrated fluorescence was calculated for each cell and used to determine the mean fluorescence intensity of each population, as well as the fluorescence intensity variability within the population. To test the variability displayed by the different promoters in the presence of antibiotics, one could simply use variance comparison. However, it is known that variance increase does not necessarily directly correspond to an increase in genetic expression heterogeneity, as variance increase could be due to mean increase itself. .In other words, mean increase can mask an increase in the variance, for example a 10% variation of a mean of 2 corresponds to ± 0.2, while a variation of 10% of a mean of 20 corresponds to ± 2, which would apparently correspond to higher variance To determine whether the increase in the variance was due to an increase in heterogeneity or merely to mean increase we performed a variance-stabilizing transformation by calculating the logarithm of gene

expression values, as it is known that genetic expression levels among a biological population tend to be well approached by a lognormal distribution [81]. This way, we can test directly the variability exhibited by the populations, independently of the mean value increment, by analyzing the variances of the logarithm of the data collected in the different sets of experiments [82]. Variability of each CWSS promoter in the presence of antibiotic, obtained after the log-transformation of the fluorescence data, was compared to the variability exhibited in the absence of antibiotic (Tables 6, 8,10,12,14 and 16). Raw genetic expression variability was represented using histograms, in which cells were split into different classes according to the fluorescence intensity displayed. The fluorescence data used to make the histograms was not subjected to variance-stabilizing transformation, in order to get a clear view over the variability displayed. The y axis represents the number of cells inside each fluorescence intensity class and the x axis the number of classes. Thus, a broad distribution evidenced in a histogram corresponds to an increased variability.

Analysis of genetic expression in MW2 GFP reporter strains

MW2 GFP reporter strains were subjected to the conditions referred previously and fluorescence microscopy images were taken to quantify fluorescence intensity displayed (Figure 12).

Data collected was presented either in bar charts showing mean fluorescence intensity exhibited by the cells (Figures 13-A, 14-A, 14-C), in histograms showing genetic expression variability of the promoters (Figures 13-B, 14-B, 14-D) or in tables showing either the summary of the mean fluorescence intensity or variance of the log-transformed data to measure variability.

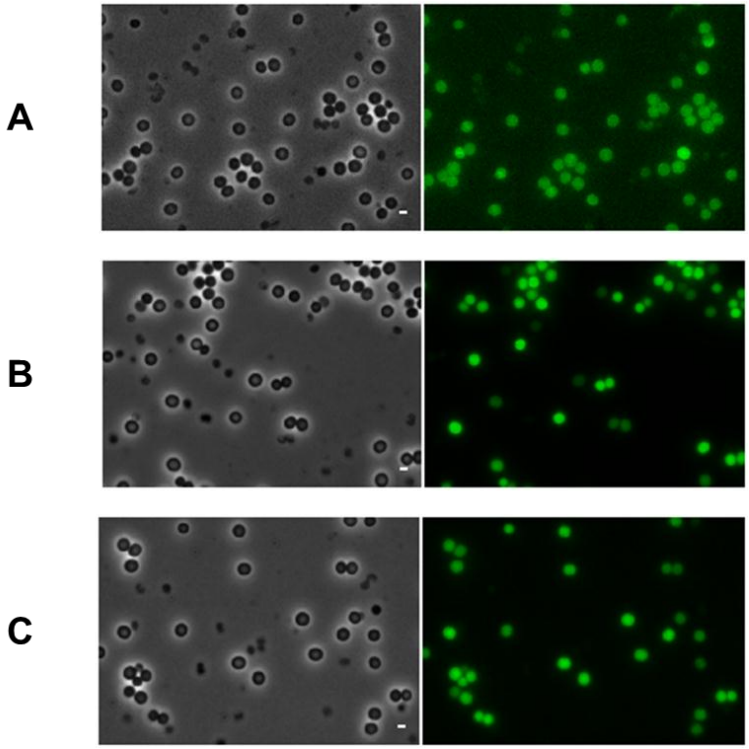


Figure 12 - Microscopy images of isogenic MW2 reporter cells exposed to ½ x MIC Oxacillin for 30min. MW2 cells expressing GFP under the control of the *pta* promoter (A), the *pbp2* promoter (B) or the *vraSR* promoter (C). For each example: left – phase contrast image; right – GFP image. Scale bar = 1µm

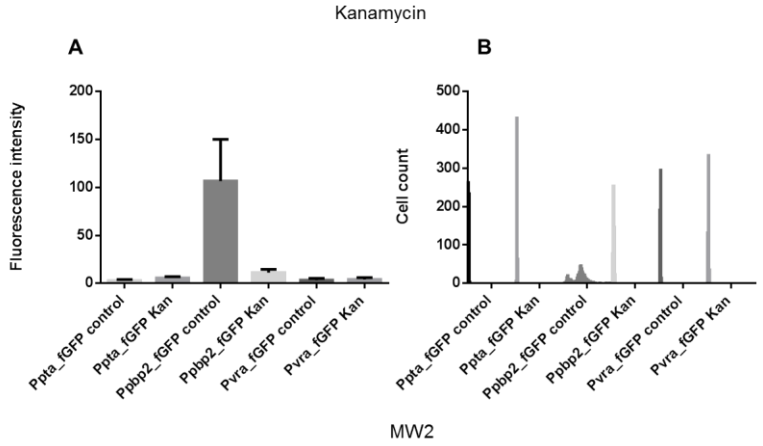


Figure 13 - Expression of *pta, vraSR* and *pbp2* promoters in Newman GFP reporter strains upon Kanamycin treatment (1 x MIC) for 1 hour. (A) For each strain, CWSS and housekeeping promoters activitywas followed by measuring the GFP fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (Kan) or absence (control) of antibiotic.(B) Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of kanamycin. Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500 cells

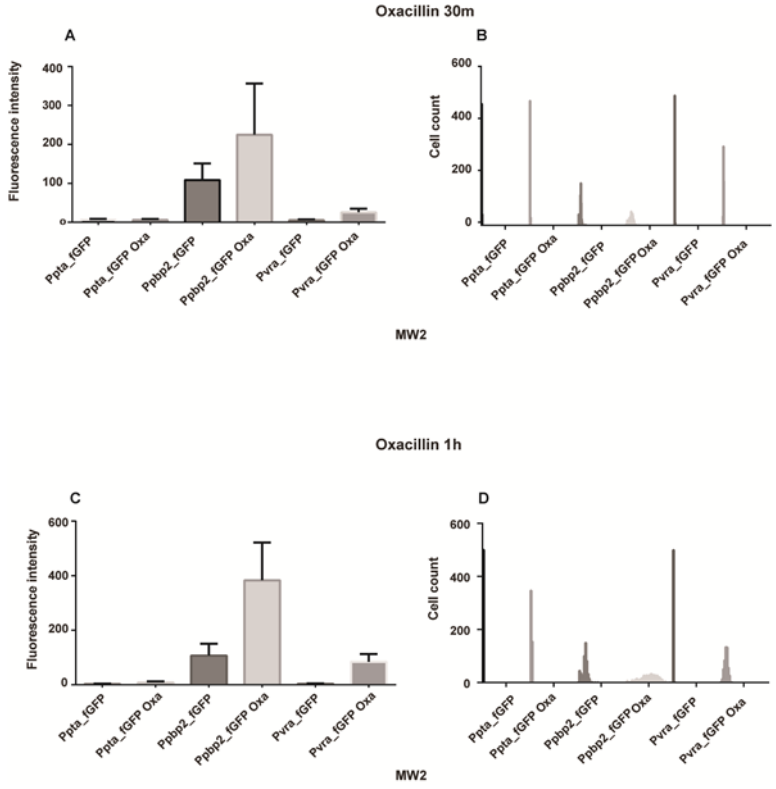


Figure 14 - Expression of *pta, vraSR* and *pbp2* promoters in MW2 GFP reporter strains upon Oxacillin treatment (1/2 x MIC) for 30 min and 1 hour. For each strain, CWSS and housekeeping promoters activity was followed by measuring the GFP fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (Oxa) or absence of antibiotic after 30min (A) or 1h (C). Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of oxacillin after 30min (B) or 1h (D). Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500 cells

Data in figures 13 and 14 regarding the activity of the different promoters determined by the mean fluorescence intensity of approximately 500 cells was summarized in table 5. MW2 reporter cells, when challenged with a cell wall targeting antibiotic, activate both *vraSR* and *pbp2* promoters, while *pta* expression remains essentially unaltered. Induction of *vraSR* and *pbp2* was observed after 30 minutes of cells exposure to oxacillin and was higher after 1 hour of antibiotic exposure. Importantly, exposure to the protein synthesis inhibitor kanamycin did not result in induction of CWSS promoters. A huge drop in *pbp2* expression could be observed upon kanamycin treatment, but this was not observed for strains Newman and COL and therefore may be a non-reproducible result.

	no antibiotic 30m	no antibiotic 1h	Kanamycin 1h	Oxacillin 30m	Oxacillin 1h
MW2					
pta	4,876	7,114	5,605	5,828	8,703
vraSR	5,032	3,343	4,134	25,305	84,140
pbp2	108,552	106,680	11,179	224,746	383,074

Table 5: Activity of the CWSS and housekeeping promoters in different conditions, measured by GFP fluorescence intensity: Average values of fluorescence intensity displayed by cells encoding the *vraSR, pbp2* and *pta* promoter fusions in the presence of different antibiotic concentrations. Fluorescence intensity data was obtained after 30 minutes or 1 hour of antibiotic exposure. Green boxes represent more than 100% fluorescence signal increment, in relation to the non-treated condition. * represent values not statistically different relatively to the non-treated condition.

Genetic expression variability evaluated through the histograms (figures 13-B, 14-B, 14-D) is displayed in table 6, in which each value represents the variance of the log-transformed data obtained for each condition, rendering the expression variability.

	no antibiotic 30m	no antibiotic 1h	kanamycin 1h	oxacillin 30m	oxacillin 1h
MW2					
pta	0,022	0,060	0,015	0,060	0,040
vraSR	0,060	0,130	0,050	0,040	0,030
pbp2	0,072	0,067	0,019	0,150	0,055*

Table 6: Variability measurement of the activity of CWSS and housekeeping promoters in different conditions. Average values were obtained by taking the variance of the log-transformed data of the fluorescence intensity. The variance of the log-transformed data renders expression variability independent from mean increase. Green boxes represent variability values 100% higher than the correspondent value showed by the non-treated condition. * represent values not statistically different relatively to the non-treated condition.

Gene expression variability of *vraSR* and *pbp2* promoters, at the single cell level, did not increase upon exposure to oxacillin. Furthermore variability in expression of *pbp2* showed a decrease in this parameter when in the presence of kanamycin. This means that, despite the presence of variability in

the system, there was no systematic variability increase correlated with the presence of cell wall targeting antibiotics. Furthermore, *pta* increased genetic expression variability in some conditions. Since both *vraSR* and *pbp2* were successfully activated in the presence of oxacillin and once the expression variability did not increase for these two promoters, it suggests that the higher heterogeneity observed is due to increase in gene expression of these promoters and the variance observed is due to increase the increase of the mean fluorescence values.

Analysis of genetic expression in Newman GFP reporter strains

Newman GFP reporter strains were subjected to the same conditions described above. Data collected was presented either in the bar charts below showing mean fluorescence intensity exhibited by the cells (Figures 15-A, 16-A, 16-C, 18-A, 18-C) and in the correspondent histograms showing genetic expression variability of the promoters (Figures 15-B, 16-B, 16-D, 18-B, 18-B).

