Impact des antibiotiques et des déterminants de résistance sur l'expression et le contrôle moléculaire des facteurs de virulence des souches nosocomiales de "Staphylococcus aureus"

LI, Dongmei

Abstract
La forte pression sélective des antibiotiques et l'augmentation croissante de la résistance à de nombreux antibiotiques ont un impact significatif sur la virulence des souches nosocomiales des staphylocoques dorés les plus adaptées au milieu hospitalier, surtout celles qui sont résistantes à la méticilline. Les souches de "S. aureus" résistantes aux fluoroquinolones deviennent plus adhérentes à des surfaces recouvertes de fibronectine après croissance en présence d'une concentration sub-inhibitrice de ciprofloxacine. Cette augmentation d'adhérence est due à deux protéines bactériennes liant spécifiquement la fibronectine, dont la synthèse est stimulée par la ciprofloxacine par l'intermédiaire du système RecA-LexA de réparation de l'ADN bactérien. Ce travail décrit la contribution du facteur de transcription alternatif Sigma B à l'augmentation de l'adhérence à la fibronectine ainsi que son interaction avec le système RecA-LexA stimulé par la ciprofloxacine. Les systèmes Sigma B et RecA-LexA ont un effet combiné sur l'adhérence de "S. aureus" à la fibronectine.

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Impact des antibiotiques et des déterminants de résistance sur l'expression et le contrôle moléculaire des facteurs de virulence des souches nosocomiales de *Staphylococcus aureus*

Thèse
présentée à la Faculté de Médecine
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pour obtenir le grade de Docteur en Médecine

par

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de

Chine

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>agr</td>
<td>accessory gene regulator</td>
</tr>
<tr>
<td>AIP</td>
<td>autoinducing peptide</td>
</tr>
<tr>
<td>asp23</td>
<td>alkaline-shock protein 23</td>
</tr>
<tr>
<td>CA-MRSA</td>
<td>community-acquired MRSA</td>
</tr>
<tr>
<td>ccrA</td>
<td>cassette chromosome recombinases A</td>
</tr>
<tr>
<td>ccrB</td>
<td>cassette chromosome recombinases B</td>
</tr>
<tr>
<td>ClfA</td>
<td>clumping factor A</td>
</tr>
<tr>
<td>ClfB</td>
<td>clumping factor B</td>
</tr>
<tr>
<td>cna</td>
<td>collagen binding protein</td>
</tr>
<tr>
<td>CPX</td>
<td>ciprofloxacin</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>Ebp</td>
<td>elastin-binding protein</td>
</tr>
<tr>
<td>EFT</td>
<td>exfoliative toxins</td>
</tr>
<tr>
<td>Fn</td>
<td>fibronectin</td>
</tr>
<tr>
<td>fnbA</td>
<td>fibronectin binding gene A</td>
</tr>
<tr>
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<td>FnBPA</td>
<td>fibronectin-binding proteins A</td>
</tr>
<tr>
<td>FnBPB</td>
<td>fibronectin-binding Protein B</td>
</tr>
<tr>
<td>GI</td>
<td>genomic islands</td>
</tr>
<tr>
<td>HA-MRSA</td>
<td>hospital-acquired MRSA</td>
</tr>
<tr>
<td>HCWs</td>
<td>healthcare workers</td>
</tr>
<tr>
<td>hla</td>
<td>alpha-hemolysin gene</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IS</td>
<td>insertion sequence</td>
</tr>
<tr>
<td>MDR</td>
<td>multidrug resistance</td>
</tr>
<tr>
<td>mec</td>
<td>methicillin resistant gene</td>
</tr>
<tr>
<td>MHB</td>
<td>mueller-hinton broth</td>
</tr>
<tr>
<td>MICs</td>
<td>minimum inhibitory concentrations</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MSCRAMM</td>
<td>microbial surface components recognizing adhesive matrix molecules</td>
</tr>
<tr>
<td>NNIS</td>
<td>the National Nosocomial Infections Surveillance system</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frames</td>
</tr>
<tr>
<td>PBP</td>
<td>penicillin binding protein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PFGE</td>
<td>pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PMMA</td>
<td>polymethylmethacrylate</td>
</tr>
<tr>
<td>Q-R</td>
<td>quinolone resistant</td>
</tr>
<tr>
<td>QRDR</td>
<td>quinolone resistance-determining region</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartate</td>
</tr>
<tr>
<td>rsb</td>
<td>regulators of Sigma B</td>
</tr>
<tr>
<td>sar</td>
<td>staphylococcal accessory regulator</td>
</tr>
<tr>
<td>SCCmec</td>
<td>staphylococcal cassette chromosome <em>mec</em></td>
</tr>
<tr>
<td>SE</td>
<td>staphylococcal enterotoxin</td>
</tr>
<tr>
<td>SigB</td>
<td>transcription factor Sigma B</td>
</tr>
<tr>
<td>spa</td>
<td>protein A</td>
</tr>
<tr>
<td>TCS</td>
<td>two-component system</td>
</tr>
<tr>
<td>TSST</td>
<td>toxic shock syndrome toxin</td>
</tr>
<tr>
<td>VISA</td>
<td>vancomycin-intermediate <em>S. aureus</em></td>
</tr>
<tr>
<td>VRSA</td>
<td>vancomycin-resistant <em>S. aureus</em></td>
</tr>
<tr>
<td>$\sigma^\text{A}$</td>
<td>primary sigma factor</td>
</tr>
<tr>
<td>$\sigma^\text{B}$</td>
<td>alternative sigma factor B</td>
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Résumé du travail en français

Les souches nosocomiales de *Staphylococcus aureus* (nom commun: staphylocoque doré) représentent la source majeure des infections hospitalières. Bien que munies d’un vaste arsenal de facteurs de virulence, les souches de *S. aureus* ne créent pas systématiquement des infections mais colonisent le plus souvent les muqueuses d’un grand nombre de porteurs sains. De plus, le spectre d’infections causées par *S. aureus* est très étendu, allant des infections cutanées mineures jusqu’à des infections systémiques sévères telles que septicémies et endocardites. Ces caractéristiques de virulence font de *S. aureus* un pathogène majeur d’infections hospitalières et communautaires. En milieu hospitalier, la forte pression sélective des antibiotiques depuis un demi-siècle a généré une augmentation croissante de résistances multiples à différentes catégories d’antibiotiques. Des observations récentes montrent également une augmentation croissante de la résistance à un ou plusieurs antibiotiques chez les souches de *S. aureus* responsables d’infections communautaires.

Parmi les différentes catégories de résistance antibiotique, celle ayant l’impact nosocomial le plus important est la résistance à la méticilline (et à l’ensemble des autres béta-lactamines et céphalosporines). Les souches de *S. aureus* résistantes à la méticilline, connues sous l’acronyme de MRSA, font l’objet d’une surveillance épidémiologique constante dans la plupart des hôpitaux du monde entier. Bien qu’une proportion importante des souches de MRSA colonisent de façon asymptomatique les muqueuses nasales des patients hospitalisés, elles sont à l’origine d’une forte proportion d’infections nosocomiales chez ces même patients. Une caractéristique importante des souches MRSA est d’associer la résistance à la méticilline avec celle à d’autres catégories d’antibiotiques (macrolides, aminosides, fluoroquinolones). Moins fréquemment, on observe chez les souches MRSA des co-résistances à la rifampicine, au co-trimoxazol, ou à des glycopeptides tels que la teicoplanine ou la vancomycine. La forte pression sélective des antibiotiques ainsi que l’augmentation croissante de résistances multiples à différentes catégories d’antibiotiques ont forcément un impact sur les caractéristiques de virulence et l’évolution des souches MRSA nosocomiales de *S. aureus* les plus adaptées au milieu hospitalier. L’éradication des souches MRSA nosocomiales est un objectif difficile à atteindre, car les méthodes de décolonisation des porteurs asymptomatiques sont peu efficaces à long terme.
En plus des antibiotiques et antiseptiques locaux auxquels ils sont exposés, les isolats nosocomiaux de *S. aureus* (incl. MRSA) doivent également affronter d’autres stress physiques ou chimiques, tels que les hautes températures, les rayons UV, les traitements alcooliques, etc. Les souches de *S. aureus* (incl. MRSA) ont développé au cours de leur évolution des systèmes efficaces de réponses coordonnées à ces différents stress, qui favorisent leur survie dans un environnement hospitalier particulièrement hostile. Parmi ces réponses coordonnées aux agents de stress, certaines leur permettent de réparer leur ADN endommagé alors que d’autres vont réparer les protéines endommagées ou dénaturées. Ces différents systèmes de réparation sont mal connus car ils ont été très peu étudiés chez les souches de *S. aureus*. Cependant, nous pouvons modéliser en partie leur fonctionnement grâce à des systèmes homologues étudiés en détail chez d’autres bactéries à Gram positif (*Bacillus subtilis*) ou Gram négatif (*Escherichia coli*). Nous pouvons également prédire leur présence grâce aux séquençages complets des génomes de plusieurs souches de *S. aureus*.

Deux systèmes permettant de réparer chez *S. aureus* les lésions de l’ADN (système SOS) ou de répondre à divers stress physico-chimiques (par l’intermédiaire du facteur de transcription alternatif Sigma B) ont été particulièrement étudiés dans mon laboratoire d’accueil. Le système SOS de réparation de l’ADN est principalement stimulé par l’exposition à des fluoroquinolones (ex. norfloxacine, ciprofloxacine, levofloxacine, moxifloxacine, etc.) ou par d’autres types de lésions de l’ADN par rayons UV ou certains agents chimiques (ex. mitomycine). Les fluoroquinolones sont utilisées très intensivement depuis une quinzaine d’années en milieu hospitalier et ambulatoire et la majorité des souches MRSA sont devenues hautement résistantes à cette classe d’antibiotiques. Le système SOS a surtout été étudié en détail chez *E. coli* et beaucoup moins chez les bactéries à Gram positif telles que *B. subtilis* ou *S. aureus*. Les homologies entre espèces bactériennes ne sont que partielles. Toutefois, la partie la plus centrale du système SOS, qui est commune à la quasi-totalité des espèces bactériennes étudiées, est le système RecA-LexA. La protéine LexA est le répresseur principal de l’ensemble des gènes du système SOS, qui ne sont presque pas exprimés à l’état de repos. A l’inverse, la protéine RecA, qui s’auto-active au contact de lésions de l’ADN, induit un clivage spontané de la protéine LexA permettant la transcription des gènes SOS précédemment réprimés par LexA.

Le système sigma B de réponse globale aux stress a comme élément central une sous-unité de l’ARN polymérase qui reconnaît spécifiquement certains gènes dont la transcription est
déclenchée par exposition à certains stress. Dans cette situation, la sous-unité sigma B va remplacer la sous-unité sigma A de l’ARN polymérase pour permettre l’expression de gènes de réponses aux stress. La reconnaissance des gènes induits par le stress se fait grâce à des changements de courtes séquences nucléotidiques des régions promotorices –35 et –10, en amont des codons initiateurs de la transcription, qui sont spécifiquement reconnues par la sous-unité sigma B associée à l’ARN polymérase. La synthèse et l’activité de la sous-unité sigma B sont contrôlées par un opéron associant le gène codant pour cette protéine avec trois autres gènes synthétisant des régulateurs contrôlant de manière stricte la concentration intracytoplasmique de Sigma B. Ces trois régulateurs de Sigma B sont RsbW (formant un complexe avec sigma B dans les conditions de repos), RsbV (inactif dans les conditions de repos, mais activé par le stress et menant à la libération de Sigma B), et RsbU qui est responsable de la transduction des signaux de stress. Bien que les quatre gènes de l’opéron Sigma B de *S. aureus* montrent une forte homologie avec ceux présents chez *B. subtilis*, la taille totale de l’opéron Sigma B de *S. aureus* est inférieure de moitié à celle de *B. subtilis*.

De nombreux facteurs de virulence ont été identifiés chez *S. aureus*. On peut les répartir arbitrairement dans plusieurs grandes catégories en fonction de leur mécanisme d’action : (i) les exotoxines ou hydrolases endommageant les tissus ou les cellules sanguines (érythrocytes, leucocytes); (ii) les adhésines, qui sont des protéines de surface permettant l’attachement de *S. aureus* à des composantes cellulaires ou extracellulaires de l’hôte (ex. fibrinogène, fibronectin, collagène, élastine, etc.) et contribuant à la colonisation de l’hôte; (iii) les composantes de surface (ex. capsule, protéine A) protégeant *S. aureus* du système immunologique de l’hôte; (iv) les superantigènes (ex. toxine du choc septique, entérotoxine, etc.) produisant des réactions immunologiques excessives chez l’hôte; (v) la formation de biofilm à la surface de certains tissus ou d’implants artificiels.

Les deux protéines étudiées dans mon travail représentent un couple d’adhésines liant la fibronectine (en anglais : fibronectin-binding protein ; abbrév. FnBPs). Ces deux protéines, respectivement FnBPA et FnBPB, ont été étudiées en détail dans mon laboratoire d’accueil depuis >10 ans, car elles jouent un rôle central dans l’attachement de *S. aureus* à la matrice extracellulaire ainsi qu’à des matériaux implantés. Plus récemment, notre laboratoire a démontré que les FnBPs jouent un rôle essentiel pour l’endocytose de *S. aureus* par des cellules non-phagocytaires (ex. cellules épithéliales et endothéliales, fibroblastes, ostéoblastes), un processus
qui permet aux staphylocoques intracellulaires d'échapper aux mécanismes de destruction phagocytaire ou antibiotique.

D’autres expériences récentes effectuées dans notre laboratoire ont démontré que les souches de *S. aureus* résistantes aux fluoroquinolones augmentent leur adhérence à des surfaces recouvertes de fibronectine après croissance en présence d’une concentration sub-inhibitrice de ciprofloxacine. Le mécanisme moléculaire responsable de cet accroissement de l’adhérence de *S. aureus* est une augmentation de la synthèse de FnBPB, elle-même due à une transcription plus intense du gène *fnbB*. Des expériences de génétique moléculaire ont démontré que la synthèse accrue de FnBPB par l’exposition à la ciprofloxacine mettait à contribution le système RecA-LexA de réparation de l’ADN bactérien.

Mon travail expérimental décrit le rôle du facteur de transcription alternatif Sigma B dans l’augmentation de la production de FnBPs, aux niveaux transcriptionnel et fonctionnel, chez des souches isogéniques de *S. aureus* résistantes aux fluoroquinolones. Une question additionnelle était d’évaluer en parallèle la stimulation de la transcription de FnBPs *via* le système RecA-LexA, en incubant les souches isogéniques de *S. aureus* avec des concentrations sub-inhibitrices de ciprofloxacine. Le but de ce protocole était de tester si ces deux systèmes d’induction de synthèse des FnBPs pouvaient être stimulés indépendamment ou interféraient l’un avec l’autre. Des mutants isogéniques d’une souche quinolone-résistante de *S. aureus* exprimant trois niveaux différents d’activité de Sigma B ont été préparés par transduction phagique. La 1ère souche montrait une inactivation complète du gène synthétisant Sigma B, la 2ème souche exprimait une faible quantité basale de Sigma B qui ne pouvait pas augmenter en présence d’un stress, alors que la 3ème souche était complètement restaurée pour sa production de Sigma B et activable par un stress. D’un point de vue morphologique, la culture sur agar de ces différentes souches a confirmé les différences phénotypiques attendues : la souche dont le Sigma B est complètement restauré a montré une pigmentation orangée beaucoup plus intense que les deux autres souches, mais une production inférieure d’exotoxine alpha-hémolytique et de protéases secrétées dans le milieu ambiant.

Les trois souches isogéniques de *S. aureus* exprimant différents niveaux d’activité de SigB ont montré une augmentation proportionnelle de la synthèse de FnBPs qui a entraîné une augmentation de leur attachement à la fibronectine. La présence accrue de FnBPs due à l’augmentation de Sigma B ou à l’exposition à la ciprofloxacine a été confirmée par cytométrie
de flux. L’accroissement de FnBPs lié à l’activité de Sigma B dans les souches isogéniques et/ou à leur exposition à la ciprofloxacine était le reflet d’une transcription accrue, testée par RT-PCR en temps réel, de leurs gènes fnbB et dans une moindre mesure fnbA. D’autres mesures de transcription de gènes par RT-PCR en temps réel ont montré une diminution importante de la transcription du gène de l’exotoxine alpha-hémolytique et à l’inverse une très forte augmentation de l’activité transcriptionnelle du gène codant pour la protéine A, chez la souche dont le Sigma B est complètement restauré par rapport aux deux autres souches. Une partie des changements transcriptionnels observés chez les gènes codant pour les FnBPs, l’exotoxine alpha-hémolytique ou la protéine A s’explique par des changements d’activité de deux de leurs régulateurs globaux : (i) le régulateur global agr a montré une activité inversement proportionnelle à l’activité du facteur Sigma B ; (ii) par contre, le régulateur global sarA a montré une activité directement proportionnelle à l’activité du facteur Sigma B, car il possède un promoteur spécifiquement activé par ce facteur de transcription.

En conclusion, le facteur alternatif de transcription Sigma B chez S. aureus régule positivement les gènes fnbB et fnbA et augmente la quantité de protéines de surface agissant comme adhésines pour la fibronectine. La stimulation des FnBPs par l’activité du facteur Sigma B est due à l’activation d’un système de régulation indépendant du système LexA-RecA stimulé par la ciprofloxacine. Cependant la stimulation combinée des deux systèmes Sigma B et RecA-LexA a un effet additif sur la production accrue de FnBPs et l’augmentation de l’adhérence de S. aureus à la fibronectine. La composition moléculaire de l’ensemble des facteurs des systèmes Sigma B et RecA-LexA reste en grande partie énigmatique et nécessitera d’autres études approfondies.

