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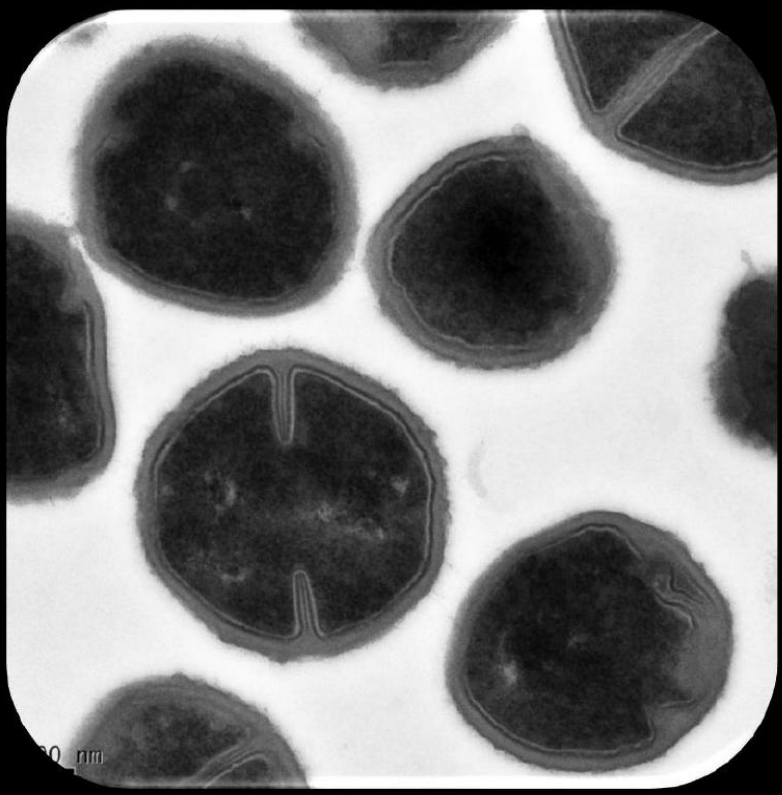
Identification of MurT-GatD enzymatic complex and insights into the biological role of this secondary modification

Teresa de Almeida Figueiredo



Dissertation presented to obtain the Ph.D. degree in Biology

Instituto de Tecnologia Química e Biológica António Xavier
Universidade Nova de Lisboa

Oeiras
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Knowledge Creation

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Abstract

Staphylococcus aureus is one of the most important contemporary human pathogens. The evolutionary "success" of this species is closely related to its remarkably capacity to acquire antibiotic resistance traits. In this perspective, it is important to extend our knowledge concerning the mechanisms of antibiotic resistance in *S. aureus* and to identify new antimicrobials targets.

Peptidoglycan is a unique and essential structure of the bacterial cell wall; its biosynthetic pathway is the target of powerful antibiotics such as β-lactams and glycopeptides. The biosynthesis of this heteropolymer, which consists of alternating disaccharide units composed of *N*-acetyl-glucosamine (Glc*N*Ac) and *N*-acetyl-muramic acid (Mur*N*Ac) crosslinked by short peptides, is a complex process that takes part in three different cellular compartments. The biosynthesis begins in the cytoplasm with the formation of the UDP-Mur*N*Ac-pentapeptide precursor. Then, in the inner face of the cell membrane, the pentapeptide precursor is linked to a membrane associated lipid carrier, which results in the formation of lipid I that, with the subsequent addition of the Glc*N*Ac, form the lipid II structure. The last stage of peptidoglycan biosynthesis includes polymerization of the disaccharide pentapetide units at the outside surface of the cytoplasmic membrane, through the coordinated action of transpeptidases and transglycosylases. During its synthesis and assembly, *S. aureus* peptidoglycan undergoes several modifications in its structure, namely the *O*-acetylation of Mur*N*Ac; the association of structures covalently linked, including teichoic acids, proteins and capsules; and the D-alanine esterification of wall teichoic acids. Furthermore, the peptidoglycan of *S. aureus* is virtually free of carboxyl groups, as the second aminoacid of the stem peptide, D-glutamic acid, is modified by amidation of its α-carboxyl group, which results in the formation of D-iso-glutamine. Although

peptidoglycan amidation is commonly present among gram-positive bacteria, its biological role is not completely clear, and the genes responsible for this modification were not identified, until now.

The first part of this Thesis describes identification of two hitherto unknown genes, *murT* and *gatD*, in the genome of the methicillin resistant *S. aureus* (MRSA) strain COL. The protein products of these two genes are responsible for the amidation of D-glutamate into D-iso-glutamine, in the peptidoglycan of *S. aureus*.

Due to their sequence similarities, the open reading frames corresponding to the *murT* and *gatD* genes were automatically annotated as a Mur ligase family-like protein and a glutamine amidotransferase, respectively. The DNA sequence analysis raised the hypothesis that *murT* and *gatD* genes are co-transcribed, since both genes are next to each other in the chromosome; they are transcribed in the same direction, and there is no promoter region upstream of the *gatD* gene. The results from reverse transcription-PCR and Northern blotting assays allowed us to conclude that *murT* and *gatD* are co-transcribed from the same promoter, forming a small operon. It is also interesting to note that and the *murT-gatD* are merged in several Gram-positive bacteria, and exist as a single gene.

In order to explore the role of these uncharacterized genes the *murT-gatD* operon was placed under the control of an inducible promoter in the background of the MRSA strain COL. In the absence of inducer, the *murT-gatD* conditional mutant produced a peptidoglycan of abnormal composition, with an increase in the amount of muropeptides containing D-glutamate instead of D-iso-glutamine in the stem peptide. This provided direct evidence for the involvement of *murT-gatD* in the amidation of *S. aureus* peptidoglycan. The previously characterized *glnRA* transposition mutant RUSA208, in which the transcription of glutamine synthetase (*glnA*)

is affected, has already shown a similar - abnormal - muropeptide composition of its cell wall. In the present work, the peptidoglycan composition of the double mutant, in which expression of both *murT-gatD* and *glnRA* are inhibited, showed absence of amidated muropeptides, strongly suggesting that their protein products are the sole determinants for the amidation of the D-glutamate residues of *S. aureus* peptidoglycan. Analysis of the cell wall precursor pool composition, in the parental and in *murT-gatD* depleted cells, revealed the presence of D-glutamate in peptidoglycam precursors of both strains, which indicated that the amidation reaction occurs in a later stage of peptidoglycan biosynthesis, most likely in the membrane phase. Further, *in vivo* complementation assays of the *murT-gatD* depleted phenotype allowed us to infer that peptidoglycan amidation requires specifically expression of *murT,* (which appears to be essential and highly specific for the recognition of the peptidoglycan precursor), and at least a basal level of *gatD*, suggesting that *S. aureus* contains other glutamine amidotransferases able to partially replace GatD activity.

The experimental data together with the analysis of aminoacid sequence, and the mechanism of action of the glutamine amidotransferases allowed us to propose that MurT and GatD together contain the domains and motifs required for the amidation of *S. aureus* peptidoglycan to occur. MurT, with its central domain similar to Mur ligases with motifs for ATP binding seems to represent the synthetase domain, responsible for the recognition of the acceptor substrate and for ATP binding, while GatD contains the glutaminase domain, catalyzing the hydrolysis of glutamine and the transfer of the resultant amino group to the acceptor substrate, the peptidoglycan precursor.

The inhibition of *murT-gatD* transcription caused impairment in growth rate, which demonstrated that peptidoglycan amidation has an important role in bacterial growth. Nevertheless, the *murT-gatD* depleted cells showed normal morphology, by electron microscopy analysis, indicating that the *murT-gatD* operon is not required for cell division and the involvement of peptidoglycan amidation in bacterial growth is completely independent of cell division. Consistent with the previous results from the *glnRA* transposition mutant RUSA208, the *murT-gatD* depleted mutant showed decreased methicillin resistance, confirming that peptidoglycan amidation is associated with the mechanism of resistance to β-lactams. Furthermore, it was found that lack of *murT-gatD* transcription caused decrease of lysozyme resistance both in cells and also in purified peptidoglycan, indicating that the reaction catalyzed by MurT and GatD proteins is directly involved in the mechanisms of resistance to lysozyme.

The second part of this Thesis provides direct experimental evidence that the MurT and GatD proteins interact physically, forming a stable enzymatic complex, and that this interaction is essential for peptidoglycan amidation to occur.

The co-transcription of *murT* and *gatD* genes and the requirement of both proteins for full *in vivo* complementation of the *murT-gatD* depletion phenotype, (as demonstrated in the first part of the Thesis), strongly suggested the existence of an interaction between MurT and GatD proteins. In the second part of the Thesis, the co-purification of MurT and GatD recombinant proteins, (from a vector expressing both *murT* and *gatD* genes), clearly showed that these proteins interact physically and form a stable enzymatic complex, needed for the amidation of *S. aureus* peptidoglycan. Additionally, several important findings were obtained through the *in vitro* analysis of lipid II amidation, using MurT-GatD and

GatD purified proteins. These findings included demonstration that: i) lipid II is a substrate for MurT-GatD catalyzed reaction; ii) that the *in vitro* amidation reaction requires the presence of both proteins; and iii) that amidation is dependent on ATP and glutamine.

The third part of this Thesis describes that peptidoglycan amidation has different impacts in the expression of methicillin and lysozyme resistance, in representative strains of the most widespread clones of MRSA.

Despite *mecA* being considered the main genetic determinant of methicillin resistance, several genes from the core genome and some related with cell wall biosynthesis, including *murT-gatD* operon, are required for the optimal expression of methicillin resistance in *S. aureus*. In the present work, different degrees of decrease in the original methicillin resistance were observed when *murT-gatD* transcription was inhibited in various MRSA genetic backgrounds, with a more pronounced effect on community acquired (CA-MRSA) related backgrounds, when compared with hospital acquired strains (HA-MRSA).

It is also interesting that these different phenotypes may be related to the capacity of the strains' genetic background to acquire and maintain the *mecA* gene, since the genetic backgrounds, previously described to be more prone to receiving *mecA,* were the ones less dependent of *murT-gatD* to express methicillin resistance. Inhibition of *murT-gatD* transcription, in a *mecA*-independent resistant strain, caused a decrease in methicillin resistance, suggesting that peptidoglycan amidation also contributes to the methicillin resistance by a *mecA*-independent pathway. Further, more two CA-MRSA genetic lineages showed a more pronounced effect on the decrease of methicillin resistance, when *murT-gatD* transcription was inhibited, as compared to inhibition of expression of MurF, an essential *S. aureus* Mur ligase. These findings highlight the proposition that MRSA can

follow different strategies, by recruitment of different housekeeping genes, for the optimal expression of methicillin resistance.