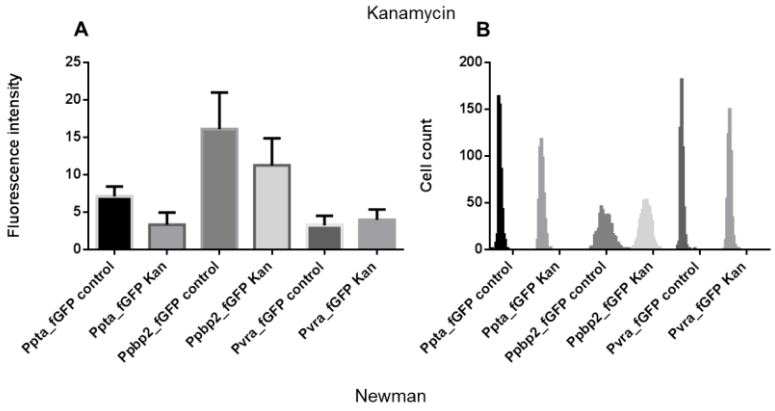


Figure 15 - Expression of *pta, vraSR* and *pbp2* promoters in Newman GFP reporter strains upon Kanamycin treatment (1 x MIC) for 1 hour. (A) For each strain, CWSS and housekeeping promoters activity was followed by measuring the GFP fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (kan) or absence (control) of antibiotic.(B) Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of kanamycin. Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500 cells

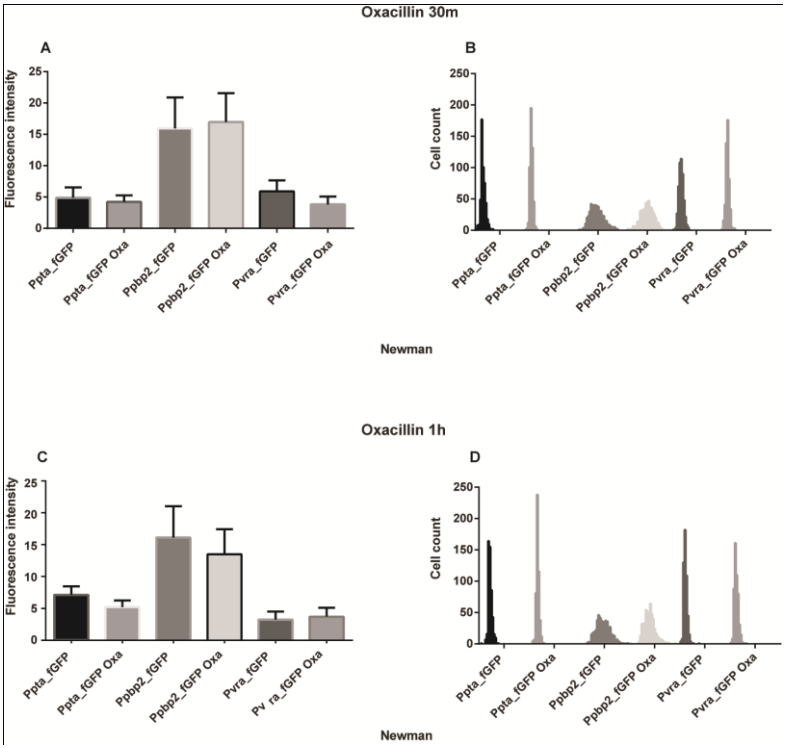


Figure 16 - Expression of *pta, vraSR* and *pbp2* promoters in Newman GFP reporter strains upon Oxacillin treatment (1/2 x MIC) for 30 min and 1 hour. For each strain, CWSS and housekeeping promoters activity was followed by measuring the GFP fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (Oxa) or absence of antibiotic after 30min (A) or 1h (C). Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of oxacillin after 30min (B) or 1h (D). Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500

In Newman, due to the low oxacillin MIC (0,2µg/ml) the amount of antibiotic added to the cell culture (1/2 x MIC) was not sufficient to trigger CWSS (Figure 17). Consequently, we opted to test also 1x MIC of oxacillin.

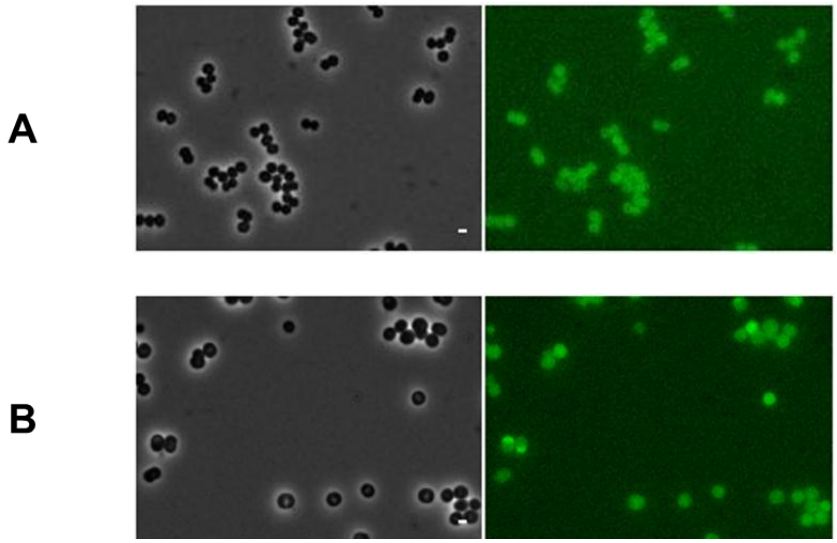


Figure 17 – Isogenic Newman cells under expressing *gfp* under the control of *vraSR* promoter, challenged with Oxacillin for 30m. (A) with ½ x MIC Oxacillin **(B)** with 1x MIC Oxacillin. ½ x MIC does not activate *vraSR* promoter while with 1x MIC, some fluorescence signal appearance can be observed. For each example: left – phase contrast image; right – GFP image. Scale bar = 1µm

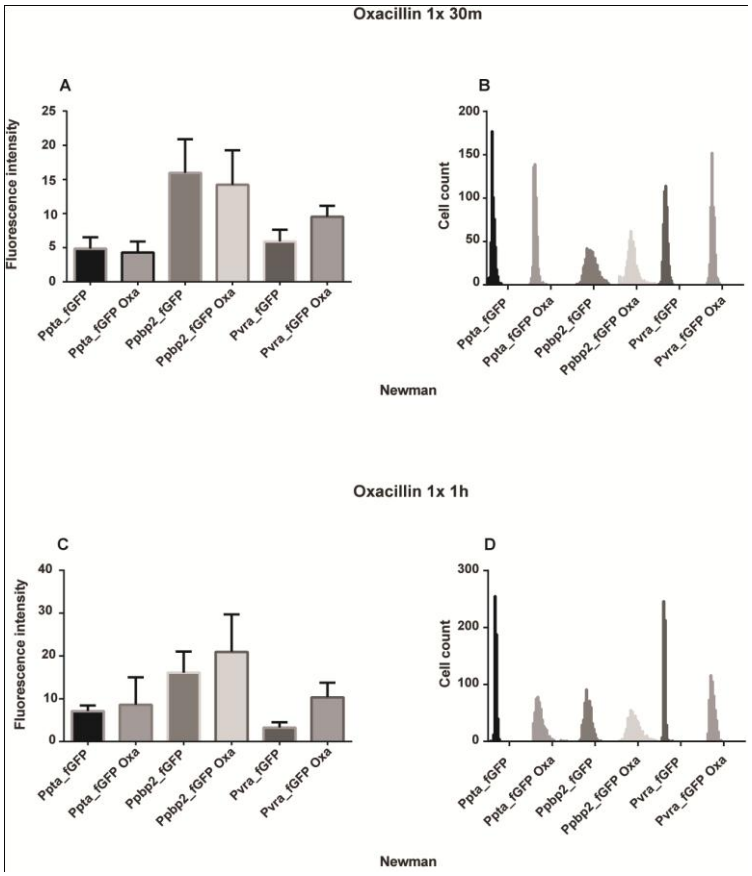


Figure 18 - Expression of *pta, vraSR* and *pbp2* promoters in Newman GFP reporter strains upon Oxacillin treatment (1 x MIC) for 30 min and 1 hour. For each strain, CWSS and housekeeping promoters activity was followed by measuring the GFP fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (Oxa) or absence of antibiotic after 30min (A) or 1h (C). Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of oxacillin after 30min (B) or 1h (D). Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500

Data displayed in the bar charts of the figures showing mean fluorescence intensity of the promoter reporters, to monitor promoters activity (figures 15-B, 16-B,16-D, 18-B and 18-D) was summarized in the table below (table 7).

	no antibiotic 30m	no antibiotic 1h	Kanamycin 1h	Oxacillin 30m	Oxacillin 1h	Oxacillin 1x 30m	Oxacillin 1x 1h
Newman							
pta	4,876	7,114	3,325	4,223	5,512	4,303	8,589 *
vraSR	5,907	3,248	3,963	3,826	3,692	9,523	10,328
pbp2	15,952	16,114	11,289	16,967	13,495	14,211	20,944

Table 7: Activity of the CWSS and housekeeping promoters in different conditions, measured by GFP fluorescence intensity: Average values of fluorescence intensity displayed by the *vraSR*, *pbp2* and *pta* promoters in the presence of different antibiotic concentrations. Fluorescence intensity data was obtained after 30 minutes or 1 hour of antibiotic exposure. Green boxes represent more than 100% fluorescence signal increment, in relation to the non-treated condition. * represent values not statistically different relatively to the non-treated condition.

It is clear that Newman, in fact, requires 1x MIC of oxacillin to successfully induce the CWSS. Both *vraSR* and *pbp2* increase their genetic expression, although only in *vraSR* that increment is more pronounced. A drop in *pbp2* genetic expression in the presence of kanamycin was also noticed in Newman background although not as marked as the one registered for MW2 strains.

Genetic expression variability exhibited by the promoters (figures 15-B, 16-B, 16-D, 18-B, 18-D), presented as the variance of the log transformed data was compiled in the table below (table 8).

	no antibiotic 30m	no antibiotic 1h	kanamycin 1h	oxacillin 30m	oxacillin 1h	Oxacillin 1x 30m	Oxacillin 1x 1h
Newman							
pta	0,028	0,008	0,069	0,020	0,009	0,034	0,270
vraSR	0,022	0,036	0,031	0,030	0,031*	0,005	0,022
pbp2	0,020	0,019	0,026	0,018	0,027	0,030	0,041

Table 8: Variability measurement of the activity of CWSS and housekeeping promoters in different conditions. Average values were obtained by taking the variance of the log-transformed data of the fluorescence intensity. The variance of the log-transformed data renders variability independent from mean increase . Green boxes represent variability values 100% higher than the correspondent value showed by the non-treated condition. * represent values not statistically different relatively to the non-treated condition. N=500 cells

Again, like the previous set of MW2 strains, the increase in genetic expression variability upon CWSS activation was not evident in Newman GFP reporter strains. The variability displayed by *vraSR* remained without substantial increase in all conditions tested, even in the presence of 1x MIC of oxacillin which revealed to trigger *vraSR* activation. Considering *pbp2* promoter expression variability, only using 1x MIC of oxacillin after 1 hour, an increase in the genetic expression variability could be observed. However, the conditions used 1x MIC clearly induce stress in the cells, probably generating unspecific variability, given that a large increase in variability was also observed for the *pta* promoter after 1 hour of oxacillin addition.

Analysis of genetic expression in COL GFP reporter strains

Data collected from COL GFP reporter strains in the different conditions tested is shown in the figures below. Figures 19-A, 20-A and 20-C present mean fluorescence intensity displayed by the cells in different settings and figures 19-B, 20-B, 20-D the variability in genetic expression in the population analyzed.