Ces observations confirment expérimentalement la notion que le milieu hospitalier a un impact significatif sur la virulence des souches nosocomiales de S. aureus, en particulier celles du groupe MRSA, qui subissent une forte pression sélective par l’usage intensif de multiples agents antimicrobiens et l’émergence croissante de nombreuses résistances antibiotiques.
English summary

The strong selective pressure of antibiotics and the increasing number of multi-drug resistant strains in hospitals have a significant impact on virulence properties of nosocomial strains of *Staphylococcus aureus*, in particular those exhibiting resistance to methicillin (MRSA). Recent experiments have shown that fluoroquinolone-resistant strains of *S. aureus* grown in the presence of subinhibitory concentrations of ciprofloxacin exhibit increased adhesion to fibronectin-coated surfaces. The molecular basis of this ciprofloxacin-promoted adhesion involves increased synthesis of two bacterial proteins (FnBPs) exhibiting specific binding to fibronectin via the contribution of the RecA-LexA DNA repair pathway. This work describes the contribution of the alternative transcription factor Sigma B (SigB), another bacterial stress response factor, to the upregulation of FnBPs as well as its interaction with the ciprofloxacin-triggered RecA-LexA pathway. Isogenic mutants of a fluoroquinolone-resistant strain of *S. aureus* exhibiting different levels of SigB activity show a proportional increase of FnBP synthesis leading to enhanced bacterial adhesion to fibronectin-coated surfaces. Upregulation of FnBPs by SigB functional activity and ciprofloxacin exposure occur via independent pathways. Nevertheless, the combined stimulation of SigB-dependent and LexA-RecA pathways leads to additive effects on upregulation of FnBPs and increased bacterial adhesion to fibronectin.
Section I. OVERVIEW OF STAPHYLOCOCCAL PATHOGENESIS AND ANTIBIOTIC RESISTANCE
A. GENERAL CHARACTERISTICS OF STAPHYLOCOCCI

Staphylococci are spherical cells about 1 micrometer in diameter (figure 1). They grow in clusters because staphylococci divide in two planes. Staphylococci are found in association with skin, skin glands, and mucous membranes of warm-blooded animals, although some species can be isolated from processed animal sources such as meat and diary products, or from environmental sources, such as soil, dust, air, and water.

Members of the genus are usually facultative anaerobes, capable of generating energy by respiratory or fermentative pathways. Most species have relatively complex nutritional requirements, usually requiring several amino acids and B vitamins. They are tolerant to high concentrations of NaCl and temperature ranging from 10°C to 45°C. Staphylococci belong to the low G + C (30 to 38 mol percent) group of the gram-positive bacterial phylogenetic group (Wilkinson, 1997).

**Taxonomy**

The genus Staphylococcus represents gram-positive, catalase-positive cocci that historically belonged to the bacterial family Micrococccaceae (Witte, 2000). Phylogenetic classification indicated that over 50% of predicted proteins encoded by the *S. aureus* genome are closely related to those in *Bacillus subtilis* and *Bacillus halodurans*. They typically contain house-keeping genes, essential for bacteria to absorb nutrients from the environment, synthesize metabolic intermediates, and multiply. They possess bacteriophages, pathogenicity islands, transposons, and insertion sequence elements distributed over the genomes (Kuroda, 2001).
Gram positive cell wall

**Figure 2.** Gram positive cell wall.

Gram positive cell wall is composed of a thick peptidoglycan (murein) layer and ribitol teichoic acids. Lipoteichoic acid, which may protrude on the surface through the peptidoglycan layer, is linked to the cytoplasm. Cell wall ribitol teichoic acid is covalently linked to the peptidoglycan layer. The cell walls of most gram-positive bacterial species have an extensive meshwork of peptidoglycan layer that is typically 15-30 nm thick. Peptidoglycan polymers are cross-linked by pentaglycine bridges between L-lysine and the terminal D-alanine, building a huge macromolecular murein sacculus around each bacterium (Beveridge, 2000).

**Basic structure of *S. aureus* genome**

The staphylococcal genome consists of a single circular chromosome generally including prophages, transposons, insertion sequences, and other variable accessory elements plus irregularly present, autonomous plasmids. *Staphylococcus aureus* (*S. aureus*) genome is about 2.8 Megabase pairs long with approximately 2600 open reading frames. The genes for antibiotic resistance in *S. aureus* are located on plasmids, transposons, or on the chromosome (Kuroda, 2001).

*S. aureus* NCTC 8325 has been the organism of choice for genetic and mapping studies of *S. aureus* chromosome. The parental strain is known to carry three prophages called φ11, φ12, and φ13. However, UV-cured (prophage minus) derivatives, such as 8325-4 (RN 450) or RN6390 have been used for most genetic studies (Iandolo, 2002).
Whole genome sequences of two health-care-associated MRSA strains, N315 (figure 3) and Mu50, isolated in Japan in 1982 and 1997, respectively, and one community-acquired MRSA strain MW2 (MW standing for midwest USA) have been published (Kuroda, 2001; Baba, 2002). The three MRSA strains have closely related nucleotide sequences (99.7% identity between N315 and Mu50, 94.8% between MW2 and N315, and 94.7% between MW2 and Mu50). General features of the N315 and Mu50 genomes are described (Kuroda, 2001). They have a low G+C content (average 33%), and possess five rDNA operons. The N315 and Mu50 genomes contain 2595 and 2697 open reading frames, respectively. Most of the nucleotide differences between strains N315 and Mu50 are due to insertion of mobile genetic elements. Both strains possess several bacteriophages, pathogenicity islands, transposons, and insertion sequence (IS) elements distributed over the genomes. Additionally,
each strain has a distinct plasmid carrying different antibiotic resistance genes (Kuroda, 2001).

**Genomic islands (GI)**

A number of GIs, defined as chromosomal regions likely acquired from other strains or species, are found on the *S. aureus* chromosome. They are named according to the chromosomal loci where the islands are integrated. Some GIs confer pathogenicity while others confer antibiotic resistance. Among these islands, the staphylococcal cassette chromosome *mec* (SCCmec; *mec* standing for methicillin resistance) element carries antibiotic resistance genes (Ito, 2003).

**B. EPIDEMIOLOGY**

Many humans are regularly colonized by *S. aureus*, and asymptomatic colonization is far more common than infection (Chambers, 2001). Colonization of the nasopharynx, perineum, or skin, particularly if the cutaneous barrier has been disrupted or damaged, may occur shortly after birth and may recur anytime thereafter. Family members of a colonized infant may also become colonized. In some epidemics of MRSA, a relatively low level of nasal carriage (3%) has been found in hospital personnel, but more recent studies indicate a higher rate of MRSA nasal carriage in health care workers for endemic MRSA situations. The sharp increase in the prevalence of MRSA in many communities has led to consider outpatients as a potential source of contamination in an institution. Populations that are more susceptible to MRSA colonization include intravenous drug users, persons with dermatologic diseases, diabetes, and persons on kidney dialysis. Young children tend to have higher colonization rates, probably because of their frequent contact with respiratory secretions. Colonization may be transient or persistent and can last for years.

**Nosocomial infections**

*S. aureus* is a major pathogen of hospital-acquired infections. Colonization of healthy carriers represents a major source of nosocomial infections. A major concern of nosocomial *S. aureus* infections is the presence of endemic isolates exhibiting resistance to methicillin and frequently to several other antibiotics. The prevalence of methicillin-resistant *S. aureus* (MRSA) has increased in many countries over the world since 1980. The first strain of MRSA
was isolated in 1961 (Jevons, 1961), two years after the introduction of methicillin. MRSA mainly reside in environments in which there is a constant antibiotic pressure such as hospitals. Once endemically present, they are difficult to control and eradicate. A striking property of MRSA is their tendency to accumulate additional unrelated resistance determinants and incorporate them into their genome. Their adaptability and ready response to antibiotic selection has led to the evolution of MRSA strains resistant to almost all commonly used antibiotics. While vancomycin is still the antibiotic of choice (and frequently of last resort) for treating MRSA infections, reports of vancomycin-intermediate *S. aureus* (VISA) in Japan in 1997 (Hiramatsu, 1997) and later in other countries caused widespread concern among physicians. In 2002, the first vancomycin-resistant *S. aureus* (VRSA) isolates, exhibiting vancomycin MICs of 1024 and 32 µg/ml, respectively, were reported in Michigan and Pennsylvania (CDC, 2002a; CDC, 2002b). Currently, there is no antibiotic class that is uniformly effective against *S. aureus* (Robinson, 2004).

**MRSA prevalence in hospitals**

Since the mid to late 1990s, the prevalence of MRSA isolates in hospitals increased in Europe, the USA, and elsewhere. In one European study performed in 25 university hospitals, one-quarter of 3051 *S. aureus* isolates were MRSA, with a geographical bias towards higher rates in southern countries such as Italy (50.5%) and Portugal (54%), and lower rates in northern European countries, including the Netherlands (2%), Austria (9%) and Switzerland (2%) (Robinson, 2004; Enright, 2003). The National Nosocomial Infections Surveillance system (NNIS) reported a 40% increase in the rate of MRSA in 1999 compared to 1994-1998 data (NNIS system report, 2000). MRSA infections are associated with increased morbidity, mortality and length of hospital stay, and represent a major financial burden on healthcare services (Nathwani, 2003; Cosgrove, 2003). Epidemiological studies confirmed that MRSA infections are more costly to manage (screening, treatment and isolation) than other types of infection (Rubin, 1999). They also highlighted the importance of hospital length of stay as a key determinant of the total cost of an episode of infection. In summary, MRSA are now endemic in many hospitals, and represent one of the leading causes of nosocomial pneumonia and surgical site infections and the second leading cause of nosocomial bloodstream infections.
Molecular structure of the methicillin resistance gene

The structural gene for methicillin resistance, \textit{mecA}, encodes a novel penicillin binding protein (PBP)-2’, which has reduced affinity for ß-lactam antibiotics (Hiramatsu, 1995; Chambers, 1988; Berger-Bächi, 2002). This gene is carried on a genetic element, staphylococcal chromosomal cassette (see below), which is a 20-67-kb DNA element which precisely inserts into the \textit{S. aureus} chromosome at the \textit{orfX} locus. SCC\textit{mec} is found in other staphylococcal species from which it is presumed to have been transferred (Ito, 2003).

The staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}) is a special family of staphylococcal genomic islands (GI) characterized by its cassette chromosome recombinases A (CcrA) and B (CcrB) (Hiramatsu, 2001; Ito, 2003; Enright, 2003).

\begin{center}
\textbf{Figure 4. Structures of four types of SCC\textit{mec}.}
\end{center}

SCC\textit{mec} is composed of two essential gene complexes, the \textit{ccr} gene complex (orange) and the \textit{mec} gene complex (gray). \textit{ccr} gene complex is composed of \textit{ccrA}, \textit{ccrB} genes which are responsible for the mobility of SCC\textit{mec} and some \textit{orfs} surrounding them. \textit{mec} gene complex is responsible for ß-lactam resistance. Other areas (light gray) of the SCC\textit{mec} are non-essential, and are divided into three regions. Type-IV SCC\textit{mec} is mostly composed of essential gene complexes (Ito, 2001).
The ccr gene complex is composed of two site-specific recombinase genes, ccrA and ccrB, responsible for the mobility of SCCmec, and surrounding orfs of unknown function. The rest of the SCCmec is designated J regions (J stands for junkyard) that contain various genes or pseudo genes whose presence does not appear essential or useful for the bacterial cell.

**Figure 4** illustrates the structure of the four SCCmec types and their subtypes. Three types of SCCmec element (type I, II, III) are found in hospital-acquired MRSA (HA-MRSA) strains and one type of SCCmec element (type IV) in community-acquired MRSA (CA-MRSA).

Type I SCCmec, a 34-kb element that was first described in 1960s MRSA isolates and does not contain any antibiotic resistance genes other than mecA.

Type II SCCmec, a 53-kb element, carries the type-2 ccr gene complex, which was identified in 1982 and is ubiquitous in Japan, Korea, and the United States. MRSA N315 and Mu50 carry type-II SCCmec, containing integrated copy of plasmid pUB110 and transposon Tn554 in the J region.

Type-III SCCmec, the largest element at 67 kb, was identified in 1985 and is prevalent in Germany, Austria, India, and other South Asia and Pacific areas, containing integrated copy of plasmid pT181, transposon Tn554, and pseudo Tn554 that encode resistance to tetracycline, erythromycin, and cadmium, respectively.

Type IV SCCmec includes four subtypes, whose sizes vary from 20 to 24 kb, and is much smaller than SCCmec types I-III. SCCmec type IVa is generally found in recently described (Okuma, 2002) community-acquired MRSA isolates (CA-MRSA). Types-IVa and -IVb SCCmec do not harbor any antibiotic resistance genes except for mecA. The community-acquired MRSA MW2 carry type-IVa SCCmec.

**Transmission of MRSA**

Recent molecular studies on the genetic origin of methicillin resistance in *S. aureus* have led to a greater understanding of the epidemiology of MRSA (Muto, 2003). De novo development of MRSA results when a strain of MSSA acquires a large genomic element SCCmec. Detailed genetic analysis of MRSA from diverse parts of the world suggests that the transfer of SCCmec from a MRSA strain to a MSSA strain occurred rarely, and therefore the worldwide emergence of MRSA mostly resulted from dissemination of only a limited number of types rather than frequent *de novo* introduction of new MRSA clones. These findings
suggest that most patients acquired their MRSA infection or colonization by transmission from other colonized patients or health care workers.

Transmission of MRSA within and between healthcare facilities has been well documented using molecular typing techniques, such as pulsed-field gel electrophoresis (PFGE). Outbreaks involving clonal spread within single facilities have been frequently reported. Geographic clustering of closely related genotypes within cities and geographic regions has been described, suggesting that spread beyond the boundaries of a single hospital may occur. Transmission of MRSA from one city to another, from country to country, and even from continent to continent has been traced to the transfer of patients infected or colonized with MRSA.

**Mechanisms of transmission**

- Healthcare workers’ (HCWs) hands is the major source of cross-transmission: in several studies, MRSA or VRE have been isolated from the hands, gloves, or both of the HCWs involved in the care of infected or colonized patients.
- HCWs’ clothes: several investigators suggested that the contaminated HCWs’ clothing may result in the transmission of microbes from patient to patient;
- Contaminated equipment, environment, and air (in the case of staphylococcal pneumonia). In one study, 42% of nurses’ gloves became contaminated with MRSA when they touched surfaces in the room of a patient with MRSA even without touching the patient.

  Prolonged hospital stay, use of broad spectrum antibiotics, greater number and longer duration of antibiotic use, stay in an ICU or burn unit, surgical wounds, poor functional status and proximity to another patient with MRSA constitute risk factors for MRSA acquisition (Muto, 2003).

**Control and preventive measures**

- Active surveillance cultures are essential to identify the reservoir for spread of MRSA and VRE infections and implement infection control guidelines. Many studies have shown that endemic and/or epidemic MRSA and VRE infections can be controlled by using surveillance cultures and contact precautions and has been proven to be effective (Pittet, 2000; Muto, 2003).
• Hand hygiene is an important measure for preventing MRSA transmission. Educating healthcare workers and patients on the importance of hand hygiene is very important for preventing MRSA transmission and has been proven to be effective (Pittet, 2000; Muto, 2003).

• Contact precautions include wearing gloves, gowns and masks (if expected contact with patient or environment), regular cleaning of equipments and items. Jernigan (Jernigan, 1996) found a 16-fold reduction in transmission when contact precautions were implemented.

• Antibiotic control may also decrease the risk of MRSA colonization. It has been shown that antibiotics in general and fluoroquinolone in particular must be used prudently in institutions where MRSA is endemic.

• Eradication of colonization in HCWs is necessary, in particular in outbreak situations.

• Information management: a hospital computer system can be used to store information regarding long-term isolation indicators for patients known to be colonized with MRSA or VRE. This was done at the University of Geneva Hospitals and resulted in a significant improvement in isolation of such patients on readmission (Pittet, 1996).
C. PATHOGENESIS OF S. AUREUS INFECTIONS

*S. aureus* expresses many potential virulence factors including (figure 5):

1. Surface proteins that promote colonization of host tissues or endocytosis;
2. Surface components that may inhibit phagocytic engulfment (capsule, protein A);
3. Membrane-damaging exotoxins that may lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin);
4. Superantigens that may lead to indirect damage of host tissues and toxic shock symptoms, such as staphylococcal enterotoxins (SE), toxic shock syndrome toxin (TSST), exfoliative toxins (EFT).

*S. aureus* has been recognized as serious pathogens for over a century, causing a wide range of diseases ranging from benign or more severe skin infection to often fatal forms of endocarditis or septic shock. The diseases spectrum of *S. aureus* includes superficial skin lesions such as boils, styes and furuncles, abscesses, and serious infections such as bacteremia, central nervous system infections, endocarditis, osteomyelitis, pneumonia, urinary track infections, and syndromes caused by exotoxins, including bullous impetigo, food poisoning, scaled skin syndrome, and toxic shock syndrome (Fred, 2000). Despite large-scale efforts to halt their spread, particularly in hospitals, *S. aureus* remain the major pathogens of community- and nosocomially-acquired bacteremia.
**Surface protein adhesins**

*S. aureus* can adhere to host extracellular matrix (ECM) components to initiate colonization. Bacterial adhesion is mediated in main part by surface protein adhesins of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family. In most cases, MSCRAMMs are covalently anchored to the cell wall peptidoglycan, in particular two fibronectin-binding proteins called FnBPA and FnBPB, a collagen-binding protein (Cna), and the fibrinogen-binding proteins clumping factor A (ClfA) and B (ClfB) (Vaudaux, 2000).