Peptidoglycan amidation appears to be more significant for lysozyme resistance in cells of CA-MRSA backgrounds, as compared with cells of HA-MRSA. The absence of significant differences in the level of lysozyme resistance of peptidoglycan, indicated that lysozyme resistance in the CA-MRSA analyzed may involve others factors that are triggered by peptidoglycan amidation.

Resumo

Staphylococcus aureus é um dos mais importantes agentes patogénicos humanos, cujo sucesso evolutivo está intimamente relacionado com a sua capacidade de adquirir resistência aos antibióticos. Por esta razão é criticamente importante alargar os nossos conhecimentos relativamente aos mecanismos de resistência aos antibióticos em *S. aureus* e identificar novos alvos antimicrobianos.

O peptidoglicano é o principal constituinte da parede celular bacteriana; as enzimas envolvidas na sua síntese são utilizadas como alvo para importantes antibióticos, como é o caso dos β-lactâmicos e dos glicopéptidos. A biossíntese deste heteropolímero, que consiste em unidades alternadas de dissacáridos compostas por *N*-acetilglucosamina (Glc*N*Ac) e por ácido *N*-acetilmurâmico (Mur*N*Ac) ligadas por pequenas cadeias peptídicas, é um processo complexo que ocorre em três compartimentos celulares distintos. A biossíntese inicia-se no citoplasma com a formação do precursor muropeptídico, o pentapéptido-Mur*N*Ac. De seguida, na face interna da membrana celular, este precursor liga-se a um transportador lipídico membranar, originando o lípido I, que por sua vez ao associar-se com uma molécula de Glc*N*Ac, origina o lípido II. A última fase da biossíntese do peptidoglicano inclui a polimerização das unidades do dissacarído pentapetídico, na face externa da membrana citoplasmática, através da ação coordenada das transpeptidases e transglicosilases. Durante a sua síntese, o peptidoglicano de *S. aureus* adquire várias modificações estruturais: é alvo de O-acetilação do Mur*N*Ac, de associação de estruturas covalentemente ligadas, como é o caso dos ácidos teicóicos, proteínas e cápsula, e de esterificação da D-alanina dos ácidos teicóicos. Para além disso, o peptidoglicano de *S. aureus* não contém grupos carboxilo, uma vez que o segundo aminoácido da cadeia peptídica, o D-glutamato, é modificado através da amidação do seu grupo

α-carboxilo em D-iso-glutamina. Apesar da amidação do peptidoglicano ser frequente em bactérias Gram-positivas, o seu papel biológico não é conhecido, e os genes responsáveis por esta modificação não estavam identificados até à realização desta Tese.

A primeira parte desta Tese descreve a identificação de dois genes, até agora desconhecidos, *murT* e *gatD*, no genoma da estirpe COL, uma estirpe MRSA (de "Methicillin Resistant *Staphylococcus aureus*). Os produtos proteicos destes dois genes são responsáveis pela amidação do D-glutamato em D-iso-glutamina, no peptidoglicano de *S. aureus*.

Devido a semelhanças de sequência, as ORFs (de "Open Reading Frames") correspondentes aos genes *murT* e *gatD,* foram automaticamente anotadas nas bases de dados como codificando uma proteína pertencente à classe das Mur ligases e uma glutamina amidotransferase, respectivamente. A análise da sequência de DNA sugeriu que os genes *murT* e *gatD* são co-transcritos, uma vez que o codão stop do *murT* e o codão de iniciação do *gatD* apenas estão separados por 4 nucleótidos, ambos os genes são transcritos na mesma direção, e não existe nenhuma região promotora antes do início do *gatD*. Os resultados de PCR por transcriptase reversa e a análise por "Northern blotting" permitiram-nos concluir que *murT-gatD* é co-transcrito a partir do mesmo promotor, confirmando que se trata de um pequeno operão. É curioso também notar que em algumas bactérias Gram-positivas estes dois genes existem como uma única região codificante, o que sugere uma interação ou complementaridade de funções entre os respectivos produtos proteicos.

Com o objetivo de investigar o papel destes genes construiu-se um plasmídeo em que o operão *murT-gatD* foi colocado sob o controlo de um promotor indutível na estirpe COL. Na ausência de indutor, este mutante

condicional do *murT-gatD* originou um peptidoglicano com uma composição alterada, nomeadamente, um aumento da quantidade de muropéptidos contendo D-glutamato em vez de D-iso-glutamina, na cadeia peptídica. Estes resultados constituíram evidências diretas para o envolvimento do *murT-gatD* na reação de amidação do peptidoglicano de *S. aureus*. O mutante de transposição RUSA208, no qual a transcrição da sintetase da glutamina (*glnA*) está afetada, também apresenta alterações idênticas na composição dos muropéptidos da sua parede celular. No presente trabalho, observou-se a ausência de precursores muropeptídicos amidados no duplo mutante, no qual a transcrição de *murT-gatD* e de *glnA* foi inibida, sugerindo que as respectivas proteínas são os únicos determinantes da amidação do D-glutamato do peptidoglicano de *S. aureus.* A análise da composição dos precursores citoplasmáticos da parede celular, na estirpe parental e na estirpe na qual *murT-gatD* não é transcrito, revelou a presença de D-glutamato em ambas, indicando que a reação de amidação ocorre numa fase posterior da biossíntese do peptidoglicano, muito provavelmente na fase lipídica. Os ensaios *in vivo* de complementação do fenótipo mutante, permitiram-nos inferir que a amidação do peptidoglicano requer a expressão de MurT, (que parece ser essencial e altamente específica para o reconhecimento do precursor do peptidoglicano), e uma expressão residual de GatD, sugerindo que em *S. aureus* existem outras glutamino amidotransferases, capazes de substituir parcialmente a atividade da proteína GatD. Os resultados experimentais, juntamente com a análise da sequência de aminoácidos, e o mecanismo de ação das glutamino amidotransferases permitiram-nos propor que as proteínas MurT e GatD, em conjunto, contêm os domínios e motivos necessários para que a amidação do peptidoglicano em *S. aureus* possa ocorrer. MurT, com o seu domínio central semelhante ao das Mur ligases e motivos para a ligação ao ATP, parece representar o domínio de sintetase, sendo responsável pelo reconhecimento do substrato recetor e pela

ligação ao ATP, enquanto a GatD contém o domínio da glutaminase, catalisando a hidrólise da glutamina e a subsequente transferência do grupo amino para o substrato recetor, o precursor do peptidoglicano.

Na ausência de indutor, observou-se a diminuição da taxa de crescimento do mutante condicional do *murT-gatD,* o que demonstrou que a amidação do peptidoglicano é importante para o crescimento bacteriano. No entanto, sob as mesmas condições, as células do mutante não apresentaram diferenças significativas de morfologia, quando foram analisadas por microscopia eletrónica, indicando que o *murT-gatD* não é necessário para a divisão celular, mas que é importante para o crescimento bacteriano, através de um mecanismo independente do processo da divisão celular. Tal como já tinha sido observado com o mutante de transposição do gene *glnA*, RUSA208, a inibição da transcrição de *murT-gatD* causou diminuição na resistência à meticilina, confirmando que a amidação do peptidoglicano está associada ao mecanismo de resistência aos β-lactâmicos. Foi também demonstrado que a reação catalisada pelas proteínas MurT e GatD está diretamente envolvida nos mecanismos de resistência à lisozima, uma vez que tanto as culturas celulares como o peptidoglicano purificado mostraram-se mais sensíveis à lisozima, quando a expressão de *murT-gatD* se encontra afetada.

A segunda parte desta Tese apresenta evidências experimentais para a existência de uma interação física entre as proteínas MurT e GatD, formando-se desta forma um complexo enzimático estável, sendo esta interação essencial para a ocorrência da amidação do peptidoglicano.

A co-transcrição dos genes *murT* e *gatD* e a necessidade da expressão das duas proteínas para a total complementação do fenótipo mutante, (como demonstrado na primeira parte desta Tese), sugeriram a existência de uma interação entre as proteínas MurT e GatD. A co-purificação das

proteínas recombinantes MurT e GatD, (através de um plasmídeo que expressa simultaneamente os dois genes), mostrou claramente que estas proteínas interagem e formam um complexo enzimático estável, necessário para a amidação do peptidoglicano de *S. aureus*. Adicionalmente, a análise da reação de amidação, *in vitro,* do lípido II, usando as proteínas MurT-GatD e GatD purificadas, permitiu demonstrar que: i) o lípido II é um substrato para a reação catalisada pelo complexo MurT-GatD; ii) que a reação da amidação, *in vitro*, necessita da presença de ambas as proteínas, e iii) que a amidação é dependente da presença de ATP e glutamina.

A terceira parte desta Tese descreve o impacto da falta de amidação do peptidoglicano na expressão da resistência à meticilina e à lisozima, em estirpes representativas dos clones mais disseminados de MRSA.

Apesar do gene *mecA* ser o principal determinante genético da resistência à meticilina, existem outros genes, localizados no cromossoma e alguns relacionados com a biossíntese da parede celular, como é o caso do operão *murT-gatD*, necessários à expressão ótima da resistência à meticilina em *S. aureus.* No presente trabalho, foi observado um impacto diferente no decréscimo da resistência original à meticilina, quando a transcrição do *murT-gatD* era inibida em vários clones MRSA, havendo um impacto maior nos clones CA-MRSA (de ”Community Acquired-MRSA”), do que nos clones HA-MRSA (de “Hospital Acquired-MRSA”).

É também interessante notar que estes diferentes fenótipos de resistência podem estar relacionados com a capacidade de cada estirpe de adquirir e manter o gene *mecA,* uma vez que os clones, anteriormente descritos como sendo mais eficientes na aquisição do gene *mecA*, foram os que apresentaram menor dependência do operão *murT-gatD* para expressar a resistência à meticilina. A inibição da transcrição dos genes *murT-gatD*

provocou um decréscimo de resistência numa estirpe resistente que não contém *mecA*, sugerindo que este operão está também envolvido na resistência à meticilina, através de um mecanismo que não necessita da presença do gene *mecA* para ser expresso. Curiosamente, duas linhagens genéticas de CA-MRSA apresentaram um efeito mais drástico na diminuição da resistência à meticilina, quando a transcrição de *murT-gatD* foi inibida, do que na ausência de expressão de uma Mur ligase essencial de *S. aureus*, a proteína MurF. Estas observações sugerem que MRSA pode desenvolver diferentes estratégias, através da utilização de diferentes genes essenciais de *S. aureus*, de forma a expressar resistência à meticilina.