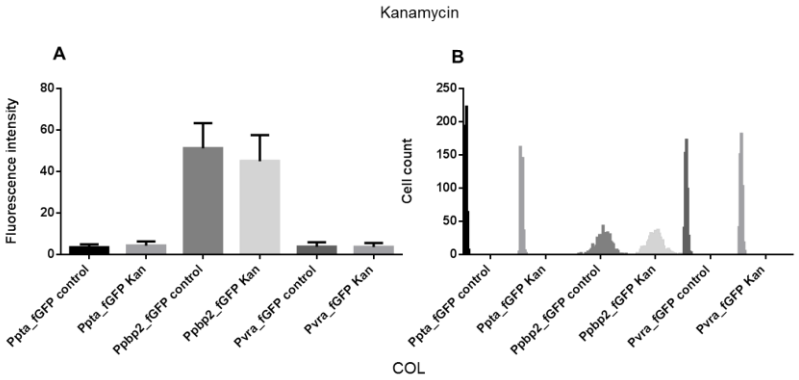


Figure 19 - Expression of *pta, vraSR* and *pbp2* promoters in COL GFP reporter strains upon Kanamycin treatment (1 x MIC) for 1 hour. (A) For each strain, CWSS and housekeeping promoters activity was followed by measuring the GFP fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (Kan) or absence (control) of antibiotic.(B) Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of kanamycin. Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500 cells

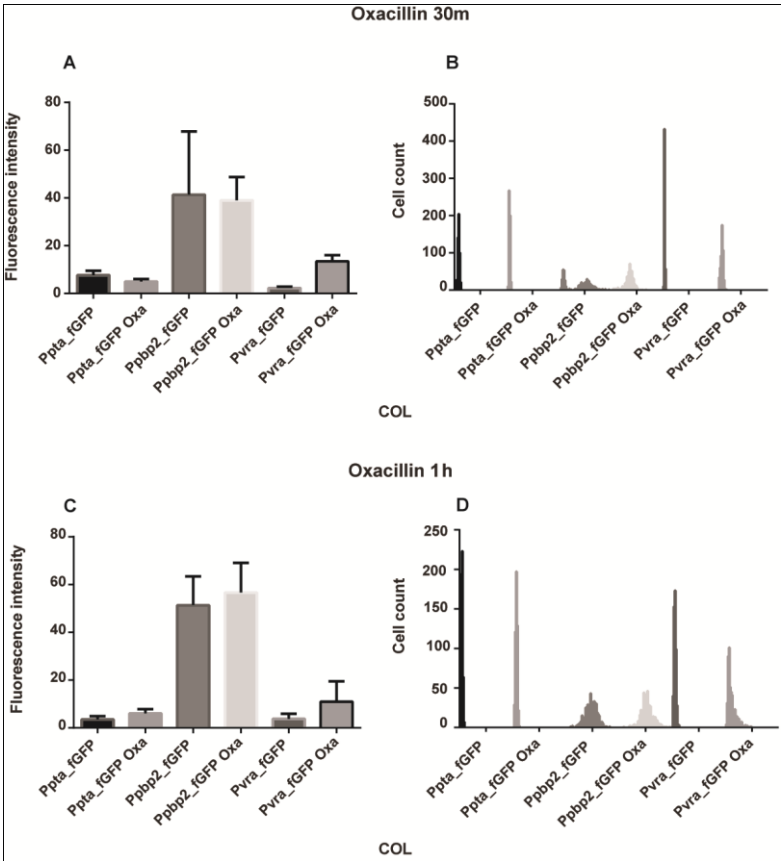


Figure 20 - Expression of *pta, vraSR* and *pbp2* promoters in COL GFP reporter strains upon Oxacillin treatment (1/2 x MIC) for 30 min and 1 hour. For each strain, CWSS and housekeeping promoters activity was followed by measuring the GFP fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (Oxa) or absence of antibiotic after 30min (A) or 1h (C). Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of oxacillin after 30min (B) or 1h (D). Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500

Data regarding mean fluorescence intensity displayed by the bar charts (figures 19-A, 20-A and 20-C) above was summarized in the table below (table 9).

COL	no antibiotic 30m	no antibiotic 1h	Kanamycin 1h	Oxacillin 30m	Oxacillin 1h
pta	7,658	3,503	4,280	4,948	6,033
vraSR	2,224	3,794	3,648	13,406	10,959
pbp2	41,326	51,284	45,035	38,985	56,662

Table 9: Activity of the CWSS and housekeeping promoters in different conditions, measured by GFP fluorescence intensity: Average values of fluorescence intensity displayed by the *vraSR, pbp2* and *pta* promoters in the presence of different antibiotic concentrations. Fluorescence intensity data was obtained after 30 minutes or 1 hour of antibiotic exposure. Green boxes represent more than 100% fluorescence signal increment, in relation to the non-treated condition. * represent values not statistically different relatively to the non-treated condition.

Like MW2, the other MRSA strain, COL activates the CWSS in the presence of ½ X MIC of oxacillin, although only *vraSR* was seen to increase its expression. However, contrary to MW2, *pbp2* genetic expression does not increase even upon *vraSR* activation. In fact, *pbp2* expression does not change substantially, similarly to what was observed for the housekeeping gene *pta*.

Genetic expression variability shown in the histograms above (figures 19-B, 20-B and 20-D) was compiled in table 10.

COL	no antibiotic 30m	no antibiotic 1h	kanamycin 1h	oxacillin 30m	oxacillin 1h
pta	0,014	0,050	0,057*	0,011	0,020
vraSR	0,029	0,098	0,096*	0,008	0,11*
pbp2	0,016	0,014	0,017	0,025	0,010

Table 10: Variability measurement of the activity of CWSS and housekeeping promoters in different conditions. Average values were obtained by taking the variance of the log-transformed data of the fluorescence intensity. The variance of the log-transformed data renders variability independent from mean increase. Green boxes represent variability values 100% higher than the correspondent value showed by the non-treated condition. * represent values not statistically different relatively to the non-treated condition.

In COL, independently of the condition tested, no substantial increment in the variability was observed. Considering these data, this may suggest that, genetic expression variability does not increase whether CWSS is activated or not. Once again, the heterogeneity observed in COL cells upon CWSS activation was due to mean increase.

Construction of *mCherry* promoter fusions

In order to corroborate our data obtained with the GFP promoter fusions, a second set of strains was made in which study of *vraSR* and *pta* or *pbp2* and *pta* can be made is the same cell. This strategy should minimize cell to cell variation. For that purpose, the *pta* promoter was fused to *mCherry* and inserted in the ectopic *spa* locus in *S. aureus* genome. The *spa* gene encodes the non-essential protein A, a surface protein responsible for immunological disguise during host colonization [83, 84], which is not required for cell wall synthesis or antibiotic resistance. Pvra_GFP and Ppbp2_GFP fusions were then transduced from the strains harboring the respective *gfp* promoter fusions into the strains that encoding the pta_mcherry fusion. The resulting strains therefore express either *vraSR* or *pbp2* promoter fused to *gfp*, in their native loci, and *pta* promoter fused to *mCherry* in *spa* locus (Figure 21).

These constructs have an internal control, since, in each cell, we can monitor the expression of the cell wall stress stimulon promoters, through GFP fluorescence, as well as that of a housekeeping gene promoter, through mCherry fluorescence (Figure 21). It is however important to notice that in order to monitor the two promoters simultaneously inside the same cell, we are have two different fluorescent proteins, with different folding kinetics, to analyze the genetic expression.

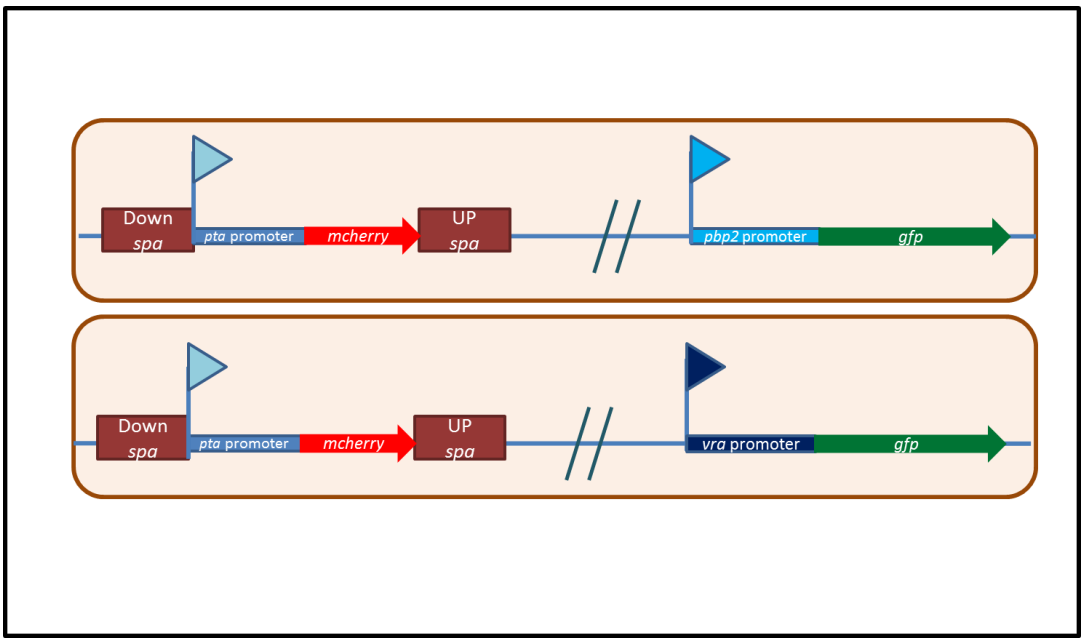


Figure 21 – Schematic representation of the chromosomal organization of strains expressing a *pta* promoter fusion to *mCherry,* from the ectopic *spa* locus, and a *vraSR* or *pbp2* promoter fusion to *gfp*, from the native chromosomal locus**.** The strains allow monitoring the cell wall stress stimulon (CWSS) promoters expression, through GFP fluorescence, awhile simultaneously following housekeeping *pta* promoter expression through mCherry fluorescence. *pta* promoter fusion was inserted in the ectopic *spa* locus while the CWSS promoter fusions were inserted in their native loci.

Analysis of genetic expression in MW2 mCherry/GFP reporter strains

MW2 GFP/mCherry reporter strains allowed us to quantify the GFP fluorescence signal, monitoring the activity of this CWSS promoter and mCherry fluorescence signal, following the activity of the housekeeping *pta* promoter, as shown in figure 22. Mean fluorescence intensity of promoter reporters is shown in figures 23-A, 24-A and 24-C and genetic expression variability in figures 23-B, 24-B and 24-D.

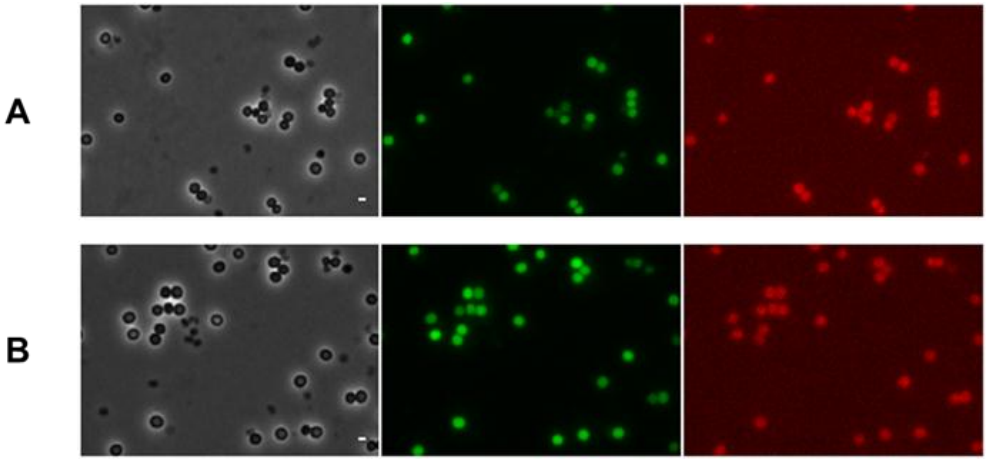


Figure 22 - Isogenic MW2 mCherry/GFP reporter cells exposed to ½ x MIC Oxacillin after 30m of exposure. (A) MW2 cells expressing *gfp* under the control of *pbp2* promoter in its native locus and *mCherry* under the control of *pta* promoter in the ectopic spa locus **(B)** MW2 cells expressing *gfp* under the control of *vraSR* promoter in its native locus and *mcherry* under the control of *pta* promoter in the ectopic spa locus. For each example: left – phase contrast image; middle – GFP image; right – mCherry image. Scale bar = 1µm