![Diagram of molecular structure of surface proteins in S. aureus](image)

**Figure 6.** Structural organization of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) proteins (a) Spa, (b) Cna, (c) ClfA and (d) FnBPA and FnBPB of *S. aureus* (Bisognano, 2000a). ‘S’ represents the signal sequence; ‘R’ represents the Ser–Asp dipeptide repeats; ‘W’ represents the wall-spanning region; and ‘M’ represents the membrane-spanning region and positively charged residues. Wr is composed of an octapeptide repeat, and Wc is a non-repeated region. ‘A, B, C, D’ represent extracellular regions. The positions of the LPXTG motif is indicated. Asterisks indicate ligand-binding domains.
The molecular structure of these *S. aureus* surface proteins is shown on figure 6. Protein A (Spa), the archetypal LPXTG-anchored wall-associated protein, can bind to the Fc domain of immunoglobulin G (IgG) and inhibit opsonophagocytosis (Foster, 1998). The four other proteins, namely Cna, FnBPA, FnBPB, and ClfA are also anchored to the cell wall by the LPXTG (Leu-Pro-X-Thr-Gly) motif and clearly promote bacterial attachment to their specific ECM ligands. Another surface protein is the elastin-binding protein (Ebp, not shown on the figure) that is not a member of the LPXTG-anchored family. *S. aureus* can bind several additional host plasma proteins and ECM components, but the bacterial components which are responsible for binding have not been characterized to the same extent (Menzies, 2003).

The role of these staphylococcal matrix-binding proteins are virulence factors was documented by *in vitro* and *ex vivo* adhesion studies of defective mutants and in experimental models of infections. ClfA-null mutants (defective in binding to fibrinogen) have reduced virulence in a rat model of endocarditis, suggesting that bacterial attachment to the sterile vegetations caused by damaging the endothelial surface of the heart valve is promoted by fibrinogen or fibrin (Moreillon, 1995). In addition, lactococci expressing recombinant FnBPA or ClfA have also increased virulence in the rat endocarditis model (Que, 2001). Similarly, mutants lacking the collagen-binding protein have reduced virulence in a mouse model for septic arthritis. Furthermore, the soluble ligand-binding domain of the fibrinogen, fibronectin and collagen-binding proteins expressed by recombinant methods can block to some extent interactions of bacterial cells with the corresponding host protein (Menzies, 2002).

Fibronectin-binding proteins (FnBPs)

*S. aureus* expresses either one or two FnBPs, FnBPA and FnBPB, which are encoded by two closely linked genes, *fnbA* and *fnbB* (Flock, 1987; Signäs, 1989; Jonsson, 1991; Greene, 1995). At the N-terminus of the protein is the A region which contains a signal sequence that is responsible for translocation of each FnBP through the cytoplasmic membrane (figure 6). Both FnBPs share approximately 45% amino acid identity and can bind specifically to fibronectin *in vitro*. The C-terminus is composed of a hydrophobic membrane-spanning region (region M) and an LPXTG motif that is a target for sortase, a transpeptidase that covalently links FnBPs and other microbial surface proteins to the cell wall peptidoglycan. The fibronectin-binding D region located in the C-terminus is composed of three tandem repeats of 37 or 38 amino acids (D1, D2, and D3) in addition to one incomplete repeat (D4).
A fifth repeat, Du, is found approximately 100 amino acid residues N-terminal to D1. Each D-repeat can bind specifically to fibronectin (Menzies, 2003; Schwarz-Linek, 2004). The D-repeats are highly conserved between FnBPA and FnBPB (about 94% identity) and homologous repeats have been found in fibronectin-binding adhesins of various streptococcal species. The B repeat region is absent in FnBPB (Menzies, 2003).

The ability to bind to the human protein fibronectin (Fn, figure 7) is a characteristic that has been reported for many pathogens (Joh, 1999). In *S. aureus*, binding to fibronectin-coated coverslips of the parental strain containing both *fnb*A and *fnb*B genes was equivalent to those of their isogenic single *fnb*A or *fnb*B mutants with a single *fnb* gene (Greene, 1995). In contrast, the double mutant of *fnb*A and *fnb*B was completely defective in adhesion.

Through binding to fibronectin in the extracellular matrix and cell surface, the FnBPs mediate adhesion and endocytosis of *S. aureus* to a variety of cultured mammalian cells including those of endothelial origin, airway epithelium, explanted biomaterials and implanted intravascular catheters (Joh, 1999; Vaudaux, 1993; Vaudaux, 1995; Greene, 1995). In a large molecular epidemiological study, adhesion to immobilized fibronectin was greater for isolates associated with orthopedic implant-associated infection than for isolates associated with nasal carriage, endocarditis, septic arthritis, and osteomyelitis (Peacock, 1999). Implanted biomaterials become coated with serum components including fibronectin and thus bacterial binding to fibronectin would be an important virulence determinant in this setting. This study demonstrated no difference in fibronectin binding between staphylococcal groups with one versus two *fnb* genes.

The *in vivo* importance of FnBPs was further tested in animal models comparing the virulence of wild-type and mutant strains deficient in FnBPs (Greene, 1995; McElroy, 2002; Menzies, 2003). Since these experiments yielded rather variable results, we can suppose that the relative importance of the FnBPs in different infections may depend on the route of initial bacterial entry and dissemination, the affected tissue and the bacterial strain used (Menzies, 2003; Schwarz-Linek, 2004).

**Role of FnBPs as invasins**

Excepting for the promotion of *S. aureus* attachment to host cells and to fibronectin-coated biomaterials which are important mediators of infection in experimental endocarditis, FnBPs also mediate uptake of *S. aureus* (endocytosis) by cultured non-professional phagocytes using host fibronectin to bridge with integrins on the cell surface. Of particular
interest is the capacity of the FnBPs to act as invasins. *S. aureus* is classically considered as an extracellular pathogen, however, many investigators have demonstrated the ability of *S. aureus* to invade non-professional phagocytes such as various epithelial and endothelial cells *in vitro* (Sinha, 1999). The uptake of staphylococci by mammalian cells may provide a safe harbor for the bacteria from most antimicrobials and from the immune response. This intracellular invasion has been shown to be FnBP-dependent and to involve fibronectin. FnBP-deficient mutants have greatly reduced uptake into host cells, and antibodies directed against fibronectin reduce staphylococcal uptake (Menzies, 2002; Sinha, 1999; Sun, 1997). In fact, FnBP alone is sufficient for invasion of host cells without the need for other staphylococcal cofactors (Sinha, 2000). The host cell receptor involved in the uptake of *S. aureus* appears to be the fibronectin-binding integrin receptor $\alpha_5\beta_1$. Fibronectin acts as a bridging molecule between the surface-bound FnBP and the host integrin $\alpha_5\beta_1$ (Massey, 2001; Sinha, 1999; Sinha, 2000; Schwarz-Linek, 2004).

**Molecular structure of fibronectin**

![Fibronectin molecular structure](image)

Fibronectin is a large ubiquitous glycoprotein found in soluble form in plasma and in an insoluble multimeric form in the extracellular matrix (Potts, 1994; Proctor, 1982). It is also one of the many host proteins deposited on the surface of implanted biomaterials. In addition to fibronectin’s numerous functions including cellular adhesion, differentiation, and tissue repair after injury, fibronectin has adhesive sites for various molecules such as heparin, collagen, fibrin, and specific integrins.

*Figure 7. Fibronectin molecular structure.*
A fibronectin molecule consists of two nearly identical polypeptide chains joined by two disulfide bonds near their carboxyl ends (figure 7). Each polypeptide chain is folded into a series of globular domains linked by short, flexible segments. The globular domains have binding sites for ECM components or for specific receptors on the cell surface. The receptor-binding domain contains the tripeptide sequence RGD (arginine-glycine-aspartate), which is recognized by fibronectin receptors (figure 7). In addition to the binding activities noted, fibronectin also has binding sites for heparin sulfate, hyaluronate, and gangliosides (glycosphingolipids that contain sialic acid groups). The major binding site in fibronectin for the S. aureus FnBPs has been localized in the N-terminal type 1 module of this host protein. In addition, several groups have found another binding site for S. aureus towards the C-terminus of fibronectin.

D. MOLECULAR CONTROL OF S. AUREUS PATHOGENESIS

S. aureus is a remarkably versatile organism. It is flexible and adaptable to its surroundings. This versatility depends on a tremendous range of adaptive or accessory gene systems. Most of the >100 genes coding virulence factors are either displayed on the bacterial surface or released into the surroundings. These enable the organism to evade host defences, to adhere to cells and the extracellular matrix, to spread within the host and to degrade cells and tissues, for both nutrition and protection. These virulence genes are sometimes designated as the virulon, even though they are not all exclusively devoted to pathogenesis (Novick, 2003).

Regulated expression of S. aureus virulence factors

The production of virulence factors is tightly regulated to enabling bacteria to thrive in a hostile host environment (Cheung, 2004).
An extensive list of the tightly regulated virulence factors is provided in Table 1 (Novick, 2003).

**Table 1.** Staphylococcal extracellular accessory proteins. Adapted from Novick, 2003.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Product</th>
<th>Activity/function</th>
<th>Timing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Superantigens</strong></td>
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<tr>
<td><em>sea</em></td>
<td>Phage</td>
<td>Enterotoxin A</td>
<td>Food poisoning, TSS</td>
<td>pxp</td>
</tr>
<tr>
<td><em>seb</em></td>
<td>chrom</td>
<td>Enterotoxin B</td>
<td>Food poisoning, TSS</td>
<td>pxp</td>
</tr>
<tr>
<td><em>sec</em></td>
<td>chrom</td>
<td>Enterotoxin C</td>
<td>Food poisoning, TSS</td>
<td>pxp</td>
</tr>
<tr>
<td><em>sed</em></td>
<td>Plasmid</td>
<td>Enterotoxin D</td>
<td>Food poisoning, TSS</td>
<td>pxp</td>
</tr>
<tr>
<td><em>eta</em></td>
<td>ETA phage</td>
<td>Exfoliatin A</td>
<td>Scalded skin syndrome</td>
<td>pxp</td>
</tr>
<tr>
<td><em>etb</em></td>
<td>Plasmid</td>
<td>Exfoliatin B</td>
<td>Scalded skin syndrome</td>
<td>pxp</td>
</tr>
<tr>
<td><em>tst</em></td>
<td>SaPI1, 2, bov1</td>
<td>Toxic shock toxin-1</td>
<td>Toxic shock syndrome</td>
<td>pxp</td>
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<tr>
<td><strong>Cytotoxins</strong></td>
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<td></td>
</tr>
<tr>
<td><em>hla</em></td>
<td>Chrom</td>
<td>α-Hemolysin</td>
<td>Hemolysin, cytotoxin</td>
<td>pxp</td>
</tr>
<tr>
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<td>Chrom</td>
<td>β-Hemolysin</td>
<td>Hemolysin, cytotoxin</td>
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</tr>
<tr>
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<td>γ-Hemolysin</td>
<td>Hemolysin, cytotoxin</td>
<td>xp</td>
</tr>
<tr>
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<td>δ-Hemolysin</td>
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<td>pxp</td>
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<td>PVL phage</td>
<td>P-V leucocidin</td>
<td>Leucolysin</td>
<td>pxp</td>
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<td><em>splA-F</em></td>
<td>Chrom</td>
<td>Serine protease-like</td>
<td>Putative protease</td>
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<td>Chrom</td>
<td>V8 protease</td>
<td>Spreading factor</td>
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<td><em>aur</em></td>
<td>Chrom</td>
<td>Metalloprotease (aureolysin)</td>
<td>Processing enzyme?</td>
<td>pxp</td>
</tr>
<tr>
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<td>Chrom</td>
<td>Cysteine protease</td>
<td>Processing enzyme?</td>
<td></td>
</tr>
<tr>
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<td>Chrom</td>
<td>Glycerol ester hydrolase</td>
<td>Spreading, nutrition</td>
<td>pxp</td>
</tr>
<tr>
<td><em>lip</em></td>
<td>Chrom</td>
<td>Lipase (butyryl esterase)</td>
<td>Spreading, nutrition</td>
<td>pxp</td>
</tr>
<tr>
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<td>Chrom</td>
<td>FAME</td>
<td>Fatty acid esterification</td>
<td>pxp</td>
</tr>
<tr>
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<td>Chrom</td>
<td>PI-phospholipase C</td>
<td>Spreading factor</td>
<td>pxp</td>
</tr>
<tr>
<td><em>nuc</em></td>
<td>Chrom</td>
<td>Nuclease</td>
<td>Nutrition</td>
<td>pxp</td>
</tr>
<tr>
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<td>Chrom</td>
<td>Hyaluronidase</td>
<td>Spreading factor</td>
<td>xp</td>
</tr>
<tr>
<td><em>coa</em></td>
<td>Chrom</td>
<td>Coagulase</td>
<td>Clotting, clot digestion</td>
<td>exp</td>
</tr>
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<td><em>sak</em></td>
<td>Phage</td>
<td>Staphylokinase</td>
<td>Plasminogen activator</td>
<td>pxp</td>
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<td><strong>Surface proteins</strong></td>
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<tr>
<td><em>spa</em></td>
<td>Chrom</td>
<td>Protein A</td>
<td>Anti-immune, anti-PMN</td>
<td>exp</td>
</tr>
<tr>
<td><em>cna</em></td>
<td>Chrom</td>
<td>Collagen BP</td>
<td>Collagen binding</td>
<td>pxp</td>
</tr>
<tr>
<td><em>fnbA</em></td>
<td>Chrom</td>
<td>Fibronectin binding protein A</td>
<td>Fibronectin binding</td>
<td>exp</td>
</tr>
<tr>
<td><em>fnbB</em></td>
<td>Chrom</td>
<td>Fibronectin binding protein B</td>
<td>Fibronectin binding</td>
<td>exp</td>
</tr>
<tr>
<td><em>clfA</em></td>
<td>Chrom</td>
<td>Clumping factor A</td>
<td>Fibrinogen binding</td>
<td>exp</td>
</tr>
<tr>
<td><em>clfB</em></td>
<td>Chrom</td>
<td>Clumping factor B</td>
<td>Fibrinogen binding</td>
<td>exp</td>
</tr>
<tr>
<td><strong>Capsular polysaccharides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>cap5</em></td>
<td>Chrom</td>
<td>Polysacch. cap. type 5</td>
<td>Antiphagocytosis?</td>
<td>pxp</td>
</tr>
<tr>
<td><em>cap8</em></td>
<td>Chrom</td>
<td>Polysacch. cap. type 8</td>
<td>Antiphagocytosis?</td>
<td>pxp</td>
</tr>
</tbody>
</table>

xp, throughout exponential phase; exp, early exponential phase only; pxp, post-exponential phase.
Expression and regulation of virulence in *S. aureus*

The coordinated regulation of extracellular and cell wall virulence determinants during growth is due to contribution of global regulatory elements in *S. aureus* infections. They are controlled by a complex regulatory network that includes some two-component systems (TCS), an alternative sigma factor $\sigma^B$, and some transcription factors. Table 2 provides a list of the most important regulatory and transcription factors identified so far.

**Table 2.** Virulence gene regulation and transcription units in *S. aureus*. Adapted from Novick, 2003.

<table>
<thead>
<tr>
<th>Regulatory unit</th>
<th>Description</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgrACDB/RNAIII</td>
<td>TCS, autoinduced by peptide</td>
<td>Regulates many extracellular and cytoplasmic protein and virulence genes</td>
</tr>
<tr>
<td>SaePQRS</td>
<td>TCS, autoinduced</td>
<td>Regulates many extracellular protein genes</td>
</tr>
<tr>
<td>ArRS</td>
<td>TCS</td>
<td>Regulates autolysis and certain virulence genes</td>
</tr>
<tr>
<td>SvrA</td>
<td>Membrane protein</td>
<td>Required for the expression of agr</td>
</tr>
<tr>
<td>SrrAB</td>
<td>TCS</td>
<td>Regulates certain virulence genes at low $P_{O_2}$</td>
</tr>
<tr>
<td>$\sigma^B$</td>
<td>Alternative sigma factor</td>
<td>Active in late exponential phase; regulates many virulence genes</td>
</tr>
<tr>
<td>SarA</td>
<td>Transcription factor</td>
<td>Contributes to agr induction under certain conditions; pleiotropic repressor</td>
</tr>
<tr>
<td>SarS</td>
<td>Transcription factor</td>
<td>Activates transcription of spa and possibly other surface proteins genes</td>
</tr>
<tr>
<td>SarT</td>
<td>Transcription factor</td>
<td>Represses transcription of hla and possibly other exoprotein genes</td>
</tr>
<tr>
<td>SarR</td>
<td>Transcription factor</td>
<td>Minor transcription factor for sarA and possibly sarS</td>
</tr>
<tr>
<td>Rot</td>
<td>Transcription factor</td>
<td>Major transcription factor for hla and other exoprotein genes</td>
</tr>
</tbody>
</table>

A simplified model presented in 1997 by Projan and Novick assumed that cell-wall proteins are actively synthesized during the exponential phase, coinciding with the tissue-binding and colonization phases of infection. These cell-wall proteins include protein A, fibrinogen-binding, fibronectin-binding, and collagen-binding proteins (table 1). During transition from exponential to postexponential phase, the expression of cell-wall proteins is repressed, while the synthesis of extracellular toxins and enzymes predominates. The combined effects of proteolytic activities (e.g. V8 protease) and toxin on host cells (e.g. alpha-toxin) and extracellular matrix components synthesized during the postexponential phase
likely facilitate the local invasion and hematogenous dissemination for infections. Figure 8 shows the time course and population density-dependent regulation of virulence factors by global regulators (Novick, 2003).