A amidação do peptidoglicano também parece ser mais importante para a expressão da resistência à lisozima em clones CA-MRSA, do que em HA-MRSA. No entanto, não se observaram diferenças significativas relativamente ao nível de resistência à lisozima do peptidoglicano purificado, o que parece sugerir que a resistência à lisozima, nos clones de CA-MRSA analisados, é determinada por fatores que estão sob o controlo de *murT-gatD*.

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Thesis Outline

Staphylococcus aureus is an important human pathogen whose clinical relevance is mainly due to its remarkable capacity to develop mechanisms of resistance to antibiotics that are being introduced into the therapeutic arsenal. In this context, it is urgent and extremely important to extend our knowledge regarding the mechanisms of antibiotic resistance in *S. aureus*, and to discover new targets for the development of alternative antimicrobials agents. Peptidoglycan, a major component of the Gram-positive bacteria cell wall, is essential for cell survival and unique to bacteria which makes the enzymatic steps of its biosynthetic pathway excellent candidates for the design of new antibiotics. Furthermore, the fact that most of the genes associated with β-lactam antibiotic resistance in *S. aureus* are important players in the biosynthesis of peptidoglycan, highlight their potential as targets for antimicrobial therapy. Despite the fact that the peptidoglycan biosynthetic pathway has been extensively studied, some features have remained unknown. The main finding of this Thesis is the identification of the so far uncharacterized *murT* and *gatD* genes, responsible for the amidation of the glutamic acid residue of the stem peptide of *S. aureus* peptidoglycan.

Chapter I provides a general introduction to some of the important aspects of the cell wall of *S. aureus*, namely its structure is described and all the enzymatic steps of the peptidoglycan biosynthesis pathway are mentioned in detail. The relevant findings about peptidoglycan hydrolases, which includes the *N*-acetylmuramidase lysozyme, are also reviewed and a current understanding of cell wall modifications is summarized. As the structure of peptidoglycan is the primary target of β-lactam antibiotics, the mode of action and the mechanisms of resistance against this class of antibiotics are also described in some detail. Finally, some relevant aspects of the molecular epidemiology of *S. aureus* are also briefly reviewed.

Chapter II entitled “Identification of Genetic Determinants and Enzymes Involved with the Amidation of Glutamic Acid Residues in the Peptidoglycan of *Staphylococcus aureus*” describes the genetic and biochemical studies performed that allowed identification of the *murT-gatD* operon as responsible for the amidation of the glutamic acid residue in the stem peptide of *S. aureus* peptidoglycan. In this chapter, evidence is described that suggests that MurT and GatD proteins have a coordinated action in order to amidate glutamic acid. The availability of a *murT-gatD* conditional mutant enabled us to conclude that amidation of *S. aureus* peptidoglycan is involved in the mechanisms of resistance to β-lactam antibiotics and to lysozyme and is important for bacterial growth.

Chapter III entitled “MurT-GatD is an enzyme complex responsible for the amidation of glutamic acid residues in the peptidoglycan precursor lipid II of *Staphylococcus aureus”* describes co-purification of MurT and GatD recombinant proteins from a vector expressing both *murT* and *gatD* genes, which allowed us to conclude that these proteins interact physically, forming a stable enzymatic complex. In this chapter, the *in vitro* lipid II amidation demonstrated that both proteins, MurT and GatD, as well as ATP and glutamine are required for the reaction to occur, and lipid II is a substrate of MurT-GatD complex.

Chapter IV entitled “Contribution of Peptidoglycan Amidation to β-Lactam and Lysozyme Resistance in Different Genetic Lineages of *Staphylococcus aureus*” shows that peptidoglycan amidation has different impacts in the level of β-lactam and lysozyme resistance, according to the genetic background of the strain. The transcription of *murT-gatD* seems to be more important for the expression of methicillin resistance in the community acquired methicillin resistant *S. aureus* (CA-MRSA), than in hospital acquired strains (HA-MRSA). On the other hand, it is also shown in this chapter that inhibition of glutamic acid amidation produces a higher impact

on lysozyme resistance in cells of CA-MRSA than in HA-MRSA, suggesting the existence of specific factors in CA-MRSA, associated with lysozyme resistance, that are triggered by *murT-gatD* expression.

Chapter V includes a general discussion of the main findings of my doctoral Thesis work. Strategies to follow up of the observations produced in this work, are also described in some detail.

Appendix I is entitled "Purification, crystallization and preliminary X-ray diffraction analysis of GatD, a glutamine amidotransferase-like protein from *Staphylococcus aureus* peptidoglycan" and it reports the crystallization of native and the selenomethionine-derivative of GatD protein.

Chapters II, IV and Appendix I describe findings that appear in the following publications:

Teresa A. Figueiredo, Rita G. Sobral, Ana Madalena Ludovice, João Manuel Feio de Almeida, Nhat K. Bui, Waldemar Vollmer, Hermínia de Lencastre, Alexander Tomasz. 2012. Identification of Genetic Determinants and Enzymes Involved with the Amidation of Glutamic Acid Residues in the Peptidoglycan of *Staphylococcus aureus.* PLoS Path. 8: e1002508. - Chapter II.

Teresa A. Figueiredo, Ana Madalena Ludovice, and Rita G. Sobral. 2014. Contribution of Peptidoglycan Amidation to β-Lactam and Lysozyme Resistance in Different Genetic Lineages of *Staphylococcus aureus*. Microb. Drug. Resist. 20: 238-49. - Chapter IV.

Diana Vieira, Teresa A. Figueiredo, Anil Verma, Rita G. Sobral, Ana Madalena Ludovice, Hermínia de Lencastre, and Jose Trincao. 2014. Purification, crystallization and preliminary X-ray diffraction analysis of

GatD, a glutamine amidotransferase-like protein from *Staphylococcus aureus* peptidoglycan. Acta Cryst. F70: 632–35. - Appendix I.

Chapter I

General Introduction

1. *Staphylococcus aureus*

1.1. General description

Staphylococci are Gram-positive cocci and were first described in 1881 by Alexander Ogston, who designated the clustered micrococci as “staphylococci” from the Greek *staphyle*, meaning bunch of grapes (247). In 1884, Rosenbach, a German surgeon, proposed the first taxonomic description of the genus *Staphylococcus* after the isolation of two types of pigmented colonies: *Staphylococcus aureus*, from the Latin *aurum* for gold colonies, and *Staphylococcus albus* (later named *Staphylococcus epidermidis*) from the Latin *albus* for white colonies (173).

All members genus *Staphylococcus* share several features: they are Gram-positive bacteria of low DNA G+C content, facultative anaerobes, glucose fermenting, and positive for catalase activity converting hydrogen peroxide to water; also, they have the capacity to grow at temperatures ranging from 15 to 45 degrees Celsius and at high saline concentrations.

Among staphylococci, *S. aureus* and *S. epidermidis* are the most important for their capacity to interact with humans. *S. aureus* can be differentiated from *S. epidermidis* by the secretion of coagulase, which converts fibrinogen to fibrin, promoting the coagulation of plasma, and also by its ability to ferment mannitol aerobic and anaerobically. *S. aureus* is the best characterized staphylococcal species. In the past decades, it has been extensively studied, mainly due to its high prevalence, virulence, and capacity to acquire mechanisms of resistance to many antibiotics.

1.2. *S. aureus* importance as a pathogen

S. aureus are mostly mutualist or commensal organisms that are frequently found colonizing the skin and mucosas of human and several animal

species. It is estimated that 20 to 25% of the healthy human population are persistent carriers and about 60% are intermittent carriers of *S. aureus* (40, 262). Despite the fact that the relationship between *S. aureus* and the host is frequently asymptomatic, the rupture of the cutaneous barrier allows bacteria to penetrate, causing disease. As a pathogen, *S. aureus* is considered an extraordinary versatile bacteria, as it can cause a wide spectrum of infections in humans ranging from skin and soft tissue infections (e.g., cellulitis, folliculitis, mastitis, impetigo, furuncles, superficial and deep skin abscesses, wound infections), to severe life-threatening diseases (e.g., pneumonia, meningitis, bacteremia, endocarditis, osteomyelitis) and toxin-mediated diseases (e.g., toxic shock syndrome, scalded skin syndrome and food poisoning) (280).

S. aureus is considered an important human pathogen not only for its capacity to easily acquire and accumulate resistance to several antimicrobials (see section 4), but also for containing a large number of virulence factors. These virulence factors can be grouped according to their role during infection: i) virulence factors that cause the attachment to the host's cells or extracellular matrix, as fibrinogen-binding proteins, coagulase, clumping factor, adhesins and biofilm related polysaccharides; ii) virulence factors that allows the bacterial evasion from host's defense, including enterotoxins, protein A and leukocidins; and iii) virulence factors involved in invasion and tissue penetration, which attack the host's cell and degrade components of extracellular matrices, such as hemolysins, α-toxin and phospholipase C (280).

The expression of the virulence factors is controlled by a complex regulatory circuit, throughout the *S. aureus* life cycle (208) and at least three major operons are involved: the *agr* (accessory gene regulator) (285, 337); the *sar* (staphylococcal accessory regulator) (43) and the *sae*

(staphylococcal accessory element) loci (3). Additionally, other regulatory systems also have an impact on virulence gene expression and these include *arlR* and *arlS* (autolysis-related locus sensor) (96) and *rot* (repressor of toxins) (303).

2. Cell wall

The bacteria cell wall envelope is a semi-rigid layer essential for the cell's structural integrity and for the maintenance of cellular shape, as it provides protection against mechanical stress or damage from osmotic pressure; the cell wall also represents a functional interface between the bacteria and the outside environment. In the last decades, this organelle has been recognized as a highly complex and dynamic structure, which is in a constant state assembly and degradation, in order to allow a variety of cellular processes, such as: cell growth and division, cellular morphogenesis, chromosome segregation, competence and virulence. The cell wall of Gram-positive bacteria is structurally different from the one of Gram-negative microbes. Since *S. aureus* is a Gram-positive bacterium, we will focus on the description of this type of wall in the next section of this Thesis.

2.1. Gram-positive cell wall

The cell wall of Gram-positive bacteria is mostly composed of peptidoglycan, consisting of up to 50% of its mass, in contrast to Gram-negative cell wall, whose peptidoglycan layer is thinner, contributing only with 10% of its mass (108). Importantly, unlike Gram-negative bacteria, Gram-positive are not protected by an outer membrane. In addition to peptidoglycan, the structure and synthesis of which is described in section 2.2 and 2.3, the Gram-positive cell wall also includes several other chemically distinct molecules such as linear polymers of polysaccharides

and proteins, most of them covalently attached to the peptidoglycan mesh. Due to their importance for *S. aureus* physiology, the teichoic acids and the penicillin binding proteins (PBPs) will be a major focus of this Thesis.