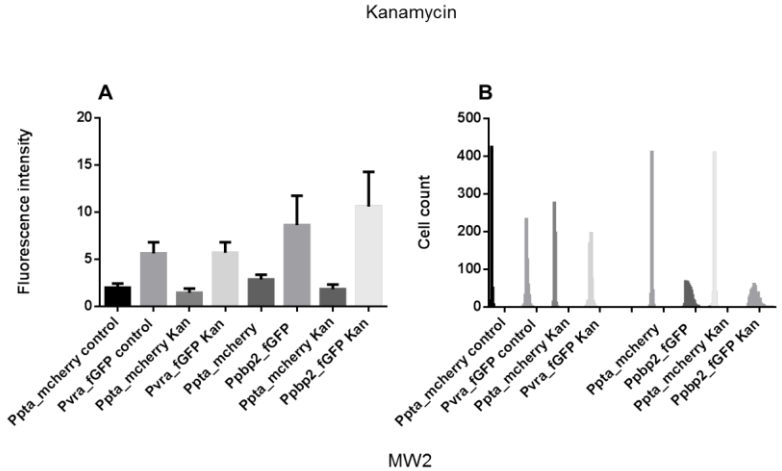


Figure 23 - Expression of *pta, vraSR* and *pbp2* promoters in MW2 GFP/mCherry reporter strains upon Kanamycin treatment (1 x MIC) for 1 hour. (A) For each strain, CWSS promoters activity was followed by measuring the GFP fluorescence signal, while housekeeping promoter activity was followed by measuring the mCherry fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (kan) or absence (control) of antibiotic.(B) Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of kanamycin. Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500 cells

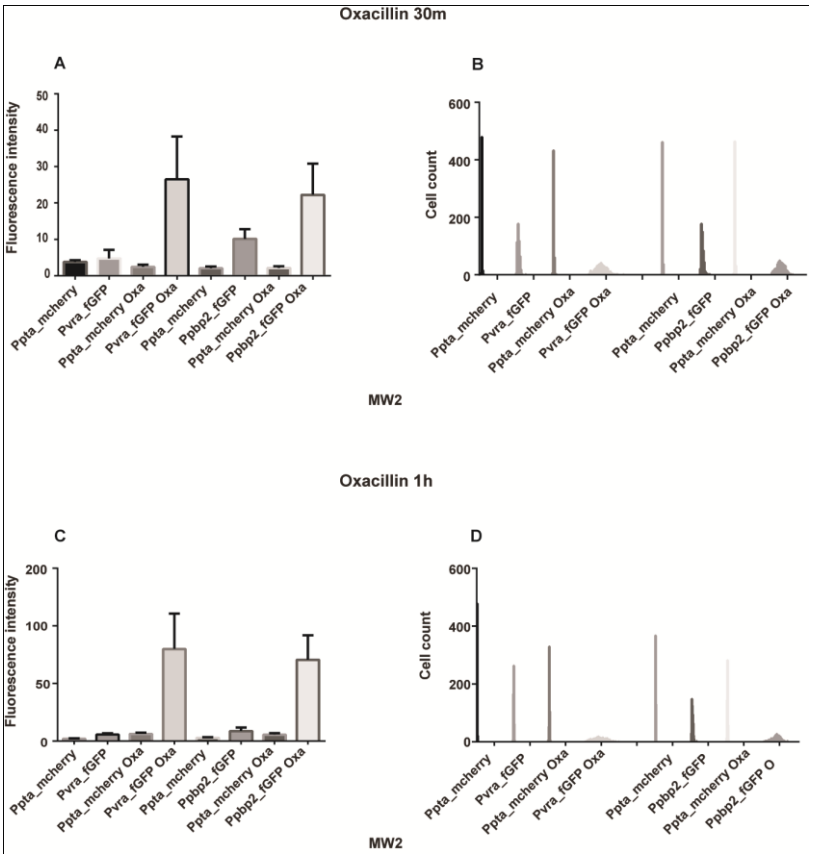


Figure 24 - Expression of *pta, vraSR* and *pbp2* promoters in MW2 GFP/mCherry reporter strains upon Oxacillin treatment (1/2 x MIC) for 30 min and 1 hour. For each strain, CWSS and housekeeping promoters activity was followed by measuring the GFP fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (Oxa) or absence of antibiotic after 30min (A) or 1h (C). Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of oxacillin after 30min (B) or 1h (D). Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500

Data of the mean fluorescence intensity of the promoter reporters shown figures 23-A, 24-A and 24-C was condensed in table 11. The activity for the CWSS and the *pta* promoter is shown for each strain.

Strain background	Promoter	no antibiotic 30m	no antibiotic 1h	kanamycin 1h	oxacillin 30m	oxacillin 1h
MW2	*pta*	2,050	2,872	1,849	2,135	5,634
	pbp2	10,132	8,645	10,819	22,227	70,597
MW2	*pta*	3,814	2,000	1,460	2,438	6,176
	vraSR	4,779	5,635	3,001	26,507	79,867

Table 11: Activity of the CWSS and housekeeping promoters in different conditions, measured by GFP and mCherry fluorescence intensity: Average values of fluorescence intensity of the CWSS and housekeeping promoters in different conditions. In each individual cell, housekeeping promoter activity was measured by following mCherry fluorescence signal while CWSS promoters activity was measured by following GFP fluorescence signal. Fluorescence intensity data was obtained after 30 minutes or 1 hour of antibiotic exposure. Green boxes represent more than 100% fluorescence signal increment, in relation to the non-treated condition. * represent values not statistically different relatively to the non-treated condition.

Results obtained with this set of strains, with the internal *mCherry* control, confirm the results described above, obtained with the strains expressing a single GFP reporter. Briefly CWSS promoters were activated with ½ x MIC of oxacillin, but not in the presence of kanamycin nor in the absence of antibiotics. Also, an increase in expression of the housekeeping *pta* promoter was observed in the strain harboring *vraSR gfp* promoter fusion after 1 hour of antibiotic exposure, but it was not consistent with what was observed with the other MW2 strain, harboring *pbp2 gfp* promoter fusion in which *pta* expression remained with a lower expression.

Data collected from histograms allowing to measure genetic expression variability (figures 23-B, 24-B and 24-D) is summarized in the following table 12.

Strain background	Promoter	no antibiotic 30m	no antibiotic 1h	kanamycin 1h	oxacillin 30m	oxacillin 1h
MW2	*pta*	0,011	0,006	0,025	0,018	0,014
	pbp2	0,011	0,021	0,023*	0,040	0,026
MW2	*pta*	0,003	0,007	0,037	0,018	0,011
	vraSR	0,057	0,008	0,088	0,090	0,040

Table 12: Variability measurement of the activity of CWSS and housekeeping promoters in different conditions. Average values were obtained by taking the variance of the log-transformed data of the fluorescence intensity. Green boxes represent variability values 100% higher than the correspondent value showed by non-treated condition. * represent values not statistically different relatively to the non-treated condition.

The data obtained with this set of MW2 strains with the internal control, corroborates the data obtained with the GFP reporter strains. The increase in the genetic expression variability does not require CWSS activation and it is not specific to the CWSS promoters.

Analysis of genetic expression in Newman mCherry/GFP reporter strains

Data obtained from Newman GFP/mCherry reporter strains is shown in the figures below. Figures 25-A, 26-A, 26-C, 27-A and 27-C display mean fluorescence intensity while figures 25-B, 26-B, 26-D, 27-B and 27-D represent genetic expression variability evaluated through the histograms.

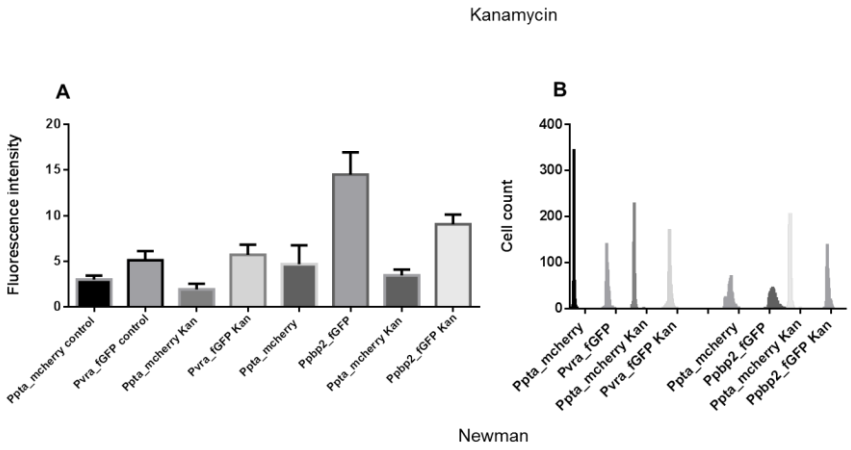


Figure 25 - Expression of *pta, vraSR* and *pbp2* promoters in Newman GFP/mCherry reporter strains upon Kanamycin treatment (1 x MIC) for 1 hour. (A) For each strain, CWSS promoters activity was followed by measuring the GFP fluorescence signal, while housekeeping promoter activity was followed by measuring the mCherry fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (Kan) or absence of antibiotic.(B) Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of kanamycin. Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500 cells

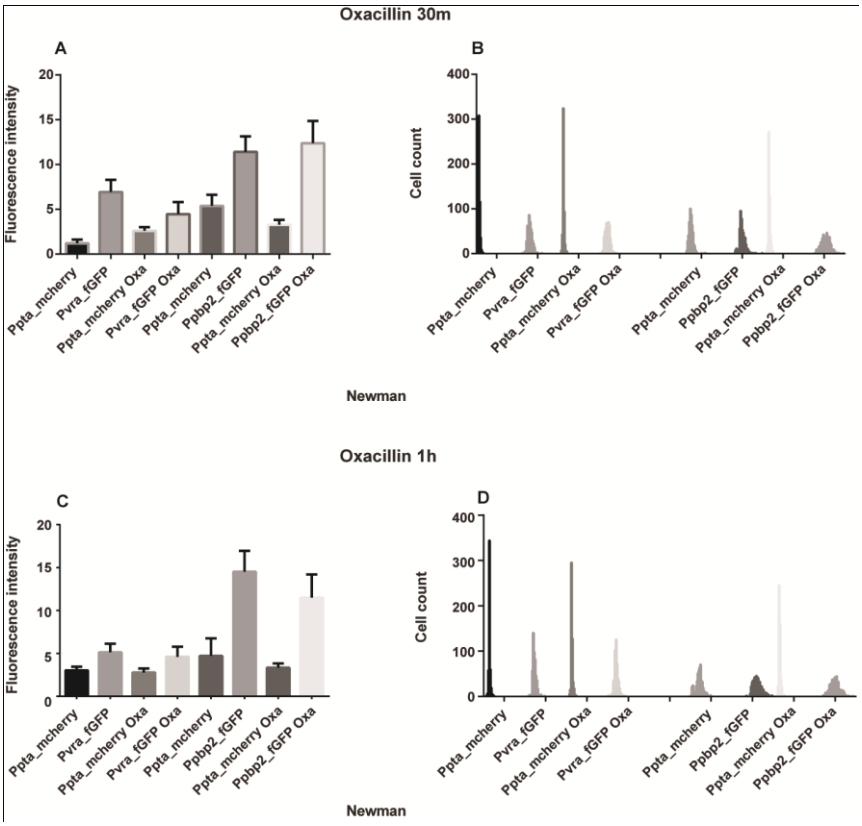


Figure 26 - Expression of *pta, vraSR* and *pbp2* promoters in Newman GFP/mCherry reporter strains upon Oxacillin treatment (1/2 x MIC) for 30 min and 1 hour. For each strain, CWSS and housekeeping promoters activity was followed by measuring the GFP fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (Oxa) or absence of antibiotic after 30min (A) or 1h (C). Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of oxacillin after 30min (B) or 1h (D). Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500