Figure 8. Regulation of virulence determinants in *S. aureus* by global regulatory loci (Cheung, 2004).

In a more recent version of this model (Cheung, 2004), the synthesis of cell surface adhesins such as fibronectin-binding proteins during the exponential phase was found to coincide with the expression of SarA and Sae, suggesting potential regulation of both *fnb* by these two loci. During transition from exponential to postexponential phase where the synthesis of cell wall proteins is repressed and extracellular toxins such as α-toxin are produced, a maximal expression of SarA and the ensuing activation of *agr* is assumed. Other complicated features of this model involve: (i) control of SarA expression by SarR, a SarA protein homolog, and SigB; (ii) control of *agr* by SarA, a quorum sensing AIP, a TCS called
ArlRS, MgrA/Rat/NorR and SvrA; (iii) activation of *agr* leading to up-regulation of another TCS system called Sae and down-regulation of a SarA homolog called Rot; (iv) eventual repression of two gene products called SarT and SarS; (v) repression of $\alpha$-toxin by SarT and activation of protein A synthesis by SarS, thus explaining the effects of *agr* activation on these target genes.

The *agr* system

![Diagram of agr system](image)

**Figure 9.** A schematic diagram of *agr* system, showing the two divergent *agr* promoters (P2 and P3) and their transcripts. Adapted from Arvidson, 2001. The P2 operon contains four genes, *agrA, B, C* and *D*. Two of these, *agrC* and *agrA* represent a two-component signal transduction pathway and the other two combine to generate a peptide AIP that is the activation ligand for the signal receptor. The function of the signaling pathway is to activate P2 and P3. The P3 transcript, RNAIII, regulates (directly or indirectly) transcription of many different genes.
The *agr* (accessory gene regulator) system is a two-component system (TCS), and involves in primary regulation of expression of the virulon in *S. aureus*. The *agr* locus was originally identified as a chromosomal Tn551 insertion resulting in decreased production of secreted toxins and increased production of coagulase and cell wall-associated protein A (Peng, 1988). **Figure 9** shows the *agr* locus, consisting of an about 3-kb locus (Novick, 1993; Novick, 1999; Novick, 2000; Arvidson, 2001), containing two divergent transcription units, driven by promoters P2 and P3. The P2 operon consists of four genes, *agrB*, *D*, *C*, and *A*. *agrA* and *agrC* represent a classical two-component signal transduction system, of which *agrC* encodes the signal receptor and *agrA* the response regulator. The other two genes, *agrB* and *D*, combine to produce a small peptide, an autoinducing peptide, AIP. The AIP is a 7-9 amino-acid internal cleavage product of AgrD. The AIP binds to the N-terminal transmembrane domain of the *agr* signal receptor, *agrC*, activating the *agr* TCS, of which AgrA is the response regulator. Activated AgrA then upregulates promoters P2 and P3. The 512-nucleotide P3 transcript, RNAIII, is the intracellular effector of the target gene regulation (Novick, 1993), and RNAIII is also an mRNA encoding the secreted δ-toxin (*hld*), although δ-toxin is not involved in the regulation of virulence genes (Janzon, 1989; Janzon, 1990).

The *agr* locus has been found in nearly all *S. aureus* strains tested. Although the *agr* locus is conserved throughout staphylococci, variations are seen in the *agrB*, *C*, and *D* sequences, resulting in the production of AIP molecules and sensors (AgrCs) with different specificities (Ji, 1997). Within *S. aureus*, four specificity groups have been identified (Ji, 1997). Mutual cross-activation of the *agr* signaling pathway is observed within each group, while mutual cross-inhibition is seen between the groups.

The *agr* locus, besides being activated by AIP and SarA, may also be activated by a positive feedback loop via the *sarT* and *sarU* pathway. In this pathway, activation of *agr* represses *sarT*. Repression of SarT leads to activation of *sarU*, an activator of *agr*. This will lead to amplification of the original *agr* signal.

The *agr* system plays an essential role in up-regulating the expression of post-exponentially expressed extracellular toxins and enzymes such as α- and β-hemolysins, enzymes (lipases, proteases, and nucleases) and toxins (toxic shock syndrome toxin and enterotoxins), and down-regulating the synthesis of cell-surface adhesions such as protein A, fibronectin-binding proteins and coagulase during transition from exponential to postexponential phase (Saravia-Otten, 1997; Dunman, 2001; Cheung, 2004). In addition, the role of the *agr* system during the infection process was supported in some studies by the
greatly attenuated virulence of *agr* mutants in some animal models of infection, including arthritis, subcutaneous abscesses, endocarditis, mastitis, and osteomyelitis (Collins, 2000). The mechanisms by which RNAIII controls the transcription of its target genes are unknown. RNAIII seems to regulate transcription in an indirect way by either sequestrating a regulatory protein, or by activating or inhibiting the translation of mRNA encoding for transcription factor (Novick, 1993; Arvidson, 2001).

**The sarA locus**

The *sarA* locus was identified in a Tn917 insertion mutant of the clinical *S. aureus* strain DB showing reduced fibrinogen binding (Cheung, 1992). The product of the *sarA* locus is a small (14.7 kDa) basic protein with predominantly alpha helical structure. SarA is transcribed from three distant promoters (P1, P2 and P3) and terminates at a common 3' end (Bayer, 1996). The P1 and P2 promoters are recognised by the vegetative sigma factor, σ^A^, and are mainly expressed during early exponential phase of growth. SarA transcription from P2 promoter is inhibited by another homologue, SarR (Manna, 2001). P3 promoter is recognised by the alternative sigma factor, σ^B^, and is induced as the cells enter the postexponential phase of growth (Bayer, 1996; Manna, 1998).

**Figure 10.** Physical map of *sar* locus. Promoter positions are labelled P1, P2, and P3. The arrows depict the *sar* transcripts. The distances between the 5' ends of the transcripts and the start codon of *sarA* are expressed by negative position numbers. P3 is σ^B^-dependent, P1 and P2 are σ^A^-dependent.
SarA binds as a dimer to AT-rich sequences distributed widely in the AT-rich staphylococcal genome, including the 5' regions of several genes. Some investigators described multiple SarA binding sites in the intergenic region between *agr* promoters P2 and P3, suggesting the possibility of co-operativity (Rechtin, 1999), whereas others have identified only one site (Morfeldt, 1996; Chien, 1998b). It seems there is no consensus on the SarA binding site.

In addition to its effect on *agr*, *sarA* appears to regulate transcription of a number of virulence genes in an *agr*-independent way. Collagen binding protein (*cna*) is supressed by *sarA* (Lindsay, 1999), transcription of protein A (*spa*) was supressed by *sarA* (Cheung, 1997), while transcription of α-toxin gene (*hla*) and *fnbA* was activated by *sarA* (Chan, 1998a; Chien, 1999; Wolz, 2000).

The importance of *sarA* in virulence has been demonstrated in some animal models of infection (Cheung, 1994; Booth, 1997). Using the GFP reporter gene, the *sarA* promoters were differently expressed *in vitro* and in a rabbit endocarditis model (Cheung, 1994). The *sarP2* promoter, which was almost silent *in vitro*, became highly activated *in vivo* (Cheung, 1994). However, there is no definitive consensus on upregulating effects of SarA. The reduced fibronectin-binding activity observed with *sarA* mutants may not be at the level of transcription and may be a consequence of increased proteolytic activity released extracellularly by these *sarA* mutants (Blevins, 2002).

A number of proteins that are homologous to SarA have been found in the *S. aureus* genome (www.ncbi.nlm.nih.gov) and confirmed by N315 and MW2 sequencing projects (Kuroda, 2001). This group of proteins, called ‘the SarA protein family’ (Cheung, 2004), includes five previously characterized SarA homologs (SarA, -R, -S, -T and -U), as well as four other as yet uncharacterized homologues SarV, SarX, SarY, SarZ. Rot, a repressor of α-toxin synthesis, also shares homology with the smaller SarA homologues (McNamara, 2000). A recent microarray analysis has revealed that Rot has broad regulatory effects on genes belonging to the *agr* regulon and generally acts counter to *agr*, downregulating genes encoding secreted proteins and upregulating surface protein genes (Said-Salim, 2003).

σ^B – alternative sigma factor B (Sigma B)

A typical bacterial RNA polymerase has six subunits whose molecular weight exceeds 4,000,000, making it one of the largest bacterial enzymes. RNA polymerase holoenzyme has two identical α subunits, two very large subunits called β and β', one ω subunit, and the σ
factor. $\alpha_2\beta'\omega$ forms the catalytically competent RNA polymerase core enzyme (E) which is a permanent part of the RNA polymerase. RNA polymerase core enzyme (E) is capable of elongation and termination of transcription, but is unable to initiate transcription at specific promoter sequences. Binding of the $\sigma$ subunit to E forms the holoenzyme (E-$\sigma$) that directs the multimeric complex to specific promoter elements and allows efficient initiation of transcription (Borukhov, 2002; Burgess, 2001). Therefore, $\sigma$ factors provide an elegant mechanism in eubacteria to allow simultaneous transcription of a variety of genetically unlinked genes, but sharing the same promoter specificities. Because $\sigma$ factors are powerful regulatory effectors, the molecular mechanisms of controlling their synthesis or activity are of fundamental importance.

In addition to the housekeeping sigma subunits, $\sigma^{70}$ or $\sigma^A$, most bacteria produce one or more additional $\sigma$ subunits, termed alternative $\sigma$ factors, which direct the respective E-$\sigma$ complex to distinct classes of promoters that contain alternative $\sigma$ factor-specific sequences. At least six alternative $\sigma$ factors are produced by the enteric bacterium Escherichia coli. Genomic sequence analysis suggests that several alternative $\sigma$ factors also exist in different pathogenic species such as Treponema pallidum (4 alternative $\sigma$ factors), Vibrio cholerae (7 alternative $\sigma$ factors), Mycobacterium tuberculosis (12 alternative $\sigma$ factors), and Pseudomonas aeruginosa (23 alternative $\sigma$ factors) (Bischoff, 2004).

$\sigma^B$ has been identified and shown to be involved in virulence, pathogenicity, and the ability to survive under extreme conditions when bacterial cells are exposed to stress conditions such as heat, ethanol, acid, salt, oxidative agents, and/or carbon depletion (van Schaik, 2004; Ferreira, 2001; Benson, 1993; Volker, 1999) in gram positive bacteria including Bacillus, Listeria, and Staphylococcus (Brody, 1998; Ferreira, 2001; Hecker, 1998; Kies, 2001; Wu, 1996; Kullik, 1997). Two alternative $\sigma$ factors, $\sigma^B$ and $\sigma^H$, have been identified in S. aureus (Kullik, 1997; Wu, 1996). $\sigma^H$ has only recently been characterized as a bona fide S. aureus sigma factor, which is involved in the transcriptional regulation of DNA competence factors (Morikawa, 2003).

The genetic organization of the S. aureus sigB operon (Kullik, 1997; Wu, 1996) closely resembles that of the well-characterized operon of the soil-borne gram-positive bacterium Bacillus subtilis. In B. subtilis, the expression of several heat-shock or general stress genes is under the control of the alternative sigma factor, $\sigma^B$ (Boylan, 1992; Boylan, 1993a; Boylan, 1993b). $\sigma^B$ activity is regulated post-translationally by a complex network of protein-protein
interactions governed by a variety of environmental or metabolic stresses such as heat shock, osmotic shock, ethanol treatment, or entry into stationary growth phase (Boylan, 1992; Boylan, 1993a; Boylan, 1993b). The proteins involved in this regulation are encoded by the *rsb* genes (regulators of Sigma B), which together with the *sigB* structural gene are located in the eight-gene $\sigma^B$ operon (Boylan, 1992; Boylan, 1993a; Boylan, 1993b). The current model for $\sigma^B$ activation in *B. subtilis* involves the regulatory proteins RsbU, RsbV and RsbW, as well as RsbR, RsbS, RsbT, and RsbX (Wise, 1995; Hecker, 1998).

![Diagram showing similarity in the organization of the operons of *S. aureus* and *B. subtilis*.](image)

**Figure 11.** Similarity in organization of the operons of *S. aureus* and *B. subtilis*. Adapted from Wu, 1996. ORFs are indicated by arrows. Similarity between the predicted products of the corresponding genes are shown with percentages.

The *S. aureus* *sigB* operon, like *B. subtilis*, comprises the genes *rsbU, rsbV, rsbW* and *sigB* (Wu, 1996; Kullik, 1997) which shows close similarities to the *B. subtilis sigB* operon, both in overall organization and in primary sequences of the gene products (figure 11). However, the *sigB* operon in *S. aureus* represents only 50% of the *sigB* operon size in *B. subtilis*, thus suggesting that this stress response pathway plays a less important physiological role in *S. aureus* compared to *B. subtilis*. 
RsbW is an anti-σ^B protein, it can form mutually exclusive complexes with σ^B or its antagonist, RsbV. RsbV is normally inactive due to phosphorylation by RsbW and thus is unable to complex with RsbW, leaving RsbW free to interact with σ^B. When σ^B binds to RsbW, it is unable to aggregate with the RNA polymerase core enzyme (E) to form an active holoenzyme (E-σ^B). Upon stress, an σ^B activator RsbU is active and phosphorylates RsbV-P. Un-phosphorylated RsbV complexes highly and specifically with RsbW, leaving σ^B free to form the holoenzyme (E-σ^B).

As in *B. subtilis*, the σ^B operon of *S. aureus* is regulated post-translationally by *rsbU*, *rsbV*, and *rsbW*. The anti-sigma factor, RsbW, binding to σ^B and inactivates σ^B during exponential phase (Miyazaki, 1999). In response to stress or starvation, RsbW is captured by the active form of the antagonist protein RsbV, leading to the release of σ^B, leaving the σ^B free to form an active σ^B holoenzyme. The activity of RsbV is controlled by its...
phosphorylation state. Stress and starvation enhance the level of non-phosphorylated RsbV, which is the form that can bind to RsbW (figure 12).

Discovering the role of $\sigma^B$ in *S. aureus* has been delayed because of the presence of an *rsbU* mutation in 8325-4 (RN6390) which has been used most frequently for molecular and physiological analyses in laboratories. 8325-4 (RN6390) has a 11-bp deletion in *rsbU* which encodes for a positive regulator of $\sigma^B$ function, and produces a strong defect in $\sigma^B$ activity (Giachino, 2001). Derivatives of 8325 strains show important differences from the strains which have a complete *rsbU*. The differences of derivatives of 8325 strains include a reduction in the lag phase of growth, a decrease in pigmentation, and decreasing starvation survival (Kullik, 1998), etc. However, *rsbU* defective strains are not totally deficient in $\sigma^B$ function (Palma, 2001), as pigment synthesis can be induced by subinhibitory clindamycin, and it is suggested that this may involve $\sigma^B$ activation via RsbP (Novick, 2003). A small fraction of *S. aureus* clinical isolates are non-pigmented and overproduce various exoproteins (Novick, 2003).

In *S. aureus*, $\sigma^B$ feeds into the global regulatory network governing the expression of virulence genes, acting mostly through other regulatory genes and transcription factors, but also acting directly on those few that have $\sigma^B$-dependent promoters. $\sigma^B$ recognizes a specific motif [GTTT(N_{14-17})GGGTAT] that has been identified for 23 different *S. aureus* genes (Gertz, 2000), including one of the three *sarA* and one of the three *sarS* promoters, plus genes encoding transport functions and others involved in generating NADH2. $\sigma^B$ also regulates the activity of other genes that do not contain a $\sigma^B$ promoter but are indirectly regulated by $\sigma^B$-dependent transcription factors.

The *S. aureus* $\sigma^B$ factor has been shown to be involved in following functions:

1. $\sigma^B$ involves in protecting the cell from various environmental stresses (Kullik, 1997; Gertz, 2000), activated by environmental stress and energy depletion (reduced ATP/ADP ratio), as well as environmental stimuli such as ethanol (Chan, 1998b) and salicylic acid (Kupferwasser, 2003).

2. $\sigma^B$ involves in influencing the expression of several global virulence regulators, including *sar* (Bischoff, 2001b; Deora, 1997; Gertz, 2000; Manna, 1998; Cheung, 1999), *sarS* (also known as *sarHI*) (Bayer, 1996; Tegmark, 2000; Nair, 2003; Bischoff, 2004), and RNAIII (Bischoff, 2001b; Horsburgh, 2002). Some studies reported that $\sigma^B$ can increase *sarA* expression while simultaneously reducing the RNAIII level in a growth phase-dependent manner (Bischoff, 2001b).
(3) Alkaline shock protein (asp23) is exclusively controlled by a functional SigB (Giachino, 2001; Gertz, 1999). $\sigma^B$ upregulates some exoprotein genes at some growth stages, in particular coa and the P3 promoter of sarA which have $\sigma^B$-dependent promoters (Miyazaki, 1999; Nicholas, 1999; Deora, 1997; Nair, 2003); clfA is transcriptionally regulated by $\sigma^B$ (Nicholas, 1999; Nair, 2003); $\sigma^B$ downregulates spa, hla (Ziebandt, 2001; Giachino, 2001; Horsburgh, 2002; Cheung, 1999; Nicholas, 1999); $\sigma^B$ also influences the expression of some other genes including lipases (Kullik, 1998; Ziebandt, 2001), proteases (Horsburgh, 2002; Karlsson, 2001; Karlsson, 2002; Ziebandt, 2001), and thermonucleases (Kullik, 1998; Ziebandt, 2001).

(4) The production of biofilms by S. aureus may be controlled by $\sigma^B$ (Rachid, 2000).