Teichoic acids (TAs). These phosphate-rich glycopolymers are present in the cell wall in roughly equal proportions to peptidoglycan, constituting about 50% of its total mass. TAs are anionic polymers with long chains, composed of alternating phosphate and alditol groups, and can be divided in two groups: i) wall teichoic acids (WTAs), long chains of ribitolphosphate units covalently attached to the peptidoglycan muramic acid, by phosphodiester bonds (277); and ii) lipoteichoic acids (LTAs), long chains of 1,3-linked glycerolphosphate residues, which are linked to the cytoplasmic membrane by a glycolipid (89). Importantly, the chemical structure of WTAs is modified in Gram-positive bacteria, namely WTAs of *S. aureus* are D-alanylated, which has implications in the bacteria mechanism of clearance by the host immune system during the infection process (see section 3).

Recent studies have indicated that TAs have a critical role in cell division and morphogenesis by controlling the biosynthesis of peptidoglycan. On the one hand, LTAs are involved in the control of autolysis rates (268) and in other important cellular processes, namely in bacterial growth, physiology and during development (for a review see reference 287). On the other hand, in *Bacillus subtilis,* the transcription of several genes from peptidoglycan biosynthesis can be induced by alterations in WTAs production (57).

In *S. aureus*, several functional roles have been attributed to WTAs, namely: i) host colonization; involvement of WTAs in bacterial adhesion to endothelial cells (363); ii) coordination of peptidoglycan synthesis; WTAs control the level of secondary cross-linking by temporally and spatially

regulating the recruitment of PBP4 to the site of cell-wall synthesis, the division septum (14); iii) β-lactam resistance mechanism; the impairment of *tarO*, which encodes a glycosyl transferase responsible for the first enzymatic step in WTAs biosynthesis (319), caused a decrease in β-lactam resistance level (39, 209); and iv) autolysis regulation; WTAs are important for the proper localization of Atl, the major staphylococcal autolysin, at the cross-wall where it performs the last step of cell division, namely the separation of daughter cells (305).

The recent discoveries regarding the role of TAs in essential cellular processes in *S. aureus*, have made the biosynthesis of these polymers as a promising antibacterial target. Actually, early results in targeting TAs biosynthesis are encouraging, and both synthetic and modifying enzymes are validated antibiotic targets in a variety of animal models (for a review see reference 260).

2.2. *S. aureus* peptidoglycan structure

The peptidoglycan (murein) sacculus is a unique and essential structural macromolecule that encompasses the entire bacterial cell, providing strength to resist the high internal osmotic pressure and maintaining cell shape (226, 241, 353). Also, it functions as a scaffold for anchoring other cell envelope components such as proteins (77) and teichoic acids (243), as was mentioned in section 2.1. This polymer consists of alternating disaccharide units, composed of *N*-acetyl-glucosamine (Glc*N*Ac) and *N*-acetyl-muramic acid (Mur*N*Ac) linked by β-1,4 glycosidic bonds, to which pentapeptide chains are attached (L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala) through the lactyl moiety of Mur*N*Ac. In *S. aureus*, most of the pentapeptide chains of the adjacent macromolecules are interlinked by pentaglycine bridges between the penultimate D-alanine of one peptide chain and the

free amino group of the L-lysine residue of an adjacent chain, accompanied by the release of the last D-alanine (Figure 1). It has been estimated that only about 20% of the terminal D-alanyl-D-alanine residues remain intact (356).

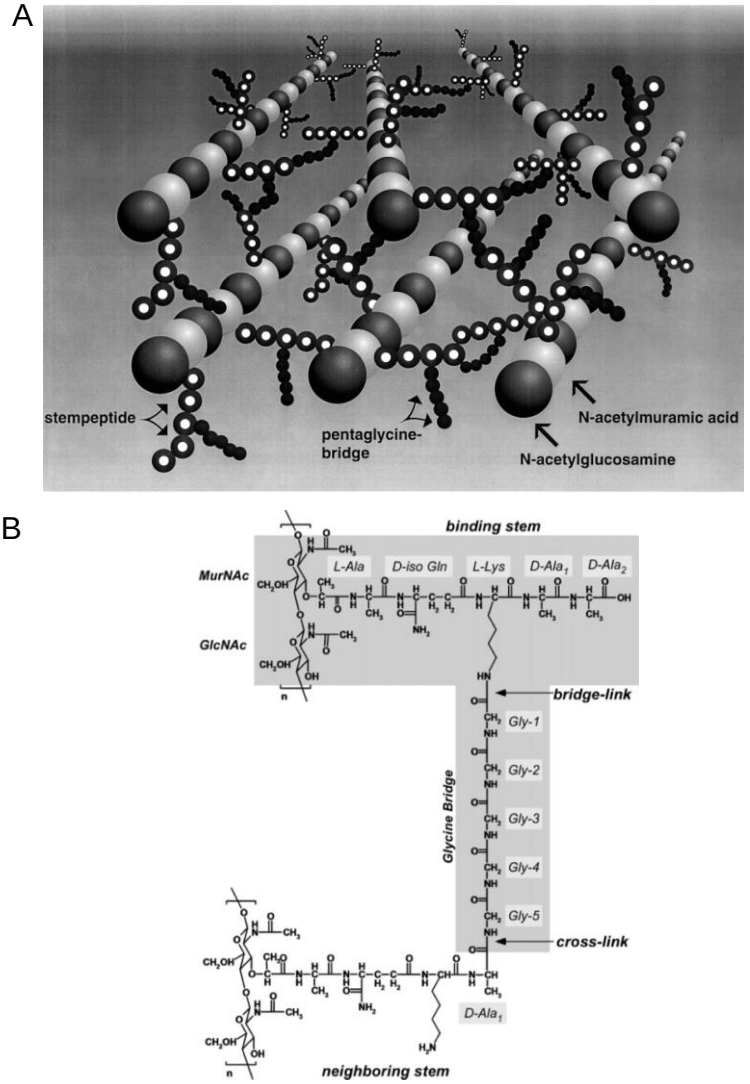

Figure 1. Structure of *S. aureus* peptidoglycan. (A) Schematic representation of the mesh-like structure of *S. aureus* peptidoglycan (adapted from reference 335); (B) Structure of a disaccharide composed of *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) attached to a pentapeptide, which is cross-linked to a second peptidoglycan molecule by a pentaglycine bridge (adapted from reference 170).

The peptidoglycan of Gram-positive and Gram-negative bacteria shows some diversity, regarding chemical structure. The most common variations are the presence of unmodified D-glutamate amino acid in the position 2 and meso-diaminopimelate residue in the position 3 of the pentapeptide structure of Gram-negative bacteria, while Gram-positive bacteria have D-iso-glutamine and L-lysine in the positions 2 and 3, respectively. Another important feature of the peptidoglycan of Gram-positive bacteria is the presence of an interpeptide bridge that is used in the cross-linking reaction, which is commonly absent in Gram-negative bacteria where the pentapeptides are directly crosslinked. The structure of this interpeptide bridge is extremely diverse in size and sequence of amino acids among bacterial species. In *S. aureus*, the interpeptide bridge is composed of five glycine residues that are added at the third amino acid (L-lysine) of the stem peptide (352).

Identification of the chemical structure of *S. aureus* peptidoglycan were performed through the analysis of the muropeptide profile by reverse-phase high performance liquid chromatography (RP-HPLC), combined with mass spectrometric analysis. About 21 distinct peaks and a “hump” of unresolved material, corresponding to oligomers with high degree of cross-linking, were identified when *S. aureus* peptidoglycan was digested with a muramidase (62) (Figure 2). The mass spectrometry analysis of these peaks revealed the existence of monomers (disaccharide pentapeptides that represent 13% of *S. aureus* muropeptides), dimers (two monomeric structures cross-linked that correspond to 20%), trimers to enneamers (40%) and even higher oligomers account for an additional 15% of the muropeptides units.

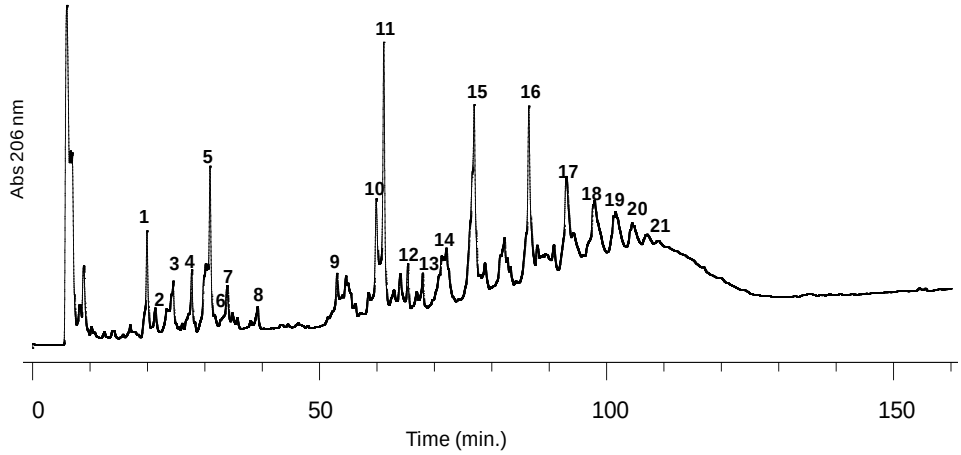

Figure 2. ***S. aureus*** **peptidoglycan composition**. Separation of *S. aureus* cell wall muropeptides by reverse-phase HPLC. Peptidoglycan of methicillin resistant *S. aureus* strain COL was isolated, digested with muramidase and the resulting muropeptides were separated by reverse-phase HPLC (adapted from reference 62).

2.3. *S. aureus* peptidoglycan biosynthesis pathway

In the last half century, the peptidoglycan biosynthesis pathway has been extensively studied, partly for its role as an antimicrobial target. The resolution of this pathway started with the pioneering work of Park and Johnson, who first reported the impact of penicillin on cell wall (259), followed by the description of specific steps of the peptidoglycan biosynthesis pathway by Strominger and collaborators (146-150).