Like the results obtained with the Newman strains that harbor the *gfp* promoter fusions, fluorescence intensity of the CWSS promoters indicated again that ½ x MIC was not sufficient to successfully activate these promoters, even during 1 hour of antibiotic exposure (Figures 26-A, 26-C)

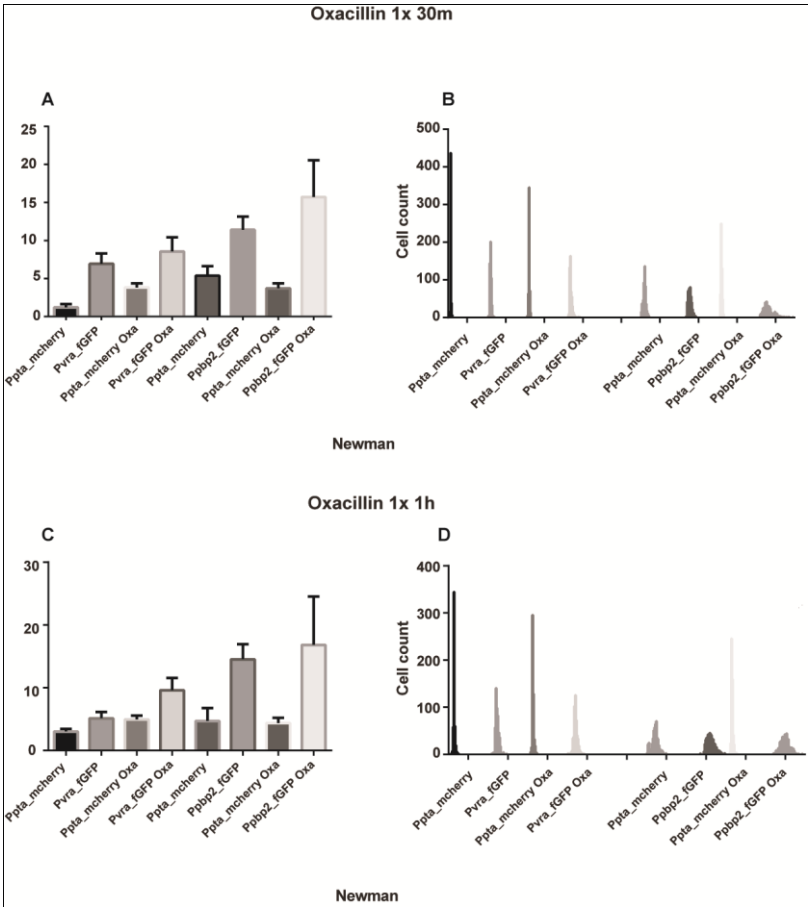


Figure 27 - Expression of *pta, vraSR* and *pbp2* promoters in Newman GFP/mCherry reporter strains upon Oxacillin treatment (1 x MIC) for 30 min and 1 hour. For each strain, CWSS and housekeeping promoters activity was followed by measuring the GFP fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (Oxa) or absence of antibiotic after 30min (A) or 1h (C). Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of oxacillin after 30min (B) or 1h (D). Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500

Mean fluorescence intensity values displayed in the bar charts above (figures 25-A, 26-A, 26-C, 27-A and 27-C) was compiled in the following table 13.

Strain background	Promoter	no antibiotic 30m	no antibiotic 1h	Kanamycin 1h	Oxacillin 30m	Oxacillin 1h	Oxacillin 1x 30m	Oxacillin 1x 1h
Newman	*pta*	5,377	4,696	3,457	3,283	3,316	3,717	4,351
	pbp2	11,411	14,507	9,065	12,367	11,481	15,708	16,811
Newman	*pta*	1,207	3,014	1,925	2,595	2,765	3,823	4,931
	vraSR	6,932	5,124	5,715	4,457	4,584	8,555	9,594

Table 13: Activity of the CWSS and housekeeping promoters in different conditions, measured by GFP and mCherry fluorescence intensity: Average values of fluorescence intensity of the CWSS and housekeeping promoters in different conditions. In each individual cell, housekeeping promoter activity was measured by following *mCherry* fluorescence signal while CWSS promoters activity was measured by following GFP fluorescence signal. Fluorescence intensity data was obtained after 30 minutes or 1 hour of antibiotic exposure. Green boxes represent more than 100% fluorescence signal increment, in relation to the non-treated condition. * represent values not statistically different relatively to the non-treated condition.

With this set of strains, although an increase in the expression of the CWSS promoters could be noticed upon oxacillin addition, mainly when using 1x MIC, it was lower than two fold. Only *pta* promoter revealed higher expression values when in the presence of oxacillin, suggesting that the increase in genetic expression observed was not specific to the CWSS promoters in presence of β-lactam antibiotics.

Genetic expression variability data shown in the histograms above (figures 25-B, 26-B, 26-D, 27-B and 27-D) is summarized in the next table 14.

Strain background	Promoter	no antibiotic 30m	no antibiotic 1h	kanamycin 1h	oxacillin 30m	oxacillin 1h	Oxacillin 1x 30m	Oxacillin 1x 1h
Newman	*pta*	0,011	0,091	0,004	0,005	0,005	0,006	0,009
	pbp2	0,004	0,005	0,002	0,008	0,011	0,024	0,037
Newman	*pta*	0,031	0,004	0,032	0,004	0,006	0,004	0,005
	vraSR	0,007	0,007	0,011	0,026	0,029	0,008*	0,006

Table 14: Variability measurement of the activity of CWSS and housekeeping promoters in different conditions: Average values were obtained by taking the variance of the log-transformed data of the fluorescence intensity. The variance of the log-transformed data renders variability independent from mean increase. Green boxes represent variability values 100% higher than the correspondent value showed by non-treated condition. * represent values not statistically different relatively to the non-treated condition.

In this set of Newman strains with the internal control, the results obtained approach what was initially hypothetically conceived. *pbp2* and *vraSR* promoters increase genetic expression variability only in the presence of cell wall targeting antibiotics. Interestingly, an increment could be observed with

antibiotic concentrations that did not trigger CWSS activation, namely when we using concentrations below the MIC in Newman, which we have showed (Figure 26) not sufficient to activate the CWSS.

Analysis of genetic expression in COL mCherry/GFP reporter strains

Regarding COL GFP/mCherry reporter strains, the mean fluorescence intensity exhibited by the cells is showed in figures 28-A, 29-A and 29-C and genetic expression variability in figures 28-B, 29-B and 29-D.

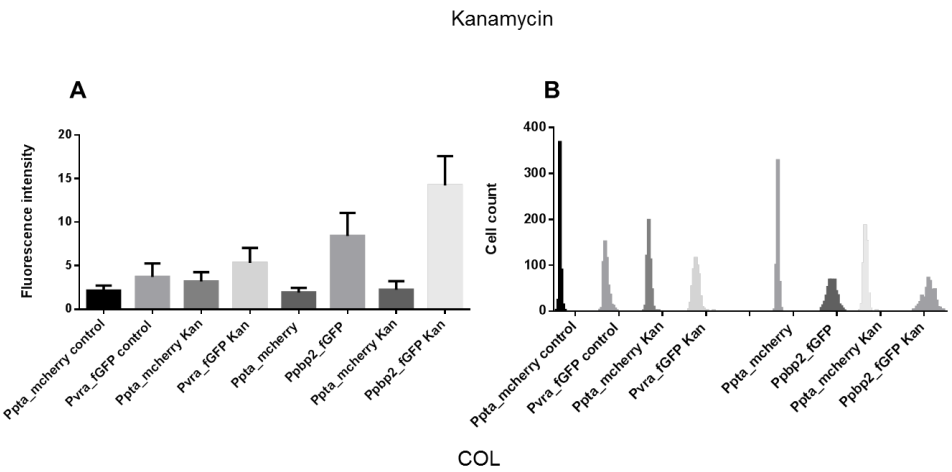


Figure 28 - Expression of *pta, vraSR* and *pbp2* promoters in COL GFP/mCherry reporter strains upon Kanamycin treatment (1 x MIC) for 1 hour. (A) For each strain, CWSS promoters activity was followed by measuring the GFP fluorescence signal, while housekeeping promoter activity was followed by measuring the mCherry fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (Kan) or absence of antibiotic.(B) Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of kanamycin. Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500 cells

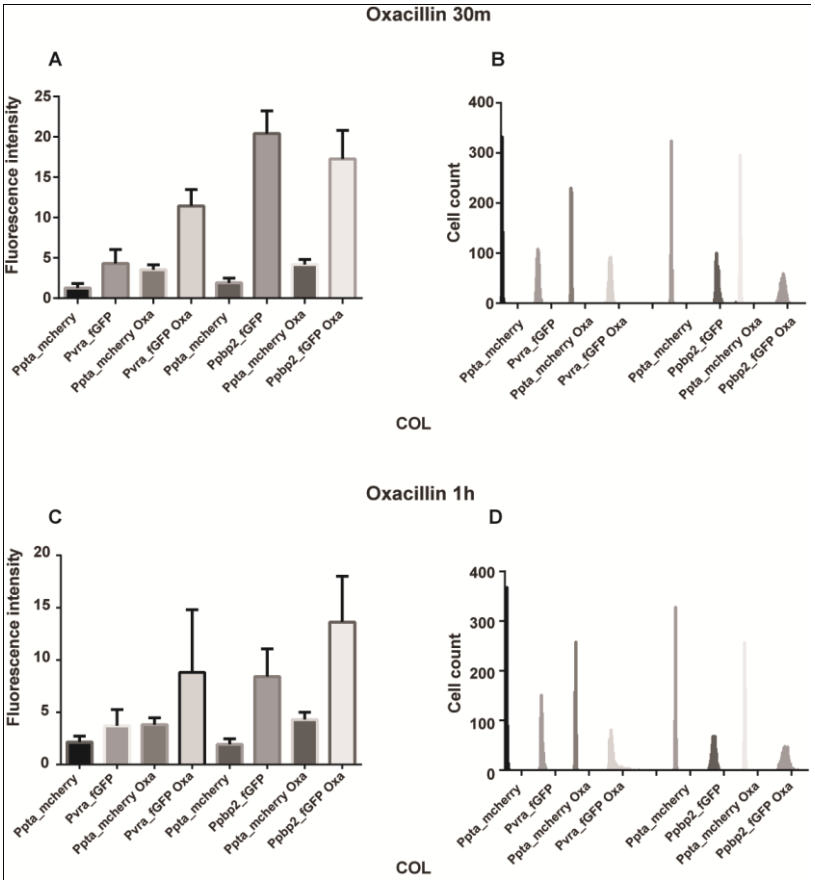


Figure 29 - Expression of *pta, vraSR* and *pbp2* promoters in COL GFP/mCherry reporter strains upon Oxacillin treatment (1/2 x MIC) for 30 min and 1 hour. For each strain, CWSS and housekeeping promoters activity was followed by measuring the GFP fluorescence signal in microscopy images. Mean fluorescence intensity was calculated in the presence (Oxa) or absence of antibiotic after 30min (A) or 1h (C). Each histogram represents the genetic expression variability exhibited by each strain in the presence or absence of oxacillin after 30min (B) or 1h (D). Cells were split into different classes according to the fluorescence intensity displayed. Bin width was chosen so that the number of bins equals the log base 2 of sample size. N=500

Mean fluorescence intensity displayed by the different isogenic COL cells shown in the bar charts above (figures 28-A, 29-A and 29-C) is condensed in the next table 15.

Strain background	Promoter	no antibiotic 30m	no antibiotic 1h	Kanamycin 1h	Oxacillin 30m	Oxacillin 1h
COL	*pta*	1,912	1,920	2,246	4,173	4,293
	pbp2	20,405	8,400	14,219	17,254	13,623
COL	*pta*	1,266	2,143	3,189	3,530	3,802
	vraSR	4,299	3,708	5,317	11,431	8,812

Table 15: Activity of the CWSS and housekeeping promoters in different conditions, measured by GFP and mCherry fluorescence intensity: Average values of fluorescence intensity of the CWSS and housekeeping promoters in different conditions. In each individual cell, housekeeping promoter activity was measured by following mCherry fluorescence signal while CWSS promoters activity was measured by following GFP fluorescence signal. Fluorescence intensity data was obtained after 30 minutes or 1 hour of antibiotic exposure. Green boxes represent more than 100% fluorescence signal increment, in relation to the non-treated condition. * represent values not statistically different relatively to the non-treated condition.