(5) The ability of S. aureus to bind to various host-cell matrix proteins such as fibrinogen and fibronectin is influenced by $\sigma^B$ (Kullik, 1998; Cheung, 1999; Ziebandt, 2001; Kies, 2001; Nair, 2003; Renzoni, 2004).

(6) Besides regulating virulence determinants, $\sigma^B$ has been suggested to contribute to expression of resistance to methicillin, vancomycin, and teicoplanin (Bischoff, 2001a; Singh, 2003; Renzoni, 2004).
E. MECHANISMS OF ACTION OF ANTIMICROBIALS

Currently available antimicrobial agents are targeting key components of cell wall synthesis, protein synthesis, RNA synthesis, DNA synthesis, or intermediary metabolism (Table 3).

Bacterial cell wall synthesis has been the target which are most extensively exploited for antimicrobial development. The components of the cell wall synthesis machinery are appealing antimicrobial targets because of the absence of counterparts in human, thereby providing intrinsic target selectivity. β-Lactams as transpeptidase inhibitors thus block the conversion of immature to mature peptidoglycan (Hooper, 2001a). Vancomycin is a glycopeptide that is known to bind tightly to the terminal D-ALA-D-ALA of the peptide side chain of the immature peptidoglycan. Thus, vancomycin produces a block at the same step as β-lactams by producing steric hindrance to transpeptidase action, thereby preventing conversion of immature to mature peptidoglycan.

Table 3. Bacterial targets of antimicrobial agents (Hooper, 2001a)

<table>
<thead>
<tr>
<th>Bacterial targets</th>
<th>Antimicrobial agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall synthesis</td>
<td>β-Lactam</td>
</tr>
<tr>
<td></td>
<td>Glycopeptides</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Aminoglycosides</td>
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<tr>
<td></td>
<td>Macrolides</td>
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<tr>
<td></td>
<td>Lincosamides</td>
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<td></td>
<td>Ketolides</td>
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<tr>
<td></td>
<td>Streptogramins</td>
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<td></td>
<td>Tetracyclines</td>
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<tr>
<td></td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td></td>
<td>Oxazolidinones</td>
</tr>
<tr>
<td>RNA synthesis</td>
<td>Rifamycins</td>
</tr>
<tr>
<td>DNA synthesis</td>
<td>Coumarins</td>
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<tr>
<td></td>
<td>Naphthyridines</td>
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<tr>
<td></td>
<td>Quinolones</td>
</tr>
<tr>
<td></td>
<td>2-Pyridones</td>
</tr>
<tr>
<td>Intermediary metabolism</td>
<td>Sulfonamides</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim</td>
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</tbody>
</table>

Mechanisms of fluoroquinolone action

The fluoroquinolones are antibacterial agents widely used in clinical medicine. In addition to nalidixic acid, the original non-fluorinated member of the quinolone drug class,
several derivatives have been developed, such as norfloxacin, menoxacin, lomefloxacin, pefloxacin, ciprofloxacin, fleroxacin, ofloxacin, levofloxacin, sparfloxacin, grepafloxacin, tosufloxacin, temafloxacin, and trovafloxacin. Some of these agents have been removed from the market because of toxicity. Initial members of this class were used largely for treatment of infections caused by facultative gram-negative bacteria, gram-positive bacteria and also against anaerobic bacteria. The multiple clinical indications for ciprofloxacin and ofloxacin, in particular, have led to extensive use of these compounds for over a decade (Hooper, 1999; Hooper, 2000).

Figure 13. Formation of fluoroquinolone complexes with topoisomerase IV and DNA gyrase on E. coli DNA as barriers to progression of the multicomponent DNA replication complex (Hooper, 2002). ParC and ParE are E. coli homologs of GrlA and GrlB S. aureus topoisomerase IV, and GyrA and GyrB are subunits of DNA gyrase. DnaB, primase, PriA, and polymerase are components of the DNA replication complex. Q represents quinolone molecules.
Dual targets

Fluoroquinolones have two major enzyme targets in bacterial cells, namely DNA gyrase and topoisomerase IV. Both targets are essential for bacterial DNA replication (Hooper, 2001a; Hooper, 2001b; Hooper, 2002).

DNA gyrase is composed of two GyrA subunits and two GyrB subunits in both E. coli and S. aureus. DNA gyrase is the only bacterial enzyme that introduces negative super-helical twists into DNA. Negatively super-twisted DNA is important for initiation of DNA replication. DNA gyrase also facilitates DNA replication by removing positive super-helical twists that accumulate ahead of the replication fork as a result of the transcription of certain genes. Topoisomerase IV are composed of two subunits of ParC and two subunits of ParD in E. coli, which are homologous to GrlA, and GrlB in S. aureus. Topoisomerase IV acts in the terminal stage of DNA replication, allowing for the decatenation (superaction) of inter-linked daughter chromosomes so that segregation into daughter cells can occur.

As shown by figure 13, fluoroquinolones interact with complexes of gyrase or topoisomerase IV with DNA, leading to trapping of the complex and blocking the progression of the DNA polymerase contributing to DNA replication. Subsequent events, as yet poorly defined, which probably involve generation of an irreparable DNA break triggered by this block, ultimately result in bacterial-cell death (Hooper, 2002).

Mechanisms of fluoroquinolone resistance

1. Alterations in drug targets

The most important mechanism of fluoroquinolone resistance is mediated through altered target enzymes. Importantly, resistance arises stepwise. The resistance mutations cluster in the "quinolone resistance-determining region" (QRDR) of GyrA. A common model shows that amino acids in the QRDR of GyrA alter the structure of the site of quinolone binding near the interface of the enzyme and DNA, and the resistance is then caused by reduced drug affinity for the modified enzyme-DNA complex (Schmitz, 2002).

The results of studies done with S. aureus indicated that, in contrast to E. coli, topoisomerase IV was the primary quinolone target in this species. Mutations in GrlA represent the first step towards acquisition of high-level resistance, but is not generally sufficient to cause clinical resistance to ciprofloxacin or other more potent fluoroquinolones.

High-level resistance requires a second mutation in GyrA besides GrlA thus increasing quinolone MICs by >16-fold compared to MICs for susceptible isolates of S. aureus.
Interestingly, most MRSA clinical isolates express high levels of fluoroquinolone resistance mediated by combined mutations in GrlA and GyrA. Table 4 shows the most common mutations in GyrA and GrlA of *S. aureus*.

**Table 4.** Common mutations in GyrA and GrlA and the corresponding MICs of ciprofloxacin for clinical isolates of *S. aureus*. Adapted from Schmitz, 2002.

<table>
<thead>
<tr>
<th>Amino acid change</th>
<th>Ciprofloxacin MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GyrA</td>
<td>ParC/GrlA</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>Ser80Phe/Tyr</td>
</tr>
<tr>
<td>Ser84Leu</td>
<td>nt</td>
</tr>
<tr>
<td>Glu88Lys</td>
<td>Ser80Phe</td>
</tr>
<tr>
<td>Ser84Leu</td>
<td>Ser80Tyr</td>
</tr>
<tr>
<td>Ser84Leu</td>
<td>Ser80Phe/Tyr</td>
</tr>
<tr>
<td>Ser84Leu</td>
<td>Glu84Lys</td>
</tr>
<tr>
<td>Ser84Leu</td>
<td>Ser80Phe/Tyr + Glu84Lys</td>
</tr>
<tr>
<td>Glu88Lys</td>
<td>Ser80Phe + Glu84Val</td>
</tr>
</tbody>
</table>

-, no mutation; nt, not tested.

**2. Increased efflux of fluoroquinolones**

All bacteria have proteins inserted into the cytoplasmic membrane that have important physiological functions as transporters of various substances into and out of the cell. Efflux pumps that can remove substances from the cytoplasm and from the cytoplasmic membrane have been found in all bacteria studied, and the general role of many such pumps is thought to remove toxic substances from the cell and its membrane (Hooper, 2002). Among these efflux pumps are those that can remove antibiotics from the cell, called multidrug resistance (MDR) pumps which can remove multiple antibiotics. MDR efflux pumps are normally present in most bacteria. Inactivation of some pumps can lead to hypersusceptibility to fluoroquinolones.

In *S. aureus*, low-level resistance to ciprofloxacin can result from increased expression of a pump called NorA (Fournier, 2000). The effect of increased NorA expression on gatifloxacin and ciprofloxacin activity was investigated. When overexpression of the NorA efflux pump was effected through a single mutation of the promoter region of the *norA* gene, the MIC of ciprofloxacin increased 2- to 4-fold (from 0.25 to 0.5–1.0 µg/ml). The same mutation was shown to cause an 8-fold increase in the MIC of norfloxacin. Overexpressing NorA, the MICs of sparfloxacin and gatifloxacin were less affected than those of ciprofloxacin and norfloxacin (Ng, 1994; Ng, 1996; Hooper, 2000; Fournier, 2000).
**SOS response**

The discovery of SOS response system came from studies on the effect of UV irradiation on *E. coli*. In *E. coli*, these potentially fatal lesions induce transcription of about 20 DNA repair genes. This is known as the SOS response (Walker, 1985). The central regulatory element of the SOS pathway is the LexA repressor, which binds to the operators of SOS genes with a high affinity, thereby inhibiting transcription. Other requirements for induction of the SOS response depend on the type of DNA damage, but genetic analyses have shown that both the RecA and RecBCD enzymes — the two initiators of dsDNA break repair and homologous recombination — are essential for SOS induction in response to dsDNA breaks (Anderson, 1998; Janion, 2001).

In contrast to *E. coli*, the major components of the SOS response pathway in *S. aureus* are mostly unknown.

**Induction of SOS response by quinolones**

The quinolones are potential inducers of the SOS regulon (Drlica, 1997), involving three major proteins in *E. coli*, RecA, RecBCD, and LexA. The early event is the generation of an inducing signal, which consist of short oligonucleotides and/or single-stranded DNA breaks. The damaged segments of nucleic acids activate RecA, which subsequently interacts with LexA protein and lead to auto-cleavage of LexA protein. LexA cleavage removes the repression of genes of the SOS regulon.
Figure 14. Model for SOS response in *E. coli* (Anderson, 1998).

(A) DNA damage produces a dsDNA break.

(B) RecBCD enzyme processes the broken DNA, degrading 3'→5' until a χ site is recognized, at which time RecBCD enzyme pauses; the 3'→5' exonuclease activity is attenuated; and RecA protein is loaded onto the χ-containing DNA strand within an ssDNA loop.

(C) The nuclease polarity is then switched, with continued degradation occurring 5'→3', leading to the production of a 3' ssDNA overhang that is coated with RecA protein.

(D) This activated RecA nucleoprotein filament that is assembled on the χ-containing ssDNA stimulates the self-cleavage of LexA repressor.

(E) SOS genes are repressed by the binding of the LexA repressor to the operator (OP). The cleaved LexA protein no longer binds the promoter, thereby derepressing transcription.
Section II. EXPERIMENTAL STUDY
A. ORIGINS AND AIMS OF THIS STUDY
Previous studies performed in the research laboratory of the Division of Infectious Diseases revealed that fluoroquinolone-resistant (Q-R) *grlA gyrA* strains of *S. aureus* grown in the presence of sub-MIC levels of ciprofloxacin (CFX) expressed increased levels of FnBPs and showed significantly higher attachment to *in vitro* fibronectin-coated or explanted polymer surfaces (Bisognano, 1997; Bisognano, 2000). This increase was selectively expressed in Q-R *grlA gyrA* double mutants, as opposed to *grlA* or *gyrA* single mutants or susceptible parental strains. The CFX-triggered response by *S. aureus* NCTC 8325 strain RA1, carrying combined *grlA gyrA* mutations, involved increased transcription of *fnbB* gene in contrast to *fnbA* gene. The response was due to increased promoter activity of the *fnbB* gene. Inhibition of this CFX-promoted response by rifampin pretreatment confirmed that it occurred at a transcriptional level (Bisognano, 2000).

A recent study (Bisognano, 2004) further analyzed the molecular mechanisms of CFX-promoted adhesion. Inactivation of *recA*, the central activator of SOS and DNA repair pathway, abolished the CFX-triggered upregulation of *fnbB* gene. The study also revealed that recombinant LexA, another key component of the SOS response, exhibited specific binding to the *fnbB* promoter region. These data provided evidence for a role of the still incompletely characterized RecA-LexA-dependent pathway in the CFX-triggered FnBPs upregulation in Q-R *S. aureus* (Bisognano, 2004).

Sigma B (σB) is a transcription factor contributing to virulence and bacterial survival under stress conditions (see σB in section II). The studies from other laboratories (Bischoff, 2001; Nair, 2003) showed that SigB could also upregulate expression of FnBPs. The aims of this experimental study were to (i) evaluate whether SigB contributed in an additive manner to the ciprofloxacin-triggered upregulation of FnBPs by using the isogenic derivatives of Q-R *grlA gyrA* strain RA1, exhibiting widely different functional levels of SigB activity; (ii) evaluate the potential contribution of global regulons *agr* and *sarA* to either the SigB- or ciprofloxacin-modulated effects on *fnbA* or *fnbB* transcription.

These results provide evidence that optimal expression of a stress response factor and triggering of a drug-induced DNA repair system may independently, but in an additive manner, significantly promote FnBPs-mediated *S. aureus* adhesion.
B. MATERIALS AND METHODS
1. Bacterial strains

Strain RA1 is a quinolone-resistant (MIC of ciprofloxacin: 32 µg/ml) mutant of *S. aureus* NCTC (National Culture Type Collection) 8325 strain (Bisognano, 2004). Like all members of the NCTC 8325 family, strain RA1 has a natural 11 bp deletion in *rsbU* gene thus yielding a defective truncated RsbU protein. Strain RA1 has one point mutation in *grlA* (Ser 80 Phe) and another point mutation in *gyrA* (Ser 84 Leu). The first mutation in topoisomerase IV (*grlA*) brings a low level of quinolone resistance (MIC of ciprofloxacin: 2 µg/ml). When the first mutation combines with the second mutation in gyrase (*gyrA*), it generates a high level of quinolone resistance (MIC of ciprofloxacin: 32 µg/ml) (Bisognano, 2004).

Strain TE1 is a Tn$^{551}$ insertion *sigB* mutant of RA1 (Estoppey, 2003). TE1 was constructed by transducing RA1 with a phage lysate of strain ALC1001 (kindly provided by Cheung, A.L.) (Cheung, 1999; Wu, 1996) carrying the *sigB* mutation.

The correct insertion of transposon Tn$^{551}$ in the *sigB* gene of TE1 was confirmed by PCR, using the primers P1 (located in the end of *rsbW* gene) and P2 (located in the beginning of Tn$^{551}$), which yielded a 832-bp product as expected (TE1, figure 1). No equivalent PCR product was found in strain RA1 (figure 1).

Strain TE2 (Estoppey, 2003) is a derivative of RA1 whose SigB functional activity was restored by transducing RA1 with a phage lysate prepared from strain *rsbU* $^+$ *V* $^+$ *W* $^+$ *sigB* $^+$ *Tc* $^+$ GP268 (kindly provided by M. Bischoff) (Giachino, 2001).

The PCR used to amplify the region of *rsbU* gene that encompassed the 11-bp deletion in *rsbU sigB* $^+$ parent RA1, which was restored in the *rsbU* $^+$ *sigB* $^+$ strain TE2, yielded a 88 bp-
product in TE2 compared to a 77 bp-product in RA1. These data confirmed that TE2 had a restored \( rsbU \) gene (figure 2).

Figure 2. (left) PCR products in \( rsbU^+ \) \( sigB^+ \) strain TE2 (88 bp) and its \( rsbU^+ \) \( sigB^+ \) parent RA1 (77 bp). (right) The positions of primers used to perform PCR.

2. Determination of ciprofloxacin susceptibility

The minimum inhibitory concentrations (MICs) of ciprofloxacin (CPX) for fluoroquinolone-resistant strains RA1, TE1, and TE2 were determined by a macrodilution method using cation-adjusted Mueller-Hinton Broth (MHB; Difco) and a standard inoculum of \( 10^6 \) CFU/ml, according to the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 2000).

3. Detection of pigmentation

The pigmentation of the \( sigB \) null strain TE1, the \( rsbU^+ \) \( sigB^+ \) parent RA1, and the \( rsbU^+ \) \( sigB^+ \) strain TE2 was observed after streaking on Mueller-Hinton agar (MHA) for 48 h at 37°C.

4. Detection of hemolysin

2 µl late-logarithmic-phase (5 h) cultures in MHB of the \( sigB \) null strain TE1, the \( rsbU^+ \) \( sigB^+ \) parent RA1, and the \( rsbU^+ \) \( sigB^+ \) strain TE2 were spotted on sheep blood agar (SBA) for 24 h at 37°C to detect \( \alpha \)-toxin, and then 24 h at 4°C to detect \( \beta \)-toxin. The clear hemolytic zones around the bacterial growth after the first 24 h of incubation at 37°C were produced by
α-toxin. The halo effects around the clear zones after the second 24 h of incubation at 4°C detected hot-cold hemolysis produced by β-toxin. Strain ATCC (American Type Culture Collection) 25923 was used as a positive control strain for β-toxin.

5. Detection of protease activity on casein agar plates

2 µl of exponential phase cultures (5 h) of the SigB-modulated strains were spotted on 2% casein agar plates for 24 h at 37°C to detect extracellular protease production (Karlsson, 2002).

6. Bacterial adhesion assay to fibronectin

The attachment properties of *S. aureus* strains RA1, TE1, and TE2 were measured using a previously described adhesion assay with polymethylmethacrylate (PMMA) coverslips coated in vitro with three different concentrations (0.5, 1, and 2 µg/ml) of purified human fibronectin (Fn) (Greene, 1995; Greene, 1996; Bisognano, 1997; Bisognano, 2000).