Peptidoglycan biosynthesis is a complex process that takes place in three stages at three different cellular compartments. The biosynthesis begins in the cytoplasm where the soluble nucleotide precursors, the UDP-Mur*N*Ac-pentapeptide precursor and the UDP-Glc*N*Ac are synthesized. The second stage takes place at the cytoplasmic membrane, and consists in the transfer of the UDP-*N*-acetylmuramyl-pentapeptide to the membrane acceptor bactoprenol, leading to the synthesis of lipid I. The subsequent addition of Glc*N*Ac from UDP-Glc*N*Ac produces lipid II. The stage II also includes synthesis of the interpeptide crossbridge, which constitutes a

distinctive structure of *S. aureus* peptidoglycan. The lipid II is then translocated from the cytoplasmic face to the external face of the membrane, where it is incorporated into nascent peptidoglycan by penicillin-binding proteins (PBPs). In the stage III, the last steps of biosynthesis take place, which include the assembly of peptidoglycan through transglycosylation and transpeptidation reactions catalyzed by PBPs (Figure 3).

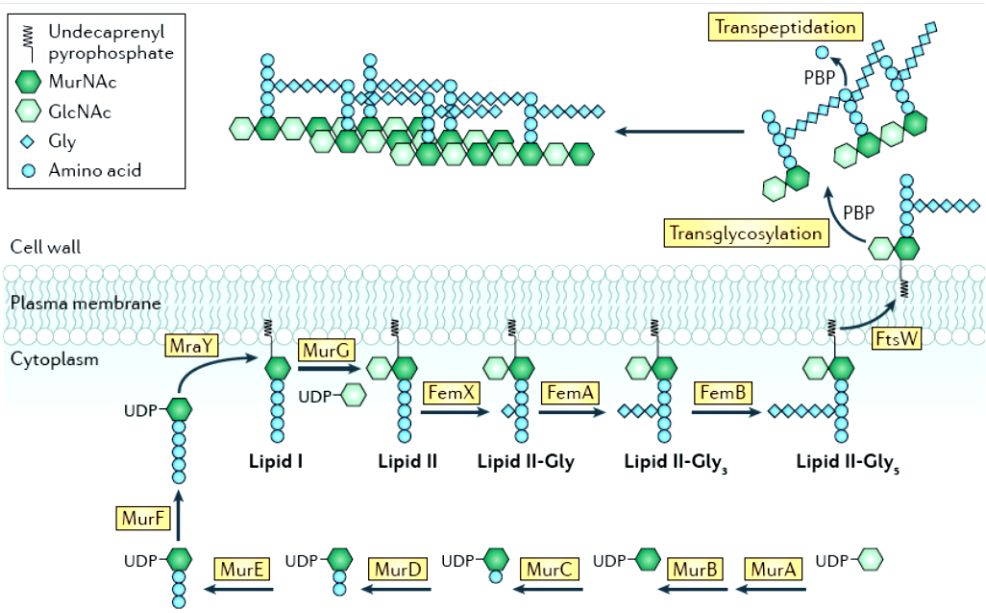

Figure 3. **The peptidoglycan biosynthesis occurs in three different locations in the cell**. In a first stage, which occurs in the cytoplasm, the peptidoglycan monomer is produced. The second stage takes place at the cytoplasmic membrane, where the UDP-Mur*N*Ac-pentapeptide precursor is linked to a membrane associated lipid carrier resulting in the formation of lipid I, which is subsequently added to Glc*N*Ac forming lipid II. The stage three includes the translocation of Lipid II from the cytoplasmic face to the external face of the membrane, where it is incorporated into nascent peptidoglycan by transglycosylation and transpeptidation reactions (adapted from reference 275).

2.3.1. Stage I: synthesis of the cytoplasmic precursor

The cytoplasmic steps that result in the formation of the peptidoglycan monomer include the following reactions: formation of UDP-Glc*N*Ac,

conversion of UDP-Glc*N*Ac into UDP-Mur*N*Ac, assembly of the stem peptide leading to UDP-Mur*N*Ac-pentapeptide (15).

Formation of UDP-*N*-acetyl-glucosamine (UDP-Glc*N*Ac). UDP-Glc*N*Ac biosynthesis from fructose-6-phosphate requires four successive enzyme activities. The first reaction, the conversion of D-fructose-6-phosphate into glucosamine-6-phosphate is catalyzed by the glucosamine-6-phosphate amidotransferase, GlmS. Then, GlmM, a phosphoglucosamine mutase, catalyses the isomerization of glucosamine-6-phosphate to glucosamine-1-P (Glc-1-P), which is modified sequentially to UDP-*N*-acetylglucosamine by acetylation and uridylation through the action of a bifunctional enzyme, GlmU. The C-terminal domain of GlmU acts as an acetyltransferase, transferring an acetyl group from acetyl-CoA to Glc-1-P yielding N-acetyl-glucosamine-1-phosphate (Glc*N*Ac-1-P), and the N-terminal domain works as an uridyltransferase, catalyzing the transfer of an uridyl group from UTP to Glc*N*Ac-1-P, which results in the formation of UDP-Glc*N*Ac (Figure 4).

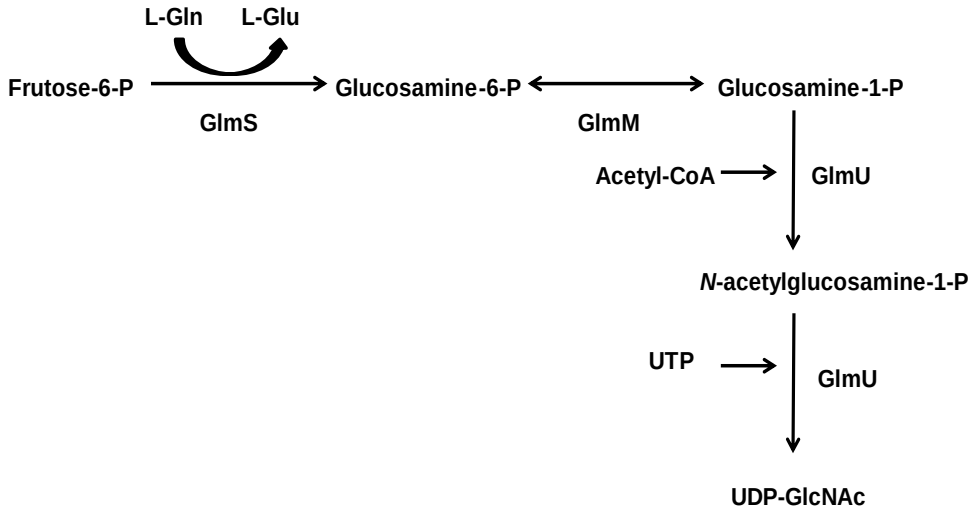

Figure 4. Formation of UDP-Glc*N*Ac. Formation of UDP-Glc*N*Ac from a fructose-6-P molecule, through the sequential catalytic action of GlmS, GlmM and GlmU enzymes.

Formation of UDP-*N*-acetyl-muramic acid (UDP-Mur*N*Ac). The formation of UDP-Mur*N*Ac from UDP-Glc*N*Ac occurs in a two-step process. First, MurA catalyses the transfer of the enolpyruvyl moiety from phosphoenolpyruvate to the 3'-hydroxyl of UDP-Glc*N*Ac, resulting in the formation of UDP-Glc*N*Ac-enolenol. Then, the enolpyruvyl moiety undergoes a reduction catalysed by MurB, yielding UDP-Mur*N*Ac. These two reactions are the first ones that occur specifically for the peptidoglycan synthesis.

Formation of the UDP-Mur*N*Ac-pentapeptide. The UDP-Mur*N*Ac-pentapeptide is formed through the stepwise assembly of five amino acids to the UDP-Mur*N*Ac. These successive reactions are ensured by four specific and essential enzymes, known as the Mur ligases enzymes (MurC, D, E and F) (316). These proteins catalyse the addition of L-alanine (MurC), D-glutamic acid (MurD), L-lysine (MurE) and dipeptide D-alanyl-D-alanine (MurF) onto the D-lactoyl group of UDP-Mur*N*Ac (Figure 5).

Biochemical studies regarding the mechanism of action of the Mur ligases have revealed that these enzymes share some characteristics: i) they share the same type of enzymatic reaction, consisting in the activation of the carboxyl group of the UDP precursor by ATP, generating an acyl phosphate intermediate and ADP, and in the nucleophilic attack by the amino group of the condensing amino acid or dipeptide, which removes the phosphate and forms a new amide bond (5); ii) they show a series of six invariant amino acid residues in addition to an ATP-binding consensus sequence, a characteristics that led to the definition of the Mur ligases as a new family of enzymes (32, 85); iii) they also share a similar three-dimensional structure based on the occurrence of three domains, a N-terminal domain primarily responsible for binding the UDP-Mur*N*Ac substrate, a central domain

involved in the binding of ATP and a C-terminal domain, associated with binding of the incoming amino acid (26, 310).

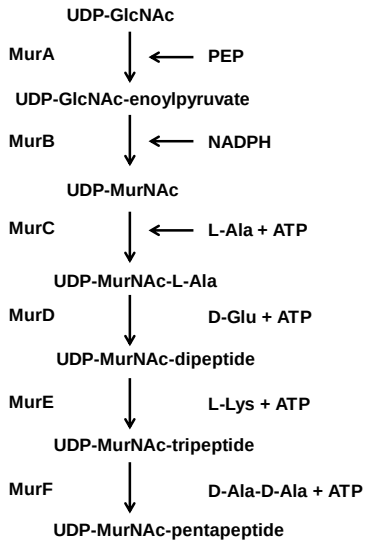

Figure 5. Formation of UDP-Mur*N*Ac-pentapeptide. Formation of UDP-Mur*N*Ac-pentapeptide by a series of consecutive peptidoglycan synthesis committed steps, which occurs in the cytoplasm.

Formation of D-alanyl-D-alanine. To synthesize the D-alanyl-D-alanine dipeptide, L-alanine is converted to D-alanine by the alanine racemase Alr. Then, the two D-alanine residues are dimerized through the action of DdlA ligase with the consumption of an ATP molecule.

2.3.2. Stage II: synthesis of peptidoglycan lipid-linked intermediates

Formation of the lipid intermediates. Once completely synthesized, the pentapeptide precursor has to be translocated across the cytoplasmic membrane in order to reach the cell wall, where the polymerization of the peptidoglycan occurs. This process begins with the transfer of the muramyl-pentapeptide from UDP-Mur*N*Ac-pentapeptide to the undecaprenyl-phosphate (or bactoprenol), a lipid carrier molecule embedded within the membrane, resulting in the synthesis of the Mur*N*ac(pentapeptide)-

phosphate-undecaprenol, also designated lipid I (124). This transfer is catalysed by the UDP-Mur*N*Ac-pentapeptide phosphotransferase, MraY (33, 143). Then, the Glc*N*ac, from the UDP-Glc*N*ac precursor, is transfered to lipid I to form a β-1,4 glycosidic bond, through the activity of a *N*-acetylglucosaminyltransferase, MurG, yielding the second lipid intermediate, lipid II.