This set of COL strains confirmed that *vraSR* was consistently activated upon oxacillin addition, contrary to the response of *pbp2,* the other CWSS promoter. *pta* showed an increase in some experiments with oxacillin.

Genetic expression variability data shown in the histograms (figures 28-B, 29-B and 29-D) above was summarized in the following table 16.

Strain background	Promoter	no antibiotic 30m	no antibiotic 1h	kanamycin 1h	oxacillin 30m	oxacillin 1h
COL	*pta*	0,024	0,021	0,049	0,007	0,010
	pbp2	0,002	0,025	0,013	0,009	0,024*
COL	*pta*	0,040	0,019	0,024	0,067	0,007
	vraSR	0,064	0,034	0,023	0,007	0,050

Table 16: Variability measurement of the activity of CWSS and housekeeping promoters in different conditions: Average values were obtained by taking the variance of the log-transformed data of the fluorescence intensity. The variance of the log-transformed data renders variability independent from mean increase. Green boxes represent variability values 100% higher than the correspondent value showed by non-treated condition. * represent values not statistically different relatively to the non-treated condition.

The data obtained with COL mCherry/GFP reporter strains points to similar results as those obtained for the other MRSA strains, constructed in the MW2 background. The increase in genetic expression variability is not restricted to the conditions where the CWSS is activated.

Discussion

In the presence of cell wall (CW) targeting antibiotics, bacteria activate what is known as the CW stress stimulon (CWSS), in order to prepare the adequate response. The VraSR regulatory system displays an essential role, as it is responsible for sensing CW damage, promptly triggering the CWSS [75]. One of the genes upregulated by the VraR response regulator encodes for the PBP2 protein, a protein involved in the last steps of cell wall synthesis [54]. In this work, we wanted to determine if the expression levels of *vraSR* are identical in all cells of an isogenic population or if these levels show variation at the single cell level, due for example to genetic noise. This variability could confer an advantage to the population if cells showing higher expression levels had a fitness cost but were more tolerant to the presence of the antibiotic. Furthermore, we wanted to determine if there was an increase in variability in gene expression of the CWSS promotes when in the presence of cell wall targeting antibiotics.

In order to determine whether *vraSR* and *pbp2* expression is heterogeneous, we monitored their expression using fluorescent reporters and compared it to the expression of the housekeeping gene *pta* [79]. The *pta* gene is essential for cell viability and encodes for the phosphotransacetylase enzyme, a protein involved in acetate and acetyl-coA metabolism, catalyzing the reversible interconversion of acetyl-CoA and acetyl phosphate [85, 86]. Given that *pta* is not involved in cell wall synthesis and is not part of the CWSS, we used it to ascertain whether the genetic expression variability exhibited by the CWSS promoters was higher or lower than genes that did not belong to the CWSS, in the presence of cell wall targeting antibiotics. Its study would therefore allow us to measure genetic expression fluctuations in a non-antibiotic inducible housekeeping gene, although keeping in mind that *pta* must not be considered representative of all staphylococcal genes.

To follow the expression of the referred genes, their promoter regions were fused to the gene encoding green fluorescent protein (GFP) or mCherry fluorescent protein, so that the fluorescence intensity could be used as a reporter for promoter activity. The *vraSR*, *pbp2* and *pta gfp* fusions were inserted in their native loci, in three different *S. aureus* strains, MW2, COL and Newman. In the studies done with this set of three strains, the control *pta* promoter was analysed in a different strain from the CWSS promoters. Although we tested all strains in the same conditions, the cells were not identical and it was formally possible (although unlikely) that the expression of the *pta* promoter fusion would affect the general level of noise in gene expression. In order to be able to study expression driven by the CWSS and the *pta* promoters in the same cell, we constructed another set of strains. For that purpose the *pta* promoter was fused to the gene encoding mCherry fluorescent protein and introduced in the ectopic *spa* locus, in the strains already harboring the *vraSR* or the *pbp2* promoter fusion to *gfp* inserted in their native loci, resulting in two strains in which the CWSS and the *pta* control promoters could be monitored in the same *S. aureus* cell. The insertion of this promoter fusion in the *spa* locus, was accomplished by replacing the *spa* gene, encoding the nonessential, cell-wall-attached protein A [84], for the *pta mCherry* promoter fusion. The choice of the *spa* locus for insertion of the *pta mCherry* fusion was based on its previous use as an ectopic locus for gene expression, reported in the

literature [69, 80]. Although Protein A is a virulence factor, responsible for binding IgG, *spa* mutants are often used in *in vitro* studies as this protein may interfere in assays that require the use of antibodies [84]. In our case, we used *spa* mutants, since there were no records of phenotypic deficiencies associated with the deletion of this gene.

Due to the fact that we aimed to test the constructed strains in the presence of different antibiotics, it was important to check if any of the constructs was interfering with antibiotic susceptibility. To do so, the antibiotic minimum inhibitory concentrations (MICs) of all constructed strains were determined and compared to the respective *wild type* parental strains, using the population analysis profile (PAP) methodology. One major difference between this method and the MIC determination via micro dilution is that the latter is less accurate in determining MICs and it assumes that, in a bacterial population, all cells display the same antibiotic susceptibility [87, 88]. However, most broth cultures of MRSA strains are composed of different subpopulations exhibiting different antibiotic resistance levels [89, 90]. Interestingly, when a highly-resistant subpopulation is re-inoculated in TSB supplemented with antibiotic, the same heterogeneous resistance pattern is obtained again [89]. Therefore, and given the fact that MW2 is an heterogeneous-resistant MRSA strain, the laborious methodology of PAPs was chosen to determine the resistance profile and the MICs of the different antibiotics for the all strains used in this study

PAP analysis showed that the introduction of the promoter fusions in the MW2, COL and Newman strains did not alter their resistance/susceptibility profile to oxacillin, vancomycin and kanamycin, validating these strains for the study of the cellular response to these antibiotics. Furthermore, we could observe the expected resistance profile for each strain tested. Resistance to the β-lactam oxacillin varied considerably between strains. MSSA strain Newman, which does not harbor the SSC*mec*, and therefore lacks the major resistance determinant PBP2A, exhibited a typical sensitive pattern, with an MIC of 0.2µg/ml. Thus, even in the presence of low concentrations of β-lactams, PBPs are acylated and the transpeptidation (TP) reaction is successfully inhibited [31, 34]. Heterogeneously-resistant community-acquired MRSA strain MW2, which harbours the mobile element SSCmec type IV [91], displayed an MIC substantially higher than Newman, and we could see subpopulations with different antibiotic susceptibilities, a phenomenon previously reported [87, 89, 90]. Homogeneously resistant hospital acquired strain COL, which encodes SSCmec type I in which the *mecA* repressor gene (*mecI*) is absent resulting in constitutive *mecA* expression [92], showed an oxacillin MIC of 300µg/ml and no subpopulations with different susceptibilities. We also determined the antibiotic resistance profiles for the aminoglycoside kanamycin. This antibiotic was chosen as a control, as it targets the 30S subunit of the ribosome, inducing perturbations in the pre-translocation complex, which should not be sensed by VraS [93]. Kanamycin PAPs revealed that we can find the same resistance pattern in all backgrounds, with Newman being slightly more resistant (MIC 5µg/ml). The existence of several resistant subpopulations might be explained by spontaneous mutations in the ribosome that prevent the antibiotic molecule from binding, allowing the cell to survive. Another explanation may reside in the so-called inoculum effect, which has been reported for ribosome-targeting antibiotics [94]. Basically, the efficacy of a given antibiotic declines with the increase of bacterial density in the inoculum, once cellular density would increase its overall turnover rate [94].

However the latter hypothesis is unlikely, since we used the standard inoculum conditions, plating bacteria at several concentrations besides undiluted stationary-phase culture samples as described in [87].

Having determined that the constructed strains were not affected in their antibiotic resistance profiles and were therefore suitable for our studies, we required a second set of preliminary experiments, to test if the constructed promoter fusions were indeed responding to the presence of antibiotics. We therefore analyzed *pta* and *vraSR* promoter activities in the absence and in the presence of oxacillin and kanamycin by SDS-PAGE in a MRSA and MSSA background. As expected, the *vraSR* promoter was only induced in the presence of the cell wall targeting antibiotic oxacillin, exhibiting a basal expression in the absence of oxacillin as well as in the presence of kanamycin, while *pta* promoter was expressed to similar levels in the presence or absence of the antibiotics in both backgrounds. We then proceeded to define which conditions would lead to cell wall stimulon activation without killing the cells in the three background strains used in this study. For that purpose, strains encoding the *vraSR* and *pta gfp* promoter fusions were tested with three different oxacillin concentrations: ¼ x MIC, ½ x MIC and 1x MIC, after 30m and 1 hour of antibiotic exposure. It is important to notice that MW2 is a heterogeneously-resistant MRSA strain displaying different oxacillin susceptibilities. Therefore, we considered the MIC as that of the resistant subpopulation (256µg/ml), a concentration that kills more than 99,99% of the cells. The two different timepoints were chosen as, according to McCallum and Utaida, these exposure times are sufficient to get a clear cell wall stimulon response [49, 53]. At ¼ x MIC of oxacillin, *vraSR* was activated only in MRSA backgrounds but not in Newman strains. Similar results were obtained for oxacillin concentrations of ½ x MIC, but in this case a slight increase in *vraSR* expression could be observed in Newman. As the oxacillin MIC for Newman is lower than for MW2 and COL (0.2ug/ml versus 256ug/ml and 300ug/ml, respectively) we can infer that *vraSR* activation requires a specific antibiotic concentration above a certain threshold to trigger any response, explaining why *vraSR* activation is not clear in Newman for concentrations below the MIC. In order words, we suggest, based on these results, that VraSR activation requires a minimum number of acylated PBPs, and the concomitant CW damage that results from this acylation. Our results suggest that this damage cannot be achieved with oxacillin concentrations below 0.1µg/ml. In this sense, it interesting to notice that at least 0.7µg/ml of oxacillin are needed to fully acylate the transpeptidase domain of PBP2 *in vitro* [95]. As explained previously in the Results section, we opted to use for further studies ½ x oxacillin MIC, a concentration that did not result in considerable cell death, but was sufficient to induce an evident *vraSR* activation. We also used 1x MIC for Newman, since no clear *vraSR* induction could be achieved with ½ x MIC.

Having determined suitable antibiotic concentrations for our studies, we measured genetic expression variability among the reporter strains. The following task was to evaluate if upon *vraSR* activation, we could detect an increase in genetic expression variability in the two CWSS promoters within isogenic populations. For that purpose, the fluorescence intensity data collected from microscopy images was subjected to a variance-stabilizing transformation, in order to measure genetic expression variability independently of mean intensity increment. Although there are several variance-

stabilizing methods [82, 96], we chose the logarithm transformation, which allows the stabilization of the variance of high level expression data [82]. As we are working with inducible systems that can reach high expression values, this seemed the more appropriate method, having a constant variance for a sufficiently large mean value, despite having also some disadvantages. One of the main disadvantages of the log-transformation method is that its performance decreases when it deals with low or near-background values, causing the inflation of their variance [82]. Consequently, we have to consider that variance inflation is one of the limitations of this particular method, when we evaluate the expression of the non-induced CWSS promoters, notably in the absence of antibiotic or in the presence of kanamycin.