Overnight cultures of *S. aureus* TE1, RA1, and TE2 were centrifuged and the pellets were suspended in 1 ml 0.9% NaCl. 20 µl each of suspended strains were mixed with 25 µl [methyl-3H]thymidine in 1 ml MHB and the cultures incubated to late-logarithmic-phase (5 h) without shaking at 37°C. The bacterial cells were metabolically radiolabeled with [methyl-3H]thymidine during the growth. When the influence of subinhibitory concentrations of ciprofloxacin on bacterial adhesion was tested, ciprofloxacin was added to 5-h cultures of quinolone-resistant strains of *S. aureus* at a concentration of 1/8 MIC of ciprofloxacin (4 µg/ml). Thereafter, the radiolabeled bacterial cells were centrifuged for 10 min at 3000 rpm and each strain was suspended in 1 ml 0.9% NaCl.

Purified human fibronectin (Fn, Chemicon, Temecula, Calif.) was solubilized in phosphate-buffered saline (PBS) at 1 mg/ml and stored at −70°C. The concentration was measured spectrophotometrically using an extinction coefficient of E_{280} = 1.28.

Polymethylmethacrylate (PMMA) coverslips (1 × 1 cm) were cleaned with 100% alcohol for 5 min and sterilized by heating for 20 min at 120°C. To optimize adsorption of fibronectin from concentrations ranging from 0.5 to 2 µg/ml, the PMMA coverslips were precoated with gelatin (1 mg/ml) for 1 h at 37°C as previously described (Greene, 1995; Greene, 1996). After being rinsed in PBS, the coverslips were incubated in duplicate for 60 min at 37°C with three different concentrations (0.5, 1, and 2 µg/ml) of fibronectin in PBS.
and then rinsed in PBS as previously described (Greene, 1995; Greene, 1996). The previous study has showed that the PMMA surfaces were coated in a dose-dependent manner with fibronectin ranging from 88 to 296 ng per coverslip when radiolabeled fibronectin was used (Vaudaux, 1993; Bisognano, 1997).

10^7 CFU of washed cultures of late-logarithmic phase thymidine-labelled cells were incubated with fibronectin-coated coverslips for 60 min at 37°C in PBS with 1 mM Ca^{2+} and 0.5 mM Mg^{2+} supplemented with human albumin (5 mg/ml) which prevented nonspecific adhesion of *S. aureus* as previously described (Greene, 1995; Greene, 1996; Vaudaux, 1984). Gelatin-coated PMMA coverslips were used as controls of adhesion to fibronectin-coated surface (Greene, 1995; Greene, 1996). At the end of the attachment period, the fluids containing unbound bacteria were removed, the coverslips were rinsed, and radioactivity on the coverslips was counted by a β-scintillation counter as previously described (Vaudaux, 1984). Bacterial adhesion data of the different strains whose cell-associated radioactivity and viable counts differed slightly (<20%) were normalized as described (Vaudaux, 2002).

Each experiment was performed three times. Relative changes in bacterial adhesion were expressed as the percent increase or decrease in attachment of strains TE1 and TE2 compared to RA1 grown in ciprofloxacin-free medium, or between exposure and no exposure to ciprofloxacin for each strain. The results were expressed as the means ± standard error of the means (SEM). The differences by strains TE1 and TE2 compared to RA1 and the differences between exposure and no exposure to ciprofloxacin in each strain were considered significant from each other when all increases or decreases accumulated for the three coating concentrations of fibronectin yielded *P* values of <0.05 by paired *t* tests (Rosner, 1990).

7. Quantification of FnBPs by flow cytometry

The surface display of fibronectin-binding proteins by the SigB-modulated and quinolone-resistant (Q-R) strains of *S. aureus* was monitored by flow cytometry of formaldehyde-fixed late logarithmic-phase cells (5 h) as previously described (Vaudaux, 2002; Francois, 2000; Hartford, 1997).

7.1. Labeling fibronectin with FITC

Purified human fibronectin (Fn) was solubilized in 0.2 M carbonate buffer (pH 9.2) at a concentration of 1 mg/ml and stored at −70°C. Fluorescein isothiocyanate (FITC) 1 mg was dissolved in 100 µl dimethyl sulfoxide (DMSO, 10 mg/ml), and protected from light.
Fibronectin was labelled with FITC by mixing 1 ml of the protein (1 mg/ml) with 10 µl of FITC for 2 h with rotating at room temperature. The reaction was stopped by adding 10 µl ethanolamine for 10 min.

7.2. Purification of Fn-FITC

Fn-FITC was purified by PD-10 column (Sephadex G-25, Pharmacia, Biotech) that was equilibrated with 15 ml PBS containing 1% human serum albumin (HSA) before use. Fn-FITC (2 ml) was loaded onto the column, and the flowthrough collected in 1.5 ml eppendorf tubes. Fn-FITC was stored at – 20°C.

7.3. Incubation of bacterial cells with FITC-Fn

Approximately 10⁸ CFU of each strain grown for 5 h at 37°C in 1 ml MHB were harvested by centrifugation at 4,000 rpm for 5 min. The bacterial cells were washed once with PBS containing 2.5% bovine serum albumin (BSA), and fixed with 0.5% (v/v) formaldehyde in PBS (pH 7.2) for 10 min at room temperature. After one wash with PBS-BSA, each bacterial cell was incubated with 200 µl of FITC-Fn in PBS-BSA for 60 min with rotating at room temperature. At the end of incubation, the bacterial cells were washed once with PBS-BSA, suspended in 500 µl of BPS-BSA, and immediately assayed for fluorescence.

7.4. Detection of fluorescence

Flow cytometric analysis was performed on a Becton Dickinson FACSscan cytometer with 488 nm excitation light and the emission was measured through a 530/20 nm bandpass filter. In general, 10,000 cells were analysed with software WinMDI 2.8, and light scatter and the fluorescent signals were collected with logarithmic amplification. The threshold settings for non-specific fluorescence were obtained by using bacterial cells incubated without FITC-Fn. The specificity of flow cytometry data was assessed by parallel analysis of the negative control strain DU5883, a mutant of 8325-4 simultaneously defective in expression of both FnBPA and FnBPB, and the positive control strain DU5883(pFNBB4) overexpressing FnBPB, respectively (Greene, 1995; Francois, 2000; Vaudaux, 2002).

Relative changes in flow cytometry data were expressed as the percent increase or decrease in FITC-fibronectin binding by strains TE1 and TE2 compared to RA1 grown in ciprofloxacin-free medium, or between exposure and no exposure to ciprofloxacin for each strain. Each experiment was performed three times, and the results were expressed as mean
percent changes ± standard errors of the means (SEM). The statistical significance of pairwise differences in FITC-fibronectin binding of isogenic strains differing in SigB functional levels or in ciprofloxacin exposure versus no exposure was evaluated by paired t tests, using P values of <0.05 with a two-tailed significance level (Rosner, 1990).

8. Total RNA extraction

Overnight cultures of the sigB null strain TE1, the rsbU sigB+ parent RA1, and the rsbU+ sigB+ strain TE2 were diluted 1/50 in 1 ml MHB and incubated to late exponential phase (5 h) at 37°C. When the influence of ciprofloxacin was tested, ciprofloxacin was added to the culture at 1/8 MIC of ciprofloxacin for each strain (4 µg/ml). At the end of incubation, the cultures were centrifuged for 5 min at 4,000 rpm. After removing supernatants carefully, the pellets were suspended in a mixture of 100% acetone and 100% ethanol (1:1) for 1 min on ice, and centrifuged for 5 min at 4,000 rpm at 4°C as previously described (Herbert, 2001; Renzoni, 2004). The treated cells were washed in N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-sucrose buffer (containing 20% sucrose), and lysed in 300 µl TES-sucrose buffer containing 200 µg/ml lysostaphin (20% [wt/vol] sucrose, 20 mM Tris [pH 7.6], 10 mM EDTA, 50 mM NaCl) for 30 min on ice. RNA extraction was performed using High Pure RNA Isolation kit (Roche Applied Science, Rotkreuz, Switzerland) according to the instruction of manufacturer. DNA was removed from the RNA preparations by treating twice with DNase I (Roche). The quantity and quality of RNA samples were analysed by the use of RNA NanoLab chip on the 2100 Bioanalyser (Agilent, Palo Alto, Calif.).

9. Real-time RT-PCR

The absence of contaminating DNA in RNA samples was verified by performing PCR using Platinum Quantitative PCR SuperMix-UDG (Invitrogen) in the TaqMan 7700 (Perkin-Elmer Applied Biosystems, Foster City, Calif.) by using primers and probe of sigB gene (table 1). Only the RNA in which no DNA was detected by PCR was used afterwards.

Steady-state mRNA levels were determined by quantitative real time RT-PCR (qRT-PCR) by use of the one-step reverse transcriptase qPCR Master Mix Kit (Eurogentec, Seraing, Belgium) in the TaqMan 7700 as previously described (Vaudaux, 2002; Renzoni, 2004). All primers and probes were designed using PrimerExpress software (version 1.5; Applied Biosystems). The primers and probes, harboring a 5′FAM (6-carboxy-fluorescein) and a
3’TAMRA (6-carboxy-tetramethyl-rhodamine) were synthesized by Eurogentec (Seraing, Belgium). sarA and spA probes, harboring a 5’FAM and 3’minor groove binder, were synthesized by Applied Biosystem (Calif). Table 1 shows the list of primers and probes used for this study.

Reverse transcription (RT) and PCR were performed using primers and probes at concentrations of 0.2 and 0.1 µM, respectively. Primers and probes specific for the sigB and hla genes were used at a uniform concentration of 0.1 µM and those specific for sarA gene were used at a concentration of 0.05 µM.

The total reaction volume was 15 µl. The mixture contained 0.3 µl primer and 0.3 µl probe, 7.5 µl of 2 × ThermoScript Reaction Mix buffer (final concentrations: 3 mM MgSO4, 0.4 µM for each dNTP), 0.3 µl ROX passive reference component (for normalization of data as a passive reference), 0.3 µl ThermoScript Plus/ Platinum Taq Enzyme Mix, 1.6 µl RNase-free water and 4 ng total RNA (8 µl). For each reaction, all the reagents except RNA sample were put into the 96-well microplate, and RNA sample was put into each reaction as a final step. The microplate was centrifuged briefly to make sure that all the reagents were at the bottom of wells.

RT-PCR was performed by 1 cycle of 30 min at 48°C and 10 min at 95°C for cDNA synthesis and pre-denaturation, and then PCR amplification of 40 cycles, using a two-step PCR cycling program of denaturation of 30 s at 95°C and annealing of 60 s at 60°C. After the last amplification cycle, samples were kept for final extension for 10 min at 72°C and immediately cooled to 4°C.

Fluorescence emission was detected by the sequence detector ABI Prism™ 7700 and analyzed with Sequence Detector™ software (version 1.7; Applied Biosystems). The mRNA levels of all target genes extracted from the different strains grown with or without ciprofloxacin were normalized on the basis of their 16S rRNA levels, which were assayed in each round of qRT-PCR as internal controls as described previously (Vaudaux, 2002; Renzoni, 2004). Data were presented as means ± SEM of at least three experiments performed in triplicate. The statistical significance of strain-specific differences and differences of each strain grown in ciprofloxacin-containing compared to ciprofloxacin-free MHB in normalized threshold cycle (C_T) values of each transcript were evaluated by paired t tests, and data were considered significant when P was <0.05 (Rosner, 1990).
Table 1. TaqMan primers and probes used in this study

<table>
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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe (5'-3')</th>
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<td>16S</td>
<td>551F-GGCAAGCGTTATCCGGAATT</td>
<td>651R-GTTTCCAATGACCCTCCACG</td>
<td>573T-CCTACGCAGCGCTTTACGCCCA&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>asp23</td>
<td>299F- GTTAACCACCTTTCATGTCTAAGATAC</td>
<td>390R- AAATTAACCTTCTCTGATGAAAGTTGATTA</td>
<td>333T- CTTCACGTGCAGCGATACCAGCAATTT&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>sigB</td>
<td>4862F-AAATTAGCAGTCATATCAACCCCATCG</td>
<td>4962R- AAAAAATCTCTTACTCTGCTGCC</td>
<td>4900T- AAAAAAGTGATTTATCCGCATCACCAG&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>fnbA</td>
<td>268F-AACAAGTTGAAGTGGCAGCC</td>
<td>341R- CCGCTACATCTGCTGATCTTGTCA</td>
<td>290T- AGAACCGCATCAGAAAGTAAGCCACGTG&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>fnbB</td>
<td>803F-CACCAGAAAACGTGGAAGCA</td>
<td>889R- TCCTGTAGTTTCTTTACTCGAATCTT</td>
<td>830T- TAGAAACTTCCGGATGTTGCTTCCCATCG&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>RNAIII</td>
<td>367F- TTCCTACTGTGTCGATAATCAA</td>
<td>436R- TGATTTCATGGCAACAGAT</td>
<td>388T- TTTACTAAGTCACCGATTGTAATAGA&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>agrA</td>
<td>34F- CAAAAGAAAGAAATCAGTTACATTATTAA</td>
<td>135R- CTAAGCAGCTCTCATAAGGATTACAG</td>
<td>83T- AAAAGCCTATGGGAATTGCCCCTGC&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>sarA</td>
<td>17F- ACATGGCAATTACAAAAATCAATGAT</td>
<td>167R- TCTTTTCTTTTTCTGGCTGATG</td>
<td>45T- CTTTGAGTTGTTATCAATGGT&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>hla</td>
<td>337F- ATGAGTAATTTTAATCTATTGGATCCGCAACGG</td>
<td>437R- AGTGATAGCAACATGGCAACATGGG3</td>
<td>385T- ACAGGAATAATGCGCGCTTTATATTGGT&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>spA</td>
<td>1914F- CAGCAAAAAATCCGACAGACTAA</td>
<td>1992R- ACAGTTTGTACCAGATGAAATGGATT</td>
<td>1945T- AGCATCGGAGTACCCCTCTGGTAC3&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>sspA</td>
<td>5’GGTTAATTCAGAGGGAATGC 3’</td>
<td>5’ ACTCTTTAACAATAAACGAGCGTGAAGA3’</td>
<td>5’ AAGCATGAGGATACCCCGTCTGAGC3’</td>
</tr>
</tbody>
</table>

<sup>a</sup> 5’FAM and 3’TAMRA labeled.

<sup>b</sup> 5’FAM Minor Groove Binder and 3’dark quencher labeled.
10. Northern blotting

10.1. Agarose/formaldehyde gel electrophoresis

All the equipments were autoclaved before using. All solutions were prepared using 0.1% diethylpyrocarbonate (DEPC) water to avoid RNase contamination. Total RNA was extracted as described in total RNA extraction. The concentration of RNA was measured by the RNA NanoLab chip on the 2100 bioanalyzer (Agilent, Palo Alto, Calif.). Sixteen µg of total RNA of each sample and a 6583-bp RNA marker (Promega) mixed with formaldehyde loading buffer were separated by a 1.8% agarose-0.66 M formaldehyde gel in $1 \times 3$-$N$-morpholine propane sulfonic acid (MOPS) running buffer for 3 h at room temperature. The voltage for electrophoresis was 90 V. MOPS buffer was prepared by 20 mM MOPS, 10 mM sodium acetate, 2 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0) and 0.1% DEPC water. The solution was adjusted to the final pH 7.0 using 2 M NaOH. The intensities of the 23S and 16S rRNA bands suggested equivalent RNA loading in all samples using ethidium bromide and UV visualization.

10.2. Blotting RNA from gel onto membrane

Blotting of RNA from gel onto positively charged nylon membranes (hybond-N+, Amersham, Switzerland) was performed by two methods. Up capillary transfer was performed using Whatman 3 MM filter paper wick method as essentially described in the protocol (Brown, 1997). The transfer was done in buffer $20 \times$ SSC ($1 \times$ SSC was 0.15 M NaCl plus 0.015 M sodium citrate) for overnight at room temperature. Another transfer method was using electroblotting system (Bio-Rad, Mini Trans-Blot electrophoretic transfer cell). The transfer was done in $1 \times$ Tris-Borate-EDTA (TBE) buffer at 400 AM for 3 h at room temperature in accordance with the protocol (Chory, 1997). The blotted membranes from both methods were fixed by UV-crosslinking (Spectro Linker, XL-100) for 1 min.

10.3. Probe labelling

The PCR fragments for probes were generated by PCR amplification using an Expand High Fidelity PCR System (Roche) in trio-thermoblock (Biometra). The template DNA was generated by using a chromosomal DNA of NCTC8325 isolated with chromosomal DNA isolation kit (Qiagen, Switzerland). The primers of 16S ribosomal RNA, $\text{sigB}$, $\text{sarA}$, and RNAIII that were used to generate probe fragments are listed in table 2. The PCR products were purified by a gel extraction kit (Qiaquick, Qiagen, Switzerland). 194-bp $\text{sigB}$, 290-bp...
and 1040-bp 16S rRNA fragments were labelled with $[\alpha^{-32}\text{P}]d\text{CTP}$ by use of a Prime-it Random Primer Labeling kit (Stratagene) according to the protocol of manufacture, and purified by use of Sephadex G-50 DNA columns (Pharmacia, Biotech) in $1 \times \text{TE buffer}$. 439-bp RNAIII probe fragment was labelled with digoxigenin (DIG)-dUTP, by use of a Dig High Prime DNA Labelling and Detection Starter kit II (Roche). The labelling efficiency for RNAIII was determined by semi-quantitative determination with the control labelled DNA (supplied in the kit).