Formation of the glycine bridge. As mentioned before, in the particular case of *S. aureus*, a peptide crossbridge composed of five glycines, connects the L-lysine of the stem peptide of one peptidoglycan strand to the D-alanine in position 4 of the adjacent strand. This bridge is formed in a sequential order by the activity of specific peptidyltransferases: FemX, adds the first glycine (296), FemA adds the glycines at the position 2 and 3 (206, 323), and FemB is responsible for the addition of the last two glycines (Figure 6). Since these enzymes require glycyl-tRNAs as glycine donors (216), which are only present in the cytoplasm membrane, the synthesis of the pentaglycine bridge must occur in the inner side of the cytoplasmic membrane, after the formation of Lipid II, the glycines acceptor structure (306). This pentaglycine structure is of great importance for *S. aureus*; besides conferring a high degree of cross-linking to peptidoglycan, it also plays a crucial role in the cell division and level of expression of methicillin resistance (127, 206).

Amidation of D-glutamate. Siewert and collaborators concluded that the D-glutamate at position 2, of the *S. aureus* stem peptide, is modified by amidation of its α-carboxyl group at the stage of the lipid-linked intermediate (313). The same study showed that L-glutamine or NH_3 is required as an amino donor and that the reaction requires ATP. Later, the analysis of Tn*551* insertion mutants (see section 5.3) allowed to conclude that the amidation of D-glutamate is important for the optimal expression of

methicillin resistance (252) and that the reaction requires the expression of glutamine synthetase (120).

The identification of the genes responsible for the D-glutamate amidation, and its impact on important biological processes in S. aureus are addressed in this Thesis.

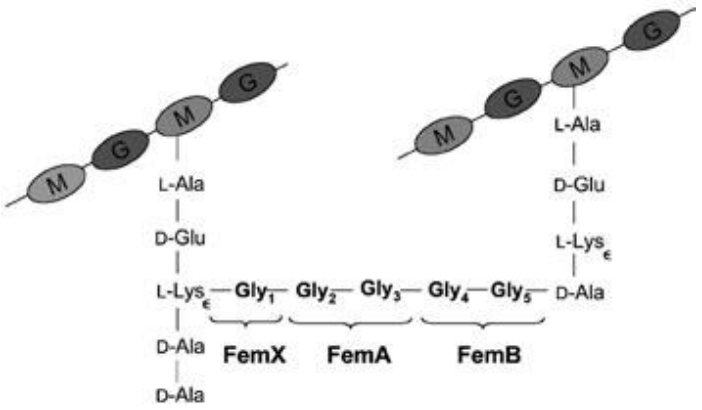

Figure 6. Three enzymes, FemX, FemA and FemB catalyse the addition of specific residues to form the *S. aureus* pentaglycine interpeptide. M: *N*-acetyl-Muramic acid; N: *N*-acetyl-Glucosamine (reproduced from reference 306).

Translocation of lipid II. The translocation of the peptidoglycan monomer to the external surface of the cytoplasmic membrane does not occur spontaneously (36, 345). In *Escherichia coli*, lipid II is translocated from the inner to the outer side of the cytoplasmic membrane by the lipid II flippase FtsW localized in the septum (231, 359). This integral membrane protein forms a size-restricted pore-like structure, which accommodates lipid II during transport across the cytoplasmic membrane, indicating that FtsW is specific for the catalysis of lipid II translocation (230). Although in *S. aureus*, the protein responsible for this process is not identified, it is likely that lipid II is translocated at the septum by a similar mechanism, as two homologues of FtsW were identified in this species.

2.3.3. Stage III: polymerization of peptidoglycan

The last stage of peptidoglycan synthesis consists of the polymerization of the monomer unit at the outside surface of the cytoplasmic membrane, through the concerted action of peptidoglycan synthetases and hydrolases.

2.3.3.1. Penicillin binding proteins (PBPs)

The PBPs catalyze the polymerization of the glycan strands (transglycosylation) and the cross-linking between peptide chains (transpeptidation). These enzymes can also hydrolyze the last D-alanine of stem pentapeptides (DD-carboxypeptidation) or cleave the cross-bridge connecting two glycan strands (endopeptidation). PBPs have been divided into two main categories: the high molecular mass (HMM) and the low molecular mass PBPs (LMM) (113).

The group of HMM PBPs is composed of multimodular proteins, and includes the major enzymes responsible for peptidoglycan polymerization and insertion into pre-existing cell envelope. Their topology consists of a cytoplasmic tail, a transmembrane anchor and two specific domains: a C-terminal penicillin-binding domain and a N-terminal domain (113). Accordingly to the structure and the catalytic function of the N-terminal domain, the HMM belong either to class A or to class B PBPs. Both classes have a C-terminal penicillin-binding domain, responsible for the transpeptidase reaction and for β-lactam binding. However, in class A HMM-PBPs, the N-terminal domain is responsible for their glycosyltransferase activity, catalyzing the elongation of uncross-linked glycan chains, whereas in class B the N-terminal is presumably associated with cell morphogenesis by interacting with other proteins involved in the cell cycle (69, 135, 376). Surprisingly, in some Gram-positive bacteria including *B. subtilis* (222), *Enterococcus faecalis* (10), and *Enterococcus*

faecium (290), it appears that these bifunctional PBPs are not essential, since strains lacking all class A PBPs are viable and have only minor differences in peptidoglycan composition. However, in *Streptococcus pneumoniae*, the deletion of class A PBP1a and class A PBP2a was lethal (139). In *S. aureus,* the PBP2 that is the only PBP of class A, is essential for viability in methicillin-susceptible strains but not to methicillin-resistant strains (273, 276).

The LMM PBPs are single-domain proteins that can catalyse both DD-carboxypeptidase and transpeptidase reactions (107).

Multiple PBPs are present in all peptidoglycan-containing bacteria, whose number varies regarding bacterial species. It seems that the number of PBPs is related with the morphology and complexity of the life cycle of the respective organism. The rod-shaped bacteria *E. coli* and *B. subtilis* have 12 (three class A, two class B and seven LMM PBPs) and 16 PBPs (four class A, six class B and six LMM PBPs), respectively; while coccoid bacteria *S. pneumoniae* and *S. aureus* have six (three class A, two class B and one LMW PBPs) and four PBPs (one class A, two class B and one LMW PBPs), respectively.

DD-transpeptidation reaction. This reaction is responsible for the cross-linking between stem peptides of different glycan strands. This process begins with the disruption of D-alanyl-D-alanine bond of the pentapeptide unit due to the nucleophilic attack of the hydroxyl group of the active serine, present in the penicillin binding domain, on D-alanyl-D-alanine bond, resulting in the formation of a serine ester-linked peptidyl enzyme intermediate. This intermediate structure is resolved by transferring the peptidyl moiety to the side chain amino group of the L-lysine residue of another peptide (107).

DD-carboxypeptidation reaction. This reaction cleaves the terminal D-alanyl-D-alanine of the peptidoglycan unit, preventing in this way that transpeptidation occurs with that particular peptide molecule. The carboxypeptidases are responsible for the control of the cross-linking degree of peptidoglycan and of the cell division process (17). The reaction mechanism is very similar to the one described for transpeptidation. It also involves the acylation of the serine residue of the catalytic domain, resulting in the cleavage of the D-alanyl-D-alanine bond and the formation of an ester-linked acyl-enzyme complex. The difference lies on the resolution of this intermediate structure, which involves in this case the transfer of the peptidyl to a water molecule, instead of the L-lysine residue of the second stem peptide (107).

DD-endopeptidation reaction. Transpeptidation and endopeptidation can be considered reverse activities, since DD-endopeptidation cleaves cross-bridges between two glycan strands.

Transglycosylation reaction. Transglycosylation consists in the elongation of the linear glycan chains. The resolution of the crystal structure of PBP2 in complex with moenomycin (structural analog of transglycosylase substrate lipid II) was crucial for the clarification of the transglycosylation mechanism. It was proposed that transglycosylase interacts both with the growing chain (donor) and lipid II (acceptor) (202). The growing glycan chain attached to the membrane lipid carrier (PP-undecaprenyl) acts as glycosyl donor and is transferred to the Glc*N*Ac moiety of lipid II with the formation of a new β-1,4 glycosidic bond (375).

2.3.3.2. Monofunctional glycosyltransferases (Mgts)

Besides the transglycosylase activity provided by the class A PBPs, bacteria may also express membrane-bound enzymes composed by a

single transglycosylase domain, capable of catalyzing only the formation of uncross-linked peptidoglycan, known as monofunctional glycosyltransferases (Mgts) (53, 71, 321, 360). Interestingly, in *B. subtilis* and *Enterococcus* spp., no Mgts were identified so far (10, 182, 290), which suggest that in both species, transglycosylases are not required for survival or that additional enzymes with transglycosylase activity are yet to be identified. Regarding *S. aureus*, significant advances have been made in order to determine the exact role of Mgts in transglycosylase activity and in expression of β-lactam resistance in this organism. In fact, *in vitro* transglycosylase activity of two Mgts was reported in *S. aureus*, MGT and SgtA (286, 332, 360). Although MGT and SgtA do not seem essential, in the absence of PBP2, the transglycosylase activity of MGT is crucial for cell viability. This indicated that either PBP2 or MGT is the sole peptidoglycan transglycosylase in *S. aureus* (286).

2.3.3.3. PBPs in *S. aureus*

As mentioned before, *S. aureus* has four native PBPs, including one bifunctional class A HMM PBP (PBP2), two class B HMM PBPs (PBP1 and PBP3) and one LMM PBP (PBP4) (106). PBP1 and PBP2 are both essential in MSSA, while PBP3 and PBP4 are not (274, 276, 355). MRSA strains have acquired an extra class B HMM PBP (PBP2A) which has low affinity to β-lactams antibiotics, being primarly responsible for the expression of β-lactam resistance (see section 5.2.4 and 5.3).

PBP2 is the major peptidoglycan transpeptidase and also the most abundant among the native PBPs, being the unique bifunctional PBP present in *S. aureus* (105). PBP2 is only essential in MSSA, since in MRSA the transpeptidase activity of PBP2 is complemented by PBP2A (276). Nevertheless, the PBP2 transglycosylase domain, although not essential, is

required for the expression of β-lactam resistance, as it cooperates with the transpeptidase domain of PBP2A to sustain cell wall biosynthesis, in the presence of a β-lactam (273) (see section 5.2.4 and 5.3).