The first set of experiments was performed using *gfp* promoter fusions. In the conditions that triggered the activation of the CWSS, there was an apparent increase in genetic expression which, however, was not verified after the log-transformation. In none of strains tested, *vraSR* exhibited an increment in expression variability, which suggests that heterogeneity observed is just due to the increment of the variance linked to mean increase. This does not mean that all cells activate *vraSR* system at the same time and with the same intensity, still maintaining the question if the cells that show higher and/or earlier *vraSR* expression are more tolerant to cell wall damage. These data also suggest that we can exclude the possibility of bistability for the VraSR regulatory system, as we did not observe a split of cells with different expression levels into two coexisting subpopulations [62]. We had raised that hypothesis because VraR positively autoregulates the *vraSR* promoter [76] and one of the mechanisms proposed to generate bistability requires a non-linear positive autoregulated gene [62]causing cells with more than a threshold amount of the regulator to accumulate even higher amounts, resulting in the bifurcation of the population into two stable states.

Regarding *pbp2*, in the case of MW2 and Newman heterogeneity of gene expression increased upon addition of oxacillin, implying that the increment in *pbp2* genetic expression variability can occur without a corresponding increase in *vraSR* variability. We also noticed that the increase in heterogeneity of *pbp2* expression was not exclusive to situations where CWSS was activated, as it was also observed for example when MW2 reporter strains cells were challenged with kanamycin. Since, *pbp2* expression is high, even without CWSS induction, we cannot argue that the variability increase was due to variance inflation effect of logarithm transformation, once this effect only occurs for low expression rates. That variance inflation effect was more likely to account for the increase in *pta* expression variability, under certain conditions, since it displays a lower expression rate than the CWSS promoters.

A second set of experiments was done using strains where the housekeeping and CWSS promoters were monitored inside the same cell, in order to corroborate data obtained with *gfp* reporter strains. This set of strains allowed for minimization of cell-to-cell variation of *pta* and CWSS promoters as we were able to monitor the two promoters inside the same cell. Essentially, the data is consistent with what was obtained with *gfp* fusions, namely the variability increase registered was not exclusively in the presence of cell wall targeting antibiotics, mainly in MRSA strains. Only in MSSA Newman the increase in genetic expression heterogeneity revealed to be specific to the presence of cell wall

targeting antibiotics but, since CWSS was not activated when using ½ x MIC, it reveals that variability increase is not specific to CWSS activation.

Taking together the results from the two sets of experiments, we can state that although CWSS promoters were induced upon a threat to the cell wall integrity, that event did not lead directly to an increase in genetic expression variability. Interestingly, some expression variability fluctuations were also registered in the housekeeping *pta* promoter in the presence of CW active antibiotics. Whether this applies particularly to this housekeeping gene or increase in genetic expression heterogeneity in the presence of CW targeting antibiotics is common to other housekeeping genes is a question that remains unclear and it can only be answered if we study other housekeeping promoters. Studying a wider number of housekeeping genes will enable us to get a clear idea of the behavior of genes that do not belong to the CWSS setting a baseline to gene expression variability to be expected for *S. aureus* genes.

Another relevant subject that remains to be explored is if there is any relation between the cell cycle and the levels of *vraSR* and *pbp2* genetic expression. For instance, *pbp2* is a protein involved specifically in cell wall synthesis [97], which occurs at specific stages of the cell cycle. It would be interesting to see if cells displayed different CWSS responses according to their cell cycle stage and, consequently, revealed different susceptibilities when challenged with antibiotics. Furthermore, it was suggested that cell cycle stage might play an important role, regarding antibiotic resistance, taking the example of persistent bacteria. It was proposed that these bacteria are in some specific part of the cell cycle, not yet determined, at the time of exposure to antibiotics [98].

Further studies are still necessary to unveil all the details concerning the relation between *vraSR* genetic expression and antibiotic tolerance. This is of major interest, since it is known that *vraSR* regulatory system plays an essential role in the bacterial defense system against potential threats, covering a wide broad of antibiotics, including the clinical useful vancomycin. This system represents therefore a keystone still to be explored and fully understood, in order to find ways to disable the mechanisms that bacteria use to cope with harmful substances. Its importance is enhanced, when we consider the development and emergence of resistant strains, namely MRSA, Vancomycin-Intermediate *Staphylococcus aureus* (VISA) or Vancomycin-Resistant *Staphylococcus aureus* (VRSA) that represent a public health burden [99, 100].

Bibliography

1. Gordon, Rachel J. and Franklin D. Lowy, *Pathogenesis of Methicillin-ResistantStaphylococcus aureusInfection.* Clinical Infectious Diseases, 2008. **46**(S5): p. S350-S359.
2. Kazakova, S.V., et al., *A clone of methicillin-resistant Staphylococcus aureus among professional football players.* N Engl J Med, 2005. **352**(5): p. 468-75.
3. Miller, L.G. and B.A. Diep, *Clinical practice: colonization, fomites, and virulence: rethinking the pathogenesis of community-associated methicillin-resistant Staphylococcus aureus infection.* Clin Infect Dis, 2008. **46**(5): p. 752-60.
4. Boucher, H., L.G. Miller, and R.R. Razonable, *Serious infections caused by methicillin-resistant Staphylococcus aureus.* Clin Infect Dis, 2010. **51 Suppl 2**: p. S183-97.
5. Chatterjee, S.S. and M. Otto, *Improved understanding of factors driving methicillin-resistant Staphylococcus aureus epidemic waves.* Clin Epidemiol, 2013. **5**: p. 205-17.
6. van Hal, S.J., et al., *Predictors of mortality in Staphylococcus aureus Bacteremia.* Clin Microbiol Rev, 2012. **25**(2): p. 362-86.
7. Klevens, R.M., et al., *Invasive methicillin-resistant Staphylococcus aureus infections in the United States.* JAMA, 2007. **298**(15): p. 1763-71.
8. Ligon, B.L., *Penicillin: its discovery and early development.* Seminars in Pediatric Infectious Diseases, 2004. **15**(1): p. 52-57.
9. Barber, M. and M. Rozwadowska-Dowzenko, *Infection by penicillin-resistant staphylococci.* Lancet, 1948. **2**(6530): p. 641-4.
10. Bondi, A., Jr. and C.C. Dietz, *Penicillin resistant staphylococci.* Proc Soc Exp Biol Med, 1945. **60**: p. 55-8.
11. Lowy, F.D., *Antimicrobial resistance: the example of Staphylococcus aureus.* Journal of Clinical Investigation, 2003. **111**(9): p. 1265-1273.
12. Shanson, D.C., *Antibiotic-resistant Staphylococcus aureus.* J Hosp Infect, 1981. **2**(1): p. 11-36.
13. Jevons, M.P., A.W. Coe, and M.T. Parker, *Methicillin resistance in staphylococci.* Lancet, 1963. **1**(7287): p. 904-7.
14. Seligman, S.J., *Penicillinase-negative variants of methicillin-resistant Staphylococcus aureus.* Nature, 1966. **209**(5027): p. 994-6.
15. Lyon, B.R. and R. Skurray, *Antimicrobial resistance of Staphylococcus aureus: genetic basis.* Microbiol Rev, 1987. **51**(1): p. 88-134.
16. Saravolatz, L.D., et al., *Methicillin-resistant Staphylococcus aureus. Epidemiologic observations during a community-acquired outbreak.* Ann Intern Med, 1982. **96**(1): p. 11-6.
17. *Moran, G. J.,et al, Methicillin-Resistant S. aureus Infections among Patients in the Emergency Department. N Engl J Med 2006. **355**: p. 666-74.*
18. Chambers, H.F., *Community-associated MRSA--resistance and virulence converge.* N Engl J Med, 2005. **352**(14): p. 1485-7.
19. Kennedy, A.D., et al., *Epidemic community-associated methicillin-resistant Staphylococcus aureus: recent clonal expansion and diversification.* Proc Natl Acad Sci U S A, 2008. **105**(4): p. 1327-32.
20. Egan, A.J. and W. Vollmer, *The physiology of bacterial cell division.* Ann N Y Acad Sci, 2013. **1277**: p. 8-28.
21. Swoboda, J.G., et al., *Wall Teichoic Acid Function, Biosynthesis, and Inhibition.* ChemBioChem, 2009. **11**(1): p. 35-45.
22. *Schleifer, K. H. and O. Kandler, Peptidoglycan Types of Bacterial Cell Walls and their Taxonomic Implications. Bacteriol Rev, 1972. **36:** p. 407-477*
23. Vollmer, W., D. Blanot, and M.A. de Pedro, *Peptidoglycan structure and architecture.* FEMS Microbiology Reviews, 2008. **32**(2): p. 149-167.
24. *Tipper, D. and J. Strominger, Chemistry and Metabolism of Macromolecules Biosynthesis of the Peptidoglycan of Bacterial Cell Walls: XII. Inhibition of cross-linking by penicillins and cephalosporins: studies in Staphylococcus aureus in vivo. J. Biol. Chem. 1968, **243**:3169-3179*
25. Lovering, A.L., S.S. Safadi, and N.C.J. Strynadka, *Structural Perspective of Peptidoglycan Biosynthesis and Assembly.* Annual Review of Biochemistry, 2012. **81**(1): p. 451-478.
26. Bugg, T.D.H., et al., *Bacterial cell wall assembly: still an attractive antibacterial target.* Trends in Biotechnology, 2011. **29**(4): p. 167-173.
27. Sharif, S., et al., *Characterization of Peptidoglycan inFem-Deletion Mutants of Methicillin-ResistantStaphylococcus aureusby Solid-State NMR†.* Biochemistry, 2009. **48**(14): p. 3100-3108.
28. de Kruijff, B., V. van Dam, and E. Breukink, *Lipid II: A central component in bacterial cell wall synthesis and a target for antibiotics.* Prostaglandins, Leukotrienes and Essential Fatty Acids, 2008. **79**(3-5): p. 117-121.
29. Pinho, M.G. and J. Errington, *Recruitment of penicillin-binding protein PBP2 to the division site of Staphylococcus aureus is dependent on its transpeptidation substrates.* Mol Microbiol, 2005. **55**(3): p. 799-807.
30. van Heijenoort, J., *Assembly of the monomer unit of bacterial peptidoglycan.* Cell Mol Life Sci, 1998. **54**(4): p. 300-4.
31. Scheffers, D.J. and M.G. Pinho, *Bacterial cell wall synthesis: new insights from localization studies.* Microbiol Mol Biol Rev, 2005. **69**(4): p. 585-607.
32. Park, W. and M. Matsuhashi, *Staphylococcus aureus and Micrococcus luteus peptidoglycan transglycosylases that are not penicillin-binding proteins.* J Bacteriol, 1984. **157**(2): p. 538-44.
33. Georgopapadakou, N.H., *Penicillin-binding proteins and bacterial resistance to beta-lactams.* Antimicrob Agents Chemother, 1993. **37**(10): p. 2045-53.
34. Malouin, F. and L.E. Bryan, *Modification of penicillin-binding proteins as mechanisms of beta-lactam resistance.* Antimicrob Agents Chemother, 1986. **30**(1): p. 1-5.
35. Lim, D. and N.C. Strynadka, *Structural basis for the beta lactam resistance of PBP2a from methicillin-resistant Staphylococcus aureus.* Nat Struct Biol, 2002. **9**(11): p. 870-6.
36. Hiramatsu, K., et al., *The emergence and evolution of methicillin-resistant Staphylococcus aureus.* Trends Microbiol, 2001. **9**(10): p. 486-93.
37. *Classification of Staphylococcal Cassette Chromosome mec (SCCmec): Guidelines for Reporting Novel SCCmec Elements.* Antimicrobial Agents and Chemotherapy, 2009. **53**(12): p. 4961-4967.