10.4. Hybridization and detection

The blotted membranes for $\text{sigB}$, $\text{sarA}$ and 16S rRNA were pre-hybridized with 200 µl Rapid-Hyb Buffer (Amersham) for 2 h at 65°C by rotating, and then hybridized with the same Rapid-Hyb Buffer containing 200 µl $\text{sigB}$, $\text{sarA}$ or 16S rRNA probe (0.125 µg $[\alpha^{-32}\text{P}]d\text{CTP}$ for each probe) for overnight at 65°C by rotating. The hybridized membrane were strictly washed first with $2 \times \text{SSC}-0.1\% \text{sodium dodecyl sulfate (SDS)}$ for 20 min at room temperature, second with $1 \times \text{SSC}-0.1\% \text{SDS}$ for 15 min at 65°C, and finally with $0.5 \times \text{SSC}-0.1\% \text{SDS}$ for 15 min at 65°C. The humid membranes were sealed in a plastic paper, and exposed to the film (Kodak-Omat AR Film) for overnight.

The blotted membrane for RNAIII was pre-hybridized in 25 ml dig-easy-hyb buffer (Dig High Prime DNA labelling and Detection Starter kit II, Roche) for 2 h at 50°C with shaking 60 rpm, and hybridized in the same dig-easy-hyb buffer containing 25 ng/ml RNAIII DIG-labelled probe for overnight at 50°C with shaking 60 rpm. The hybridized membrane was strictly washed first with $2 \times \text{SSC}-0.1\% \text{SDS}$ for 15 min at room temperature with shaking 60 rpm, second with $1 \times \text{SSC}-0.1\% \text{SDS}$ for 15 min at 37°C, and finally twice with $0.5 \times \text{SSC}-0.1\% \text{SDS}$ for 15 min at 50°C with shaking 60 rpm. After washing briefly with $1 \times$ washing buffer (supplied in the kit) for 5 min, the membrane was incubated with block solution for 60 min, and antibody solution (anti-digoxigenin-AP, 75 mU/ml) for 60 min at room temperature. Following washing the membrane with $1 \times$ washing buffer two times for 15 min, and equilibrated with $1 \times$ detection buffer for 5 min at the room temperature, the membrane was carefully covered with 1 ml chemiluminescent substrate for alkaline phosphatase (CSPD, ready to use) and incubated for 5 min at room temperature and then for 10 min at 37°C. The membrane was immediately exposed to the film (Kodak Biomax MR film) for 20 – 30 min.
**Table 2.** Specific primers used to generate the probes for Northern blotting.

<table>
<thead>
<tr>
<th>genes</th>
<th>Up primer</th>
<th>Down primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>5'-GGTAGAGCCTCCCTTCGG-3'</td>
<td>5'-CCGGCAGTCAAGT TAGAGT GC-3'</td>
</tr>
<tr>
<td>sigB</td>
<td>5'-GATATTATGGGCAACAAGATGACC-3'</td>
<td>5'-GCCGT TTCTCTGAAGTGCTGATACAT-3'</td>
</tr>
<tr>
<td>sarA</td>
<td>5'-ATGATTGCTTTGAGTTTATCAAT-3'</td>
<td>5'-ACTCAATAATGATT CGATT TTT TTA-3'</td>
</tr>
<tr>
<td>RNAIII</td>
<td>5'-GTCATTATACGATTAGTAC-3'</td>
<td>5'-GGTTAATTAAGTGGGATG-3'</td>
</tr>
</tbody>
</table>
C. RESULTS
1. MICs of ciprofloxacin for the Q-R strains

The MICs of ciprofloxacin for \(\text{sigB}\) null strain TE1, \(rsbU\ \text{sigB}^+\) parent RA1, and \(rsbU^+\ \text{sigB}^+\) strain TE2 were identical: 32 µg/ml. We decided to use 1/8 MIC (4 µg/ml) of ciprofloxacin in all the experiments when ciprofloxacin influence was tested, since this sub-MIC of ciprofloxacin was shown previously to optimally promote FnBPs without significantly affecting bacterial growth rate (Bisognano, 2000)

2. Phenotypic characteristics of SigB-modulated strains

(1) Pigmentation

The orange-yellow pigment staphyloxanthin has been shown to be positively regulated by SigB activity. \(\Delta rsbUW\text{sigB}\) mutants of genetically distinct wild-type \(S.\ \text{aureus}\) strains COL and Newman were reported to be almost unpigmented, whereas a \(rsbU\)-complemented strain of NCTC8325 derivative BB255 has orange pigment (Kullik, 1998; Giachino, 2001). The pigmentation of three SigB-modified strains was clearly influenced by their SigB activity: \(\text{sigB}\) null strain TE1 had no colour, \(rsbU\ \text{sigB}^+\) parent RA1 had a pale yellow colour, and \(rsbU^+\ \text{sigB}^+\) strain TE2 had a bright orange colour (figure 1).

![Figure 1. Production of pigmentation: sigB null strain TE1, no colour; rsbU sigB + parent RA1, pale yellow; rsbU + sigB + strain TE2, bright orange.](image)

(2) Hemolysin activity

Alpha-hemolysin activity has been shown to be negatively regulated by SigB activity, since inactivation of \(\text{sigB}\) leads to upregulation of \(hla\) (Cheung, 1999). Hemolysin activities of the SigB-modified strains TE1, RA1, and TE2 were recorded by examining the lysed zones around spotted colonies grown on sheep blood agar (SBA) (figure 2). After incubation for 24 h at 37°C on SBA, the \(\text{sigB}\) null strain TE1 and \(rsbU\ \text{sigB}^+\) parent RA1 produced clear zones of complete hemolysis (average 12 mm) around the bacterial colonies, indicating production
of α-toxin, while much smaller clear hemolytic zones (7 mm) were seen around colonies of \( rsbU^+ \text{ sig}B^+ \) strain TE2. The hot-cold hemolysis reflecting β-toxin and yields halo effects around the clear zones. The exact sizes of hemolytic zones specifically due to β-toxin was difficult to estimate for the different SigB-modulated strains, but strain TE2 had much smaller halo zones than strain TE1 and RA1 (figure 2). ATCC (American Type Culture Collection) 25923 was used as a positive control strain of β-toxin (figure 2).

![Figure 2. Hemolysins of strains TE1, RA1, and TE2 on SBA. The clear zones (short arrow) around bacterial colonies were produced by α-toxin. The halo effects (long arrow) around the clear zones were produced by β-toxin. ATCC25923 was used as a positive control strain of β-toxin.](image)

(3) Protease activity

Protease activity has been shown to be indirectly suppressed by SigB functional level, since expression of the protease is repressed by SigB-dependent SarA (Karlsson, 2002). Protease activities of the SigB-modulated strains TE1, RA1, and TE2 were recorded by examining the lysed zones around spotted colonies grown on casein agar (figure 3). After incubation for 24 h at 37°C, the \( \text{sig}B \) null strain TE1 and \( rsbU \text{ sig}B^+ \) parent RA1 produced clear zones (average 10 mm) around the bacterial colonies, indicating production of protease, while no zones were seen around colonies of \( rsbU^+ \text{ sig}B^+ \) strain TE2 (figure 3). The results confirmed the previous study that SigB activity negatively regulates protease production (Karlsson, 2002).

![Figure 3. Protease activities of \( \text{sig}B \) null strain TE1, \( rsbU \text{ sig}B^+ \) parent RA1, and \( rsbU^+ \text{ sig}B^+ \) strain TE2 on casein agar plate.](image)
3. Modulation of *S. aureus* adhesion on fibronectin-coated surfaces by SigB levels and ciprofloxacin exposure

**Strains grown in ciprofloxacin-free medium**

Following growth in ciprofloxacin-free medium, the attachment of *sigB* null strain TE1, averaged over the three fibronectin coating concentrations, was significantly lower (29 %, *P* < 0.01) than that of its *rsbU* *sigB*+ parent RA1. In contrast, the average adhesion of *rsbU*+ *sigB*+ strain TE2 increased significantly (31 %, *P* < 0.01) compared to its parent RA1 (figure 4). Thus, the respective adhesion values of strains TE1, RA1, and TE2, increased as a function of the predicted increase in their SigB functional activity.

**Effect of ciprofloxacin**

In contrast, upregulation of bacterial attachment by growth in the presence of 1/8 MIC of ciprofloxacin (4 µg/ml) in the different strains was not significantly influenced by modulation of their SigB functional levels. The average ciprofloxacin-promoted increase in adhesion, over the three fibronectin coating concentrations, was significant (*P* < 0.01) and almost equivalent for *sigB* null strain TE1 (62 %), *rsbU* *sigB*+ parent RA1 (62 %), and *rsbU*+ *sigB*+ strain TE2 (77 %), respectively (figure 4).
Figure 4. (up) Dose-response curves of adhesion to fibronectin-coated coverslips of sigB null strains TE1, rsbU sigB+ parent RA1, and rsbU+ sigB+ strain TE2 grown either in the absence or presence of 4 µg/ml (1/8 MIC) of ciprofloxacin (CFX), expressed as CFU numbers of adherent S. aureus on coverslips coated with fibronectin.

(down) Percent changes in average adhesion to fibronectin-coated coverslips of TE1 and TE2, grown either in the absence or presence of 4 µg/ml (1/8 MIC) of ciprofloxacin (CFX), compared to RA1 adhesion in ciprofloxacin-free medium. Values represent means ± SEMs (error bars) of individual adhesion data cumulated over the three fibronectin coating concentrations, in three experiments.

*, results significantly different ($P < 0.05$) for each strain grown in ciprofloxacin-containing MHB from those in ciprofloxacin free MHB.

**, results significantly different ($P < 0.05$) from those of strain RA1 grown in ciprofloxacin-free medium.
4. Effect of *S. aureus* binding to FITC-fibronectin by SigB levels and ciprofloxacin exposure

Surface display of FnBPs including FnBPA and FnBPB in the SigB-modified strains was analyzed by flow cytometry.

**Strains grown in ciprofloxacin-free medium**

Following growth in the ciprofloxacin-free medium, the binding of FITC-labelled fibronectin by *rsbU* *sigB* strain TE2 increased significantly (127 %, *P* < 0.05) whereas the binding of *sigB* null strain TE1 decreased significantly (38 %, *P* < 0.05) compared to their *rsbU sigB* parent RA1 (figure 5). Thus, the respective binding values of FITC-labelled fibronectin by the SigB-modulated strains TE1, RA1, and TE2, increased as a function of the predicted increase in their SigB functional activity.

**Effect of ciprofloxacin**

After growth in the ciprofloxacin-containing medium, the binding of FITC-fibronectin by three SigB-modulated strains was significantly (*P* < 0.05) higher than that of the strains grown in the ciprofloxacin-free medium (figure 5). The relative fluorescent levels of the *sigB* null strain TE1, *rsbU sigB* parent RA1, and *rsbU sigB* strain TE2 increased 80 %, 105 %, and 132 %, respectively, regardless of their SigB functional levels.
Figure 5. Binding of FITC-labelled fibronectin by sigB null strain TE1, rsbU sigB⁺ parent RA1, and rsbU⁻ sigB⁺ strain TE2 grown either in the absence or presence of 4 µg/ml (1/8 MIC) of ciprofloxacin (CFX), expressed as percentages of rsbU sigB⁺ parent RA1 fluorescent level in ciprofloxacin-free medium performed at least three experiments. DU5883 (pFNBB4) and DU5883 were positive and negative controls, respectively.

*, results significantly different (P < 0.05) for each strain grown in ciprofloxacin-containing MHB from those in ciprofloxacin-free MHB.

**, results significantly different (P < 0.05) from those of strain RA1 grown in ciprofloxacin-free medium.
5. Assessment of sigB transcripts and SigB functional levels in the SigB-modulated strains

Strains grown in ciprofloxacin-free medium

The sigB transcript levels detected by quantitative RT-PCR were strongly (13.8-fold) elevated in rsbU+ sigB+ strain TE2 compared to its rsbU sigB+ parent RA1 (figure 6, up).

Unexpectedly, sigB transcript levels recorded in sigB null strain TE1 were found equivalent to those assayed in RA1, despite the presence of the transposon in the sigB gene. Because of sigB primers and probe selected for RT-PCR were located shortly after Tn551 insertion (figure 6, down right), I tried to detect the exact size of transcripts of TE1 originated from P_A and P_B compared to those in strains RA1 and TE2 by Northern affinity blotting. Northern blotting showed the presence in TE1 of two much higher molecular sigB transcripts, 7.6-kb rsbUVWsigB (including whole Tn551) from promoter A and 7.0-kb rsbVWsigB (including whole Tn551) from promoter B, compared to those recorded in TE2 or RA1 (figure 6, down). The larger size of sigB transcripts in sigB null strain TE1 reflects the presence of Tn551-encoded elements (5.1 kb). Besides the presence of a strong 1.9-kb sigB transcript in RA1 and TE2, another weaker 2.5-kb transcript was present on the original Northern blotting film both in TE2 and RA1, but was too weak in RA1 to be seen in the printed figure (figure 6, down).

Transcript levels of the alkaline-shock protein 23 (asp23) gene which is frequently used as a marker for SigB functional activity showed a 10.3-fold increase in rsbU+ sigB+ strain TE2 compared to its rsbU sigB+ parent RA1. A striking finding was the 100-fold decrease in the asp23 transcript levels recorded in sigB null strain TE1 compared to RA1, providing indirect evidence for a strong reduction of SigB functional levels TE1 compared to RA1 (figure 6, up).

Ciprofloxacin effect

Ciprofloxacin exposure led to a significant (P < 0.05) increase in sigB transcript levels of strain RA1, but not TE2. At the opposite, ciprofloxacin exposure led to significantly (P < 0.05) increased asp23 levels in strain TE2, but not RA1 (figure 6, up).
Figure 6. (up). Steady state mRNA levels of sigB (left panel) and asp23 (right panel) genes of sigB null strain TE1 and rsbU+ sigB+ strain TE2, grown in the absence or presence of 4 µg/ml (1/8 MIC) of ciprofloxacin (CFX), expressed as percentages of those of rsbU sigB+ parent RA1 grown in ciprofloxacin-free medium. mRNA levels were determined by real-time RT-PCR and normalized on the basis of their 16S rRNA levels. Values are the means + SEMs of at least three experiments performed in triplicate.

*, results significantly different ($P < 0.05$) for each strain grown in ciprofloxacin-containing compared to ciprofloxacin-free MHB.

**, results significantly different ($P < 0.05$) from those of strain RA1 grown in ciprofloxacin-free medium.

asp23 transcripts levels of sigB null strain TE1 were too small to be visualized (see text).

(down). Northern blot (left) of sigB transcripts in sigB null strain TE1, rsbU+ sigB+ strain TE2, and rsbU sigB+ parent RA1. 16 µg total RNA isolated from strains grown for 5 h was loaded in each lane. (right) Schematic representation of sigB operon in S. aureus. The primers and probe used for RT-PCR and the probe used for Northern blotting, open reading frames, putative promoters, and the transcription terminal are indicated.
6. Upregulation of \textit{fnbA} and \textit{fnbB} transcripts by SigB levels and ciprofloxacin exposure

Since quantitative adhesion and surface display of FnBPs were upregulated by restoration of functional SigB activity as well as by subinhibitory levels of ciprofloxacin, we assayed the steady-state \textit{fnbA} and \textit{fnbB} mRNA levels of each SigB-modulated strain by real time quantitative RT-PCR (qRT-PCR).

\textbf{Strains grown in ciprofloxacin-free medium}

After growth in ciprofloxacin-free medium, \textit{fnbA} mRNA levels were equivalent in \textit{sigB} null strain TE1 and \textit{rsbU sigB}+ parent RA1, but significantly \((P < 0.01)\) increased by 4-fold from RA1 to \textit{rsbU+ sigB}+ strain TE2. In similar conditions, \textit{fnbB} mRNA levels were also equivalent in strains TE1 and RA1, but sharply \((P < 0.01)\) increased by 12-fold from RA1 to TE2 (figure 7).

\textbf{Effect of ciprofloxacin}

In contrast, the relative increases in \textit{fnbA} and \textit{fnbB} transcript levels triggered in the different strains by growth in the presence of 4 \(\mu\text{g/ml}\) of ciprofloxacin were not correlated with their respective SigB functional levels. The ciprofloxacin-mediated increases in \textit{fnbB} transcripts in \textit{sigB} null strain TE1, \textit{rsbU sigB}+ parent RA1, and \textit{rsbU+ sigB}+ strain TE2 were 5.0-fold, 5.8-fold, and 2.8-fold, respectively, being significantly \((P < 0.01)\) greater than those when they grown in the absence of ciprofloxacin. The \textit{fnbA} transcripts increased significantly \((P < 0.01)\) 2.5-fold, 1.9-fold, and 1.7-fold in \textit{sigB} null strain TE1, \textit{rsbU sigB}+ parent RA1, and \textit{rsbU+ sigB}+ strain TE2, respectively, when they grown in the presence of ciprofloxacin compared to those in the absence of ciprofloxacin (figure 7).

Steady-state \textit{fnbB} mRNA levels increased very sharply (about 35 fold) from \textit{sigB} null strain TE1 when grown in the ciprofloxacin-free medium to \textit{rsbU+ sigB}+ strain TE2 when grown in the ciprofloxacin-containing medium (figure 7).
Figure 7. Steady-state levels of fnbA (left panel) and fnbB (right panel) transcripts of strains sigB null strain TE1 and rsbU+ sigB+ strain TE2, grown in the absence or presence of 4 \( \mu \)g/ml (1/8 MIC) of ciprofloxacin (CFX), expressed as percentages of those of rsbU sigB+ parent RA1 grown in ciprofloxacin-free medium. mRNA levels were determined by real-time RT-PCR and normalized on the basis of their 16S rRNA levels. Values represent the means \( \pm \) SEMs of at least three experiments performed in triplicate.