PBP1 was shown to be essential in MSSA and MRSA strains (263, 355), and to be intimately related with the mechanism of cell division, playing an important role in septum's formation (263). Interestingly, in contrast to PBP2 (276), the essential function of PBP1 could be not replaced by PBP2A (263), suggesting that the essential role of PBP1 in cell division is not dependent of its transpeptidase activity. PBP1 was proposed to have a dual role in cell cycle of *S. aureus*: it is an essential protein in the initiation of the septation and also it acts as a transpeptidase to generate a critical signal for cell separation at the end of cell division (264).

PBP3 is the less studied staphylococcal PBP and although it was suggested to be involved in septation (249), its exact role remains to be determined.

PBP4, the only LMM-PBP, was characterized to have both DD-carboxipeptidase and transpeptidase activities *in vitro* (180). Later, PBP4 was described as being directly involved in secondary cross-linking of the peptidoglycan (128, 373). It seems that PBP2 and PBP4 cooperate in order to produce the high cross-linking level that characterizes *S. aureus*. This process starts with the primary cross-linking, catalysed by PBP2, which results in the formation of dimeric, trimeric, and tetrameric muropeptides. Then, these muropeptides constitute the substrate of PBP4, with the subsequent formation of more complex oligomers. On the other hand, it was also shown that PBP4 plays a crucial role the mechanism of β-lactam resistance in community-acquired MRSA (CA-MRSA) (225).

Regarding their cellular localization, PBP1, PBP2 and PBP4 localize at the septum (14, 263, 271), which is consistent with the fact that peptidoglycan synthesis occurs only at this site (271). So far, the localization of PBP3 remains unknown. The recruitment of each PBP to the division site occurs by different mechanisms. Since PBP1 is essential for cell division through a mechanism that does not require its transpeptidase activity, it seems that this PBP is part of the divisome and, probably, is recruited to the division site by an unidentified divisome protein through a mechanism that does not require the PBP transpeptidase domain (263, 264). Regarding PBP2, the delocalization of this protein can be observed when its substrate (lipid II) is eliminated, suggesting that PBP2 is recruited to the division site after binding to lipid II (272). Interestingly, PBP2 becomes dispersed over the cell surface, if its transpeptidase active site is acylated. However, in the presence of PBP2A, the lack of transpeptidase activity does not result in delocalization of PBP2, indicating that acylated PBP2 can be maintained in place by functional PBP2A (272). The recruitment of PBP4 to the septum is regulated by an unidentified intermediate of WTAs synthesis (14). As WTAs are attached to peptidoglycan, its synthesis probably occurs at the division septum after peptidoglycan assembly has been initiated by PBP1 and PBP2. Thus, it most likely that PBP4 is recruited to the septum later than PBP1 and PBP2, in order to allow the incorporation of polysaccharides and proteins into the cell wall, which may be hampered if peptidoglycan were to become highly cross-linked at an earlier stage (14).

2.3.3.4. Peptidoglycan hydrolases

In order to allow the incorporation of new murein units and also to promote the separation of the two daughter cells, old peptidoglycan must be degraded and removed by the action of peptidoglycan hydrolases such as *N*-acetylglucosaminidases, *N*-acetylmuramidases, *N*-acetylmuramyl-L-

alanine amidases, lytic transglycosylases and endopeptidases (Figure 7). Importantly, these enzymes are also associated with cell wall turnover, lysis induced by cell wall synthesis inhibitors, establishment of competence for genetic transformation and bacterial pathogenicity processes (for a review see reference 136).

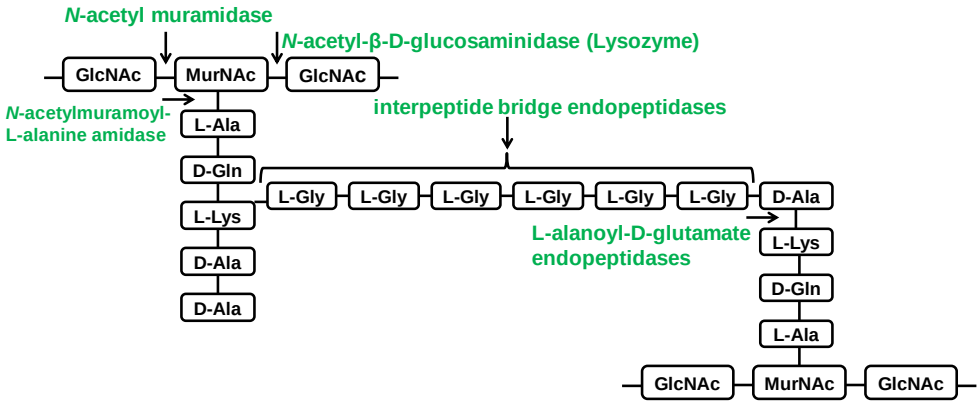

Figure 7. Murein hydrolase targets within *S. aureus* peptidoglycan. The peptidoglycan is composed of interlinked glycan chains containing alternating subunits of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). Pentapeptide chains, attached to Mur*N*Ac, are cross-linked to stem peptides from adjacent muropeptide strands as indicated. Different murein hydrolases that cleave the various bonds within peptidoglycan have been identified and include *N*-acetyl muramidase, *N*-acetyl-β-D-glucosaminidase (Lysozyme), *N*-acetylmuramoyl- L-alanine amidase, L-alanoyl-D-glutamate endopeptidases, and interpeptide bridge endopeptidases (adapted from reference 327).

The analysis of the pattern of autolytic enzymes in *S. aureus,* obtained by zymograme assays revealed the presence of more than 20 bacteriolytic bands, suggesting that *S. aureus* produces several peptidoglycan hydrolases (324). Although at least 13 genes were identified as responsible for the expression of known or putative peptidoglycan hydrolases, only three genes, *atl*, *sleI*, and *lytM*, and their products have been characterized. Atl, the major staphylococcal autolysin, is a bifunctional protein initially synthesized as a 138 kDa protein that undergoes proteolytic cleavage to produce two independent hydrolases, a 62-kDa *N*-acetylmuramyl-L-alanine amidase and a 51-kDa *N*-acetylglucosaminidase (176, 254). Atl is important

for separation of daughter cells after cell division, cell wall recycling, and for biofilm formation (27, 31, 126, 328). Both amidase and glucosaminidase proteins localize at the equatorial ring on the staphylococcal cell surface that marks the future cell division site (374). Curiously, the mechanism by which Atl-hydrolases are targeted to the equatorial surface ring is based on an avoidance strategy by WTAs, which prevents binding of Atl. As WTAs are abundant in the old cell wall but not at the cross-wall region, Atl is able to bind to this region (305). Sle1 is a 32 kDa protein with *N*-acetylmuramyl-L-alanine activity and is also involved in cell separation after division in *S. aureus* (126). In fact, Sle1 together with Atl were considered the main, if not the only, lytic enzymes responsible for cell separation after division in *S. aureus* (161). LytM is a 32 kDa protein with glycylglycine endopeptidase activity, being able to hydrolyze the glycyl-glycine bonds of *S. aureus* cross-bridges. It appears that LytM plays a role in cell growth as it is distributed uniformly on the cell surface (282, 283). Other peptidoglycan hydrolases with *N*-acetyl-muramyl-L-alanine amidase activity were described in *S. aureus* including LytH (33 kDa), LytA (23 kDa) and LytN (46 kDa). Importantly, despite their role in cell separation, the inactivation of any of these genes and respective protein products, or even the deletion of both genes, is not lethal for the cell, suggesting that other genes with similar functions should exist (161).

Since many of these enzymes are able to cause cell wall disruption, their activities and expression must be strongly regulated to avoid undesired lysis. In *S. aureus*, the regulation of autolytic activity involves several two-component signal transduction systems and global regulators. The autolysis in *S. aureus* can be negatively controlled by MgrA (144, 205), ArlRS (195), LytSR (118), and SarA (100, 210). On the other hand, *walkr* (78), *agr* (100) and *cidABC* (289) are able to regulate positively the autolytic activity. Additionally, it was shown that perturbations of *S. aureus* cell wall

synthesis resulted in a strong transcriptional repression of several peptidoglycan hydrolases, which provided evidence for close regulation between cell wall synthetic and hydrolytic enzymes (8).

2.3.3.4.1. Lysozyme: the host *N*-acetylmuramidase

Lysozyme, a powerful antibacterial protein, is among the most and best-studied enzymes. It is present in a large number of organisms and various biological fluids such as tears, breast milk, respiratory and saliva secretions, as well as in cells of the innate immune system including neutrophils, monocytes, macrophages, and epithelial cells, being involved in the initial host defensive response against bacterial infection (191).

The protective role of lysozyme for opportunistic pathogens was demonstrated in a study wherein killing of group B streptococci and *Pseudomonas aeruginosa* was enhanced by over- expression of lysozyme in the lungs of transgenic mice (4). On the other hand, deletion of both genes that encode two important lysozymes in mice resulted in a defective clearance of *P. aeruginosa* and *Klebsiella pneumoniae* from the mice lower airways (47).

In fact, lysozyme is an important player in the host's response against invading microorganisms, since it targets the bacterial cell wall and is associated with the removal of high molecular weight peptidoglycan fragments that remain after cell lysis. The persistence of these fragments, within the cell, may often cause physiological problems in the host, even after the majority of the viable bacteria have been destroyed (29; for a review see reference 353). Beside its antimicrobial activity, lysozyme has other important functions: it can be an enhancer of the phagocytic activity of polymorphonuclear leukocytes and macrophages (174, 333), and can stimulate proliferation and antitumor functions of monocytes (190).

The ability of lysozyme to cause bacterial lysis relies on its *N*-acetylmuramidase activity since it hydrolyses the β-1,4 glycosidic bonds between Mur*N*Ac and Glc*N*Ac residues of peptidoglycan, resulting in its degradation and consequently causes the lysis of bacterial cells (269, 304).

Most pathogenic bacteria are resistant to lysozyme, such as *S. pneumoniae*, *Streptococcus suis*, *Listeria monocytogenes*, *S. aureus*, *Neisseria gonorrhoeae, E. faecalis,* and *Helicobacter pylori*. The mechanisms of resistance to lysozyme are mainly due to peptidoglycan modifications, which hamper recognition by the enzyme. In the particular case of Gram negative bacteria, they are generally resistant to lysozyme because their outer membrane prevents access of the secreted enzyme to the peptidoglycan. The modifications of cell wall associated with lysozyme resistance are described in section 3.