38. Wu, S.W., H. de Lencastre, and A. Tomasz, *Recruitment of the mecA gene homologue of Staphylococcus sciuri into a resistance determinant and expression of the resistant phenotype in Staphylococcus aureus.* J Bacteriol, 2001. **183**(8): p. 2417-24.
39. Zong, Z., *Characterization of a complex context containing mecA but lacking genes encoding cassette chromosome recombinases in Staphylococcus haemolyticus.* BMC Microbiol, 2013. **13**: p. 64.
40. Pinho, M.G., et al., *Massive reduction in methicillin resistance by transposon inactivation of the normal PBP2 in a methicillin-resistant strain of Staphylococcus aureus.* Microb Drug Resist, 1997. **3**(4): p. 409-13.
41. Pinho, M.G., H. de Lencastre, and A. Tomasz, *An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10886-91.
42. Pinho, M.G., et al., *Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in Staphylococcus aureus.* J Bacteriol, 2001. **183**(22): p. 6525-31.
43. Hengge-Aronis, R., *Recent insights into the general stress response regulatory network in Escherichia coli.* J Mol Microbiol Biotechnol, 2002. **4**(3): p. 341-6.
44. Jordan, S., M.I. Hutchings, and T. Mascher, *Cell envelope stress response in Gram-positive bacteria.* FEMS Microbiol Rev, 2008. **32**(1): p. 107-46.
45. Green, D.W., *The bacterial cell wall as a source of antibacterial targets.* Expert Opin Ther Targets, 2002. **6**(1): p. 1-19.
46. Wolf, D., et al., *Cell envelope stress response in cell wall-deficient L-forms of Bacillus subtilis.* Antimicrob Agents Chemother, 2012. **56**(11): p. 5907-15.
47. Dengler, V., et al., *Induction kinetics of the Staphylococcus aureus cell wall stress stimulon in response to different cell wall active antibiotics.* BMC Microbiol, 2011. **11**: p. 16.
48. Muthaiyan, A., et al., *Transcriptional profiling reveals that daptomycin induces the Staphylococcus aureus cell wall stress stimulon and genes responsive to membrane depolarization.* Antimicrob Agents Chemother, 2008. **52**(3): p. 980-90.
49. Utaida, S., et al., *Genome-wide transcriptional profiling of the response of Staphylococcus aureus to cell-wall-active antibiotics reveals a cell-wall-stress stimulon.* Microbiology, 2003. **149**(Pt 10): p. 2719-32.
50. Mascher, T., J.D. Helmann, and G. Unden, *Stimulus perception in bacterial signal-transducing histidine kinases.* Microbiol Mol Biol Rev, 2006. **70**(4): p. 910-38.
51. Kuroda, M., et al., *Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in Staphylococcus aureus.* Mol Microbiol, 2003. **49**(3): p. 807-21.
52. Kuroda, M., K. Kuwahara-Arai, and K. Hiramatsu, *Identification of the up- and down-regulated genes in vancomycin-resistant Staphylococcus aureus strains Mu3 and Mu50 by cDNA differential hybridization method.* Biochem Biophys Res Commun, 2000. **269**(2): p. 485-90.
53. McCallum, N., et al., *Mutational analyses of open reading frames within the vraSR operon and their roles in the cell wall stress response of Staphylococcus aureus.* Antimicrob Agents Chemother, 2011. **55**(4): p. 1391-402.
54. Yin, S., R.S. Daum, and S. Boyle-Vavra, *VraSR two-component regulatory system and its role in induction of pbp2 and vraSR expression by cell wall antimicrobials in Staphylococcus aureus.* Antimicrob Agents Chemother, 2006. **50**(1): p. 336-43.
55. Belcheva, A. and D. Golemi-Kotra, *A close-up view of the VraSR two-component system. A mediator of Staphylococcus aureus response to cell wall damage.* J Biol Chem, 2008. **283**(18): p. 12354-64.
56. Boyle-Vavra, S., et al., *VraT/YvqF is required for methicillin resistance and activation of the VraSR regulon in Staphylococcus aureus.* Antimicrob Agents Chemother, 2013. **57**(1): p. 83-95.
57. Fabret, C., V.A. Feher, and J.A. Hoch, *Two-component signal transduction in Bacillus subtilis: how one organism sees its world.* J Bacteriol, 1999. **181**(7): p. 1975-83.
58. Gardete, S., et al., *Role of VraSR in antibiotic resistance and antibiotic-induced stress response in Staphylococcus aureus.* Antimicrob Agents Chemother, 2006. **50**(10): p. 3424-34.
59. McAdams, H.H. and A. Arkin, *Stochastic mechanisms in gene expression.* Proc Natl Acad Sci U S A, 1997. **94**(3): p. 814-9.
60. Dong, D., et al., *Gene expression variations are predictive for stochastic noise.* Nucleic Acids Res, 2011. **39**(2): p. 403-13.
61. Elowitz, M.B., et al., *Stochastic gene expression in a single cell.* Science, 2002. **297**(5584): p. 1183-6.
62. Dubnau, D. and R. Losick, *Bistability in bacteria.* Mol Microbiol, 2006. **61**(3): p. 564-72.
63. Balaban, N.Q., et al., *Bacterial persistence as a phenotypic switch.* Science, 2004. **305**(5690): p. 1622-5.
64. Gefen, O. and N.Q. Balaban, *The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress.* FEMS Microbiol Rev, 2009. **33**(4): p. 704-17.
65. Cozens, R.M., et al., *Evaluation of the bactericidal activity of beta-lactam antibiotics on slowly growing bacteria cultured in the chemostat.* Antimicrob Agents Chemother, 1986. **29**(5): p. 797-802.
66. Monk, I.R., et al., *Transforming the Untransformable: Application of Direct Transformation To Manipulate Genetically Staphylococcus aureus and Staphylococcus epidermidis.* mBio, 2012. **3**(2).
67. Wolz, C., et al., *Agr-independent regulation of fibronectin-binding protein(s) by the regulatory locus sar in Staphylococcus aureus.* Mol Microbiol, 2000. **36**(1): p. 230-43.
68. Memmi, G., et al., *Staphylococcus aureus PBP4 is essential for beta-lactam resistance in community-acquired methicillin-resistant strains.* Antimicrob Agents Chemother, 2008. **52**(11): p. 3955-66.
69. Pereira, P.M., et al., *Fluorescent reporters for studies of cellular localization of proteins in Staphylococcus aureus.* Appl Environ Microbiol, 2010. **76**(13): p. 4346-53.
70. Sambrook, J., E. Fritsch, and T. Maniatis, *Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York..* 1989.
71. Kraemer, G.R.I., J.J.,, *High-Frequency Transformation of Staphylococcus aureus by Electroporation.* Curr Microb, 1990. **21**: p. 373-376.
72. Oshida, T. and A. Tomasz, *Isolation and characterization of a Tn551-autolysis mutant of Staphylococcus aureus.* J Bacteriol, 1992. **174**(15): p. 4952-9.
73. Wurch, T., F. Lestienne, and P.J. Pauwels, *A modified overlap extension PCR method to create chimeric genes in the absence of restriction enzymes.* Biotechnology techniques, 1998. **12**: p. 653-657.

74. Fisher, A.C. and M.P. DeLisa, *Laboratory evolution of fast-folding green fluorescent protein using secretory pathway quality control.* PloS one, 2008. **3**: p. e2351.
75. Utaida, S., *Genome-wide transcriptional profiling of the response of Staphylococcus aureus to cell-wall-active antibiotics reveals a cell-wall-stress stimulon.* Microbiology, 2003. **149**(10): p. 2719-2732.
76. Belcheva, A., V. Verma, and D. Golemi-Kotra, *DNA-binding activity of the vancomycin resistance associated regulator protein VraR and the role of phosphorylation in transcriptional regulation of the vraSR operon.* Biochemistry, 2009. **48**(24): p. 5592-601.
77. Brown, T.D., M.C. Jones-Mortimer, and H.L. Kornberg, *The enzymic interconversion of acetate and acetyl-coenzyme A in Escherichia coli.* J Gen Microbiol, 1977. **102**(2): p. 327-36.
78. Castano-Cerezo, S., et al., *An insight into the role of phosphotransacetylase (pta) and the acetate/acetyl-CoA node in Escherichia coli.* Microb Cell Fact, 2009. **8**: p. 54.
79. Enright, M.C., et al., *Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus.* J Clin Microbiol, 2000. **38**(3): p. 1008-15.
80. Pereira, A.R., et al., *The Holliday junction resolvase RecU is required for chromosome segregation and DNA damage repair in Staphylococcus aureus.* BMC Microbiol, 2013. **13**: p. 18.
81. Limpert, E., W.A. Stahel and M. Abbt, *Log-normal Distributions across the Sciences: Keys and Clues*. Bioscience, 2001. **51**(5): p. 341-52
82. Durbin, B.P., et al., *A variance-stabilizing transformation for gene-expression microarray data.* Bioinformatics, 2002. **18 Suppl 1**: p. S105-10.
83. Foster, T.J., *Immune evasion by staphylococci.* Nat Rev Microbiol, 2005. **3**(12): p. 948-58.
84. Navarre, W.W. and O. Schneewind, *Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope.* Microbiol Mol Biol Rev, 1999. **63**(1): p. 174-229.
85. Campos-Bermudez, V.A., et al., *Functional dissection of Escherichia coli phosphotransacetylase structural domains and analysis of key compounds involved in activity regulation.* FEBS J, 2010. **277**(8): p. 1957-66.
86. Morya, V.K., V. Dewaker, and E.K. Kim, *In silico study and validation of phosphotransacetylase (PTA) as a putative drug target for Staphylococcus aureus by homology-based modelling and virtual screening.* Appl Biochem Biotechnol, 2012. **168**(7): p. 1792-805.
87. Tomasz, A., S. Nachman, and H. Leaf, *Stable classes of phenotypic expression in methicillin-resistant clinical isolates of staphylococci.* Antimicrob Agents Chemother, 1991. **35**(1): p. 124-9.
88. Wootton, M., et al., *A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in Staphylococcus aureus in a UK hospital.* J Antimicrob Chemother, 2001. **47**(4): p. 399-403.
89. Hartman, B.J. and A. Tomasz, *Expression of methicillin resistance in heterogeneous strains of Staphylococcus aureus.* Antimicrob Agents Chemother, 1986. **29**(1): p. 85-92.
90. Okuma, K., et al., *Dissemination of new methicillin-resistant Staphylococcus aureus clones in the community.* J Clin Microbiol, 2002. **40**(11): p. 4289-94.
91. Liu, Y., et al., *Unexpected diversity of staphylococcal cassette chromosome mec type IV in methicillin-resistant Staphylococcus aureus strains.* J Clin Microbiol, 2010. **48**(10): p. 3628-34.
92. Arede, P., et al., *The anti-repressor MecR2 promotes the proteolysis of the mecA repressor and enables optimal expression of beta-lactam resistance in MRSA.* PLoS Pathog, 2012. **8**(7): p. e1002816.
93. Feldman, M.B., et al., *Aminoglycoside activity observed on single pre-translocation ribosome complexes.* Nat Chem Biol, 2010. **6**(1): p. 54-62.
94. Tan, C., et al., *The inoculum effect and band-pass bacterial response to periodic antibiotic treatment.* Mol Syst Biol, 2012. **8**: p. 617.
95. Chambers, H.F., M.J. Sachdeva, and C.J. Hackbarth, *Kinetics of penicillin binding to penicillin-binding proteins of Staphylococcus aureus.* Biochem J, 1994. **301 (Pt 1)**: p. 139-44.
96. Rocke, D.M. and B. Durbin, *Approximate variance-stabilizing transformations for gene-expression microarray data.* Bioinformatics, 2003. **19**(8): p. 966-72.
97. Leski, T.A. and A. Tomasz, *Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of Staphylococcus aureus: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A.* J Bacteriol, 2005. **187**(5): p. 1815-24.
98. Gefen, O. and N.Q. Balaban, *The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress.* FEMS Microbiology Reviews, 2009. **33**(4): p. 704-717.
99. Moravvej, Z., et al., *Update on the global number of vancomycin-resistant Staphylococcus aureus (VRSA) strains.* Int J Antimicrob Agents, 2013. **42**(4): p. 370-1.
100. Taiwo, S.S., *Methicillin resistance in Staphylococcus aureus: a review of the molecular epidemiology, clinical significance and laboratory detection methods.* West Afr J Med, 2009. **28**(5): p. 281-90.