*, results significantly different \((P < 0.05)\) for each strain grown in ciprofloxacin-containing compared to ciprofloxacin-free MHB.

**, results significantly different \((P < 0.05)\) from those of parent strain RA1 grown in ciprofloxacin-free medium.
7. Influence of SigB levels and ciprofloxacin exposure on *agr* transcripts levels

The global regulator *agr* has been shown to play an essential role in down-regulating the synthesis of cell-surface adhesions such as FnBPs and protein A (Saravia-Otten, 1997; Dunman, 2001; Cheung, 2004). To detect any potential change in *agr* activity that might contribute to either the SigB-mediated or/and the ciprofloxacin-triggered upregulation of *fnb* genes, we assayed *agr* RNAII and RNAIII levels in *sigB* null strain TE1, *rsbU sigB* parent RA1, and *rsbU*+ *sigB*+ strain TE2.

**Strains grown in ciprofloxacin-free medium**

RNAII levels of three strains grown in ciprofloxacin-free medium were almost equivalent in TE1 and RA1, but showed a slight but not significant decrease in TE2 compared to RA1 and TE1. RNAIII levels were also similar in strains TE1 and RA1 grown in ciprofloxacin-free medium, but showed a significant 79% decline in TE2 compared to RA1, as confirmed by Northern blotting analysis (**figure 8**).

**Effect of ciprofloxacin**

Ciprofloxacin exposure led to a significant (*P* < 0.05) but slight (< 2-fold) increase in RNAII levels for strains RA1 and TE2, but not TE1. At the opposite, ciprofloxacin exposure led to significantly (*P* < 0.01) increased RNAIII levels in strain TE2 only, but not TE1 or RA1 (**figure 8**).
Figure 8. (up) Steady-state levels of RNAII and RNAIII of sigB null strain TE1 and rsbU+ sigB+ strain TE2, grown in the absence or presence of 4 µg/ml (1/8 MIC) of ciprofloxacin (CFX), expressed as percentages of those of rsbU sigB+ parent RA1 grown in ciprofloxacin-free medium.
mRNA levels were determined by real-time RT-PCR and normalized on the basis of their 16S rRNA levels.
Values represent the means + SEM of three experiments performed in triplicate.
*, results significantly different (P < 0.05) for each strain grown in ciprofloxacin-containing compared to ciprofloxacin-free MHB.
**, results significantly different (P < 0.05) from those of strain RA1 grown in ciprofloxacin-free medium.
(down) Northern blotting of RNAIII in strains TE1, RA1, and TE2. 16S rRNA was used as a RNA loading control. 16 µg total RNA isolated from strains grown to 5 h was loaded in each lane.
8. Impact of SigB levels and ciprofloxacin exposure on sarA transcripts levels

We next evaluated whether the global regulator sarA could contribute to either the SigB-mediated or the ciprofloxacin-triggered upregulation of fnb genes.

Strains grown in ciprofloxacin-free medium

While the overall sarA steady-state mRNA levels assayed by qRT-PCR of three strains grown in ciprofloxacin-free medium were equivalent in sigB null strain TE1 and rsbU sigB+ parent RA1, they showed a slight (53%) but significant \( P < 0.01 \) increase in rsbU+ sigB+ strain TE2 compared to its parent RA1 (figure 9, up).

Since the sarA locus is composed of three overlapping transcripts of 0.58, 0.84, and 1.15 kb, initiated from the P1, P3, and P2 promoters, respectively, I also performed Northern blotting analysis to analyze the SigB-dependent P3-driven transcript. Figure 9 (down) demonstrates a stepwise increase in the SigB-dependent P3-driven transcript from strain sigB null strain TE1 to parent RA1, and from RA1 to rsbU+ sigB+ strain TE2, as a function of the predicted increase in their SigB functional activity, and as expected from previous studies (Bischoff, 2004; Bischoff, 2001; Deora, 1997; Gertz, 2000; Manna, 1998).

Effect of ciprofloxacin

Ciprofloxacin exposure led to a significant \( P < 0.05 \) ca. 2-fold increase in sarA transcript levels in all strains TE1, RA1, and TE2 (figure 9, up).
Figure 9. (up) Steady-state levels of sarA transcripts of sigB null strain TE1 and rsbU+ sigB+ strain TE2, grown in the absence or presence of 4 µg/ml (1/8 MIC) of ciprofloxacin (CFX), expressed as percentages of those of rsbU sigB+ parent RA1 grown in ciprofloxacin-free medium. mRNA levels were determined by real-time RT-PCR and normalized on the basis of their 16S rRNA levels. Values represent the means + SEMs of at least three experiments performed in triplicate.

*, results significantly different ($P < 0.05$) for each strain grown in ciprofloxacin-containing compared to ciprofloxacin-free MHB.

**, results significantly different ($P < 0.05$) from those of strain RA1 grown in ciprofloxacin-free medium.

(down) Northern blotting (left) of sarA transcripts in sigB null strain TE1, rsbU sigB+ parent RA1, and rsbU+ sigB+ strain TE2. 16S rRNA probe was used as a RNA loading control. 16 µg of total RNA isolated from strains grown for 5 h was loaded in each lane. (right) Schematic presentation of sarA locus. The sarA probe used for Northern analysis, promoters, and the transcripts are indicated.
9. Impact of SigB levels and ciprofloxacin exposure on \textit{hla} and \textit{spa} transcript levels

Since previous reports demonstrated a strong impact of \textit{agr}, \textit{sarA}, and \textit{sigB} regulons on the expression of \(\alpha\)-hemolysin and protein A (Arvidson, 2001; Bischoff, 2004; Cheung, 2004; Norvick, 2003), we assayed \textit{hla} and \textit{spa} transcript levels of these target genes by qRT-PCR in the SigB-modulated strains grown in the absence or presence of ciprofloxacin.

Strains grown in ciprofloxacin-free medium

Striking differences were found in \textit{hla} and \textit{spa} transcript levels of \textit{rsbU}\textsuperscript{+} \textit{sigB}\textsuperscript{+} strain TE2 compared to \textit{sigB} null strain TE1 and \textit{rsbU} \textit{sigB}\textsuperscript{+} parent RA1. In TE2, \textit{hla} transcript levels were < 10\% of the nearly equivalent levels recorded in TE1 and RA1 (Figure 10, left). These strain-specific differences in \textit{hla} mRNA levels likely accounted for the strongly reduced hemolytic zones produced on sheep blood agar by strain TE2 compared to RA1 and TE1 (Figure 2).

An inverse situation was found for \textit{spa} transcript levels that were increased by > 25-fold in TE2 compared to the low, but nearly equivalent levels recorded in TE1 and RA1 (Figure 10, right).

Effect of ciprofloxacin

While ciprofloxacin exposure led to a significant increase in \textit{hla} transcript levels of strain TE2, but not RA1 and TE1, an inverse situation was seen with \textit{spa} transcripts whose levels were elevated in strains RA1 and TE1, but not TE2.
Figure 10. Steady-state levels of hla and spa transcripts of sigB null strain TE1 and rsbU+ sigB+ strain TE2, grown in the absence or presence of 4 µg/ml (1/8 MIC) of ciprofloxacin (CFX), expressed as percentages of those of rsbU sigB+ parent RA1 grown in ciprofloxacin-free medium.

mRNA levels were determined by real-time RT-PCR and normalized on the basis of their 16S rRNA levels. Values represent the means + SEMs of three experiments performed in triplicate.

*, results significantly different (P < 0.05) for each strain grown in ciprofloxacin-containing compared to ciprofloxacin-free MHB.

**, results significantly different (P < 0.05) from those of strain RA1 grown in ciprofloxacin-free medium.
D. DISCUSSION AND CONCLUSION
Growing evidence suggests that expression and surface display of FnBPs in *S. aureus* are regulated by a complex network of global regulators, transcription factors, and stress response pathways (Bischoff, 2004; Bischoff, 2001b; Bisognano, 2000; Bisognano, 2004; Blevins, 2002; Huesca, 2002; Karlsson, 2001; Saravia-Otten, 1997; Savolainen, 2001; Vaudaux, 2002; Vaudaux, 1998; Wolz, 2000; Xiong, 2004). Besides the previously reported growth-phase and quorum sensing-controlled effects of *agr* and *sarA* regulons on transcription of *fnb* genes and/or FnBP surface display (Blevins, 2002; Saravia-Otten, 1997; Wolz, 2000; Xiong, 2004), a variety of environmental or/stressful conditions may also alter expression of fibronectin adhesins. These diverse situations include switching to small-colony variant phenotypes (Vaudaux, 2002), acquisition of methicillin resistance (Rice, 2001; Savolainen, 2001; Vaudaux, 1998), emergence of teicoplanin resistance (Renzoni, 2004), or exposure of fluoroquinolone-resistant *S. aureus* to subinhibitory levels of ciprofloxacin (Bisognano, 1997; Bisognano, 2000; Bisognano, 2004). Except for the indirect impact of the methicillin resistance element, which does not affect *fnb* transcription but is believed to interfere with FnBP surface display via production of the *pls* surface protein (Huesca, 2002; Savolainen, 2001), all other conditions mentioned above were shown to regulate *fnb* transcription (Bisognano, 1997; Bisognano, 2000; Bisognano, 2004; Vaudaux, 2002). The upregulation of FnBP expression by specific environmental and stressful stimuli including fluoroquinolone exposure may play a significant role in promoting *S. aureus* attachment and colonization of host tissues or implanted biomaterials (Vaudaux, 2000).

The production of isogenic derivatives of the fluoroquinolone-resistant *grlA gyrA* double mutant strain RA1 of *S. aureus*, displaying widely different levels of SigB activity, allowed to explore the potential interaction of the SigB-mediated and ciprofloxacin-triggered pathways. Combination of transcriptional and phenotypic assays provided indirect though consistent evidence that the SigB-mediated and ciprofloxacin-triggered responses involved separate regulatory networks, whose characterization is still incomplete.

We recently reported the contribution of a RecA-LexA pathway on the ciprofloxacin-triggered induction of fibronectin binding via selective upregulation of the *fnbb* gene which did not require any functional *agr* or *sarA* activities (Bisognano, 2004). This study extends those previous findings by showing that the SigB functional activity does not interfere with the ciprofloxacin-triggered transcriptional and phenotypic responses. While the strong induction of the *fnbb* gene by a sub-MIC level of ciprofloxacin was confirmed in the SigB-
modulated derivatives of strain RA1, the \textit{fnbA} gene was also induced though to a much lower extent. The ciprofloxacin-triggered \textit{fnbA} induction may possibly result from the longer exposure of each strain with ciprofloxacin in this study compared to the shorter 20-min exposure in the previous report which failed to significantly induce \textit{fnbA} (Bisognano, 2004). Further studies are required to elucidate the molecular basis of the differential ciprofloxacin-triggered \textit{fnbA} versus \textit{fnbB} upregulation.

In contrast to the ciprofloxacin-triggered responses, those promoted by genetic modulation of SigB functional activity appear more complex at both transcriptional and phenotypic levels, as supported by a recent microarray-based analysis of the \textit{S. aureus} SigB regulon (Bischoff, 2004). A previous report also showed that GP268, a strain carrying an intact \textit{sigB} operon as the \textit{rsbU} \textit{sigB} strain TE2, had a higher fibronectin surface display than NCTC8325 derivative BB255 and its \textit{ΔrsbUVWsigB} mutant (Bischoff, 2001). In this study, the higher \textit{fnbA} and \textit{fnbB} mRNA levels that were detected in strain TE2 compared to the \textit{sigB} null strain TE1 and \textit{rsbU sigB} parent RA1 were, at least partly, confirm a recent report showing a higher level of \textit{fnbA} in a SigB-restored strain by Northern blotting (Nair, 2003).

In our experimental conditions, similar transcript levels were recorded for either \textit{fnbA} or \textit{fnbB} when comparing the \textit{sigB} null TE1 with its \textit{rsbU sigB} parent RA1. In contrast, there was a sharp though disproportionate increase in \textit{fnbB} versus \textit{fnbA} transcript levels assessed in the \textit{rsbU} + -restored strain TE2 compared to its \textit{rsbU sigB} parent RA1. The \textit{fnbA} and \textit{fnbB} transcriptional dose-response data contrasted with the smoother increase in FnBP surface display from strain TE1 to TE2 via RA1 as recorded by bacterial adhesion and flow cytometry assays. The molecular basis of these contrasted data is not understood and its elucidation will require improved understanding of the SigB-controlled pathway and its interactions with other regulatory networks controlling expression of fibronectin adhesins. Since expression of extracellular proteases was shown to be downregulated by high SigB and \textit{sarA} functional levels (Karlsson, 2002) and upregulated by \textit{agr} (Novick, 2003), we assayed in our set of SigB-modulated strains the levels of extracellular proteases that could potentially alter the half-life of surface-exposed FnBPs (Karlsson, 2001, McGavin, 1997). The levels of proteolytic activity recorded on supernatants from 5-h cultures were too low to allow assessment of strain-specific differences. While qRT-PCR data indicated a decreased expression of the V8 protease gene \textit{sspA} (data not shown) in the SigB-restored strain TE2 compared to non-restored isogenic derivatives, the extracellular protease release seems to be too marginal to play a major role in 5-h cultures. Previous reports have shown that production
of proteases mainly occurs during the late exponential and postexponential phases of growth (Horsburgh, 2002; Karlsson, 2001; Karlsson, 2002). Thus, our initial hypothesis that strain-dependent differences in production and extracellular release of proteases may account for the lack of correlation between fnb transcription and fibronectin adhesion was not supported by our experimental data. This discrepancy between fnb transcript levels and fibronectin binding, which was also previously reported (Saravia-Otten, 1997), may possibly results from differences in fnbA or fnbB mRNA decay between strain TE2 and RA1 or TE2, or from saturating amounts of cell wall-anchored FnBP molecules that may be displayed on bacterial cell surfaces.

The potential contribution of the major global regulators agr and sarA to increased levels of both fnbB transcripts in the rsbU⁺-restored strain TE2 compared to RA1 and TE1 was also evaluated. It should be emphasized that transcript levels from global regulators and their putative target genes from 5-h cultures grown without shaking cannot be directly compared with those from cultures grown with rotatory shaking whose growth rates and final biomass are much higher. In contrast to agr RNAII levels that were equivalent in all three strains, RNAIII levels were reduced by less than one order of magnitude in strain TE2 compared to RA1 and TE1, thus confirming the previously reported downregulation of the agr response regulator in strains displaying fully functional levels of SigB compared to their SigB-defective derivatives (Bischoff, 2001; Horsburgh, 2002). While the decreased RNAIII levels in strain TE2 compared to RA1 and TE1 may explain, at least in part (Dunman, 2001), the changes recorded on hla and spa transcript levels of the former compared to the latter strains, the molecular details of the RNAIII-mediated downregulation of each fnb gene are still unknown. Since no selective effect of RNAIII on fnbB compared to fnbA transcript levels has yet been reported, it is likely that other global regulatory systems or transcription factors may play a significant role in this complex regulatory process (Arvidson, 2001; Cheung, 2004; Norvick, 2003).

The impact of SigB functional levels on activities of the sarA regulon is controversial. While some studies indicate a SigB-promoted upregulation of sarA transcription (Bischoff, 2004; Bischoff, 2001a; Gertz, 1999) and translation (Gertz, 2000), other studies indicate no change in SarA protein levels (Horsburgh, 2002) or even a SigB-promoted downregulation of sarA (Cheung, 1999). These conflicting observations may potentially arise from either technical variables or significant differences in the genetic backgrounds of examined strains. In our study, overall sarA transcript levels determined by real-time RT-PCR were increased
by 2-fold in rsbU+ -restored strain TE2 compared to RA1 and TE1. Nevertheless, it is unlikely that increased sarA transcript levels may account for increased fnbB mRNA levels, in particular because sarA was previously established to upregulate fnbA but not fnbB transcription (Wolz, 2000; Xiong, 2004).

Previous studies reported that hla was negatively regulated by σB, and sigB mutants expressed higher α-hemolysin activities than their respective wild-type mutants (Cheung, 1999; Nicholas, 1999; Kullik, 1998). In this study, rsbU+ sigB+ strain TE2 expressed much lower hla mRNA level (6% of RA1) and yielded much smaller hemolytic zones on sheep blood agar than RA1 and TE1, which is consistent with the previous studies (Bischoff, 2004; Cheung, 1999). In contrast, previous studies reported that spa was negatively regulated by both sarA and agr at transcriptional (Dunman, 2001) and translational (Chien, 1999) levels. The high increase (>25-fold) in spa mRNA level of rsbU+ sigB+ strain TE2 compared to TE1 and RA1 cannot be explained by the sole contribution of agr and sarA and likely may be due to some other unknown regulatory mechanisms.

In conclusion, these results provide evidence that optimal expression of a stress response factor and triggering of a drug-induced DNA repair system may independently, but in an additive manner, lead to an impressive 30-fold increase in fnbB transcript levels and promote S. aureus attachment to fibronectin. Ongoing studies performed in our laboratory also provide preliminary evidence that increased FnBP expression in the SigB-restored strain TE2 grown in the presence of ciprofloxacin can upregulate their uptake by non-professional phagocytes (Renzoni, unpublished data). Further studies of SigB-regulated pathways using combined approaches of transcription profiling, targeted mutagenesis, and functional assays, are required to better understand how multiresistant clinical isolates may take benefit of the highly flexible regulation of S. aureus colonization and virulence.
Section III. REFERENCES


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