Importantly, lysozyme has two different antimicrobial activities. Besides the muramidase activity, lysozyme can also act as a cationic antimicrobial peptide (CAMP), which results in its ability to cause disruption of the cytoplasmic membrane. The CAMP activity of lysozyme was demonstrated using catalytically inactivated lysozyme, peptides isolated from digested lysozyme, and synthetic lysozyme-derived peptides (79, 141, 142, 184). *S. aureus* has two different resistance mechanisms to lysozyme, which are related to the muramidase and CAMPs activities of lysozyme (129). Both mechanisms will be described in section 3.

The CAMPs are present in mucous membranes and skin and are responsible for the killing capacity of phagocytic cells. They are small positively charged peptides with hydrophobic residues (189), which allow interactions with bacterial cytoplasmic membrane, that usually contains negatively charged phospholipids. In fact, membrane damage is probably the main CAMPs' mechanism of action; it involves firstly the interaction

between the cationic groups of the peptide with the anionic groups of the membrane lipids, and secondly the integration of the peptide into the hydrophobic core of the membrane. On the other hand, several authors assume that lysozyme as a CAMP is not only acting as a membrane permeabilization agent, but also it activates autolytic wall enzymes of Gram-positive bacteria thus causing cell lysis (110, 111, 362). It has been shown that the degree of D-alanylation of teichoic acids, modification that confers resistance to CAMPs action, affects the autolysis rate (46, 90, 243). In line with these findings, it was demonstrated in *S. aureus* that CAMPs activate autolytic enzymes (129).

Bacteria have developed several strategies to resist to CAMPs (for a review see reference 267). One of these mechanisms is based on modifications of their cell surfaces to reduce the net negative charge of the cell envelope and therefore reduce the affinity of CAMPs for the bacterial membrane (83, 265). The cell wall modifications involved in the resistance to CAMP activity of lysozyme will be described in the section 3.

3. Cell wall modifications

The normal glycan strands of bacterial peptidoglycan consist of alternating unmodified residues of β-1,4-linked Mur*N*Ac and Glc*N*Ac. However, there are no known examples of bacterial species that contain exclusively unmodified peptidoglycan polymerized from lipid II, as mature glycan strands become modified or linked to other polymers after their insertion into the cell wall (for a review see reference 350). Several secondary modifications were described and although their functional roles are not completely understood, their presence affects the hydrolysis of peptidoglycan and its enlargement during cell growth. Furthermore, these alterations avoid the recognition of bacteria by host factors, which

contribute to the bacteria resistance to host defense factors such as lysozyme. Actually, the occurrence of modifications in cell wall components, which leads to lysozyme resistance, are not surprising since bacteria are likely to encounter host lysozymes during infection, thus the development of resistance to these and other defense mechanisms confers a selective advantage to bacteria, being crucial for maintaining bacterial viability. The more frequent variations are in the glycan strands, that include their *N*-deacetylation and *O*-acetylation, the D-alanine esterification of teichoic acids, and the modification of membrane phospholipids with L-lysine.

***N*-deacetylation**. The presence of *N*-deacetylated glucosamine (Glc*N*) was first described in the peptidoglycan of *Bacillus cereus* (9), and soon after *N*-deacetylated muramic acid residues (Mur*N*) were identified in the *Bacillus anthracis* peptidoglycan (379). The existence of *N*-deacetylated Glc*N* was further extended to several other bacterial species including *S. pneumoniae* (248, 351), *Lactobacillus fermentum* (201) and *L. monocytogenes* (30), while *N*-deacetylated Mur*N* was identified in small quantities only in *S. pneumoniae* (351) and *Micrococcus lysodeiktikus* (138).

The gene which encodes a peptidoglycan Glc*N*Ac deacetylase, *pgdA*, was first identified in *S. pneumoniae* (351); later, several *pgdA* homologues were identified in other pathogenic species including *S. suis* (91, 92), *L. monocytogenes* (30), and *H. pylori* (358). The deacetylation reaction most likely occurs on polymerized peptidoglycan, since deacylated precursors were not detected in bacteria with deacylated peptidoglycan and also the deacetylases have a predicted extracytoplasmic localization (351).

The presence of deacetylated peptidoglycan strongly reduces the lysozyme activity, as interactions between the acetyl groups of glycan strands and amino acids of the lysozyme molecule are crucial for substrate binding (28, 349). The impact of deacetylated peptidoglycan on lysozyme activity, and

consequently on virulence of important human pathogens, has been analyzed in mouse infectious models. In *S. pneumoniae*, a *pgdA* mutant exhibited significantly reduced virulence in an intraperitoneal mouse model, indicating that PgdA is a putative virulence factor (351). Interestingly, in *S. suis* the deletion of *pgdA* caused a severe impairment in the bacteria ability to persist in the blood. It was also observed an increase of *pgdA* expression upon interaction of the bacterium with neutrophils in vivo as well as in vitro (92), which suggests that deacetylation is an adaptative response to escape the immune clearance mechanisms.

The inactivation of *pgdA* in *L. monocytogenes* revealed the key role of peptidoglycan deacetylation in virulence, as the mutant was very sensitive to the bacteriolytic activity of lysozyme and rapidly destroyed within macrophages (30). Similar findings were observed when *pgdA* homologue was inactivated in *H. pylori* (357). It seems that both lysozyme activities, the muramidase and the CAMP activities, are affected by the deacetylation of peptidoglycan, since a *L. monocytogenes pgdA* mutant was more susceptible not only to mutanolysin (a muramidase) but also to lysozyme as a CAMP (279). It was also evident that *pgdA* mutant was more prone to autolysis, suggesting that some of the peptidoglycan hydrolases (autolysins), in particular those cleaving in the glycan strands, have different functional activities against fully acetylated or deacetylated peptidoglycan. If the hydrolases' activity is affected by the state of acetylation of the peptidoglycan, the separation of the daughter cells should also be altered. However, in *S. pneumoniae* the *pgdA* deletion did not affect cell separation (351).

***O*-acetylation**. The *O*-acetylation occurs specifically at the C-6 hydroxyl group of muramoyl residues of peptidoglycan. Recently, the *O*-acetylation of Glc*N*Ac residues was described in *B. anthracis* and also in *Lactococcus plantarum* (25, 183). *O*-acetylated peptidoglycan was discovered more than

50 years ago in the cell walls of *E. faecalis* (2), and since then this modification has been found in 49 other species of bacteria, both Gram positive and Gram negative, including *S. aureus*, *B. anthracis* and *H. pylori* (19, 44). Similarly to *N*-deacetylation, peptidoglycan *O*-acetylation is known to be a maturation event, occurring within the peptidoglycan sacculus following its synthesis and assembly (44). *oatA* from *S. aureus* was the first peptidoglycan *O*-acetyltransferase gene identified (21). Meanwhile, homologues of *oatA* have been described in several other Gram positive bacteria including *S. pneumoniae* (56), *B. cereus, B. anthracis* (183), and *Lactococcus lactis* (347).

O-acetylation turns the peptidoglycan resistant to the hydrolytic activity of lysozyme (38). As the binding of peptidoglycan to lysozyme involves a large H-bonding network between the C-6 hydroxyl moieties of three Mur*N*Ac residues and amino acids from the active site of the enzyme (28), the steric hindrance caused by the *O*-acetylation of the C-6 group results in a weaker affinity of the enzyme for the modified substrate (Figure 8).

In fact, *O*-acetylation of peptidoglycan contributes to lysozyme resistance of pathogenic Gram-positive bacteria such as *S. aureus* and *S. pneumoniae,* as *oatA* mutants of both species showed more sensitivity to lysozyme (21, 56). Additionally, a correlation was found between pathogenicity, lysozyme resistance and the occurrence of *O*-acetylation in the peptidoglycan of staphylococcal species (19).

During infection, bacteria are normally lysed by the immune system, and the resulting peptidoglycan fragments rapidly degraded by hydrolytic enzymes. Since human lysozyme is unable to digest the *O*-acetylated peptidoglycan fragments, the persistence of these fragments in the host may lead to inflammation and eventually rheumatoid arthritis (94). Several studies demonstrated *in vivo* that the persistence of peptidoglycan in a host

is directly due to the high degree of *O*-acetylation (299). Therefore, it appears that, as in the case of *N*-deacetylation, *O*-acetylation prevents the recognition of peptidoglycan fragments by host factors such as peptidoglycan recognition proteins.

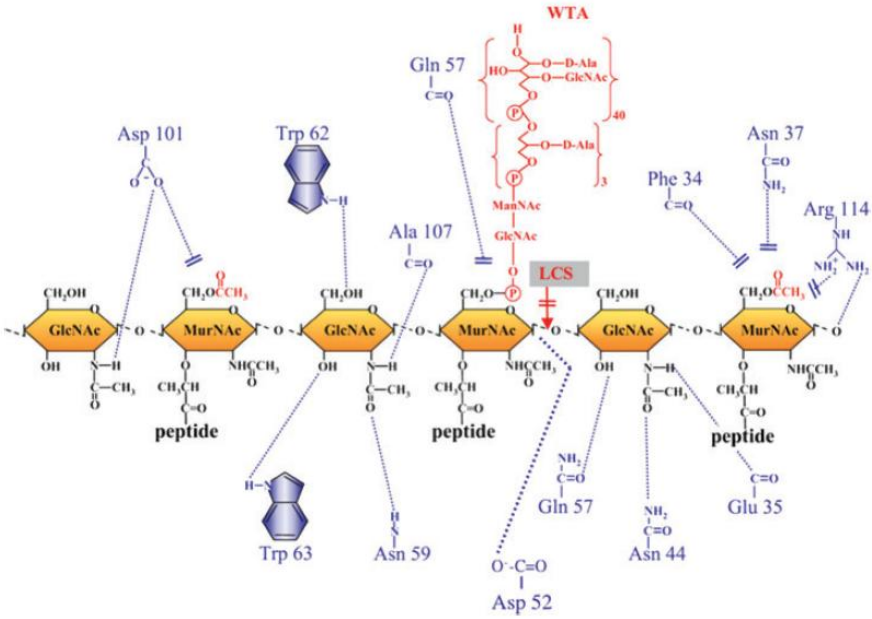

Figure 8. Proposed interaction of the aminoacids of the binding groove of the egg-white lysozyme and staphylococcal peptidoglycan, based on the structure of reference 28. The C6-OH group of muramic acid in staphylococci is modified by *O*-acetylation and serves also as an anchor for wall teichoic acid (WTA). Five amino acid residues of lysozyme binding groove (Asp101, Gln57, Phe34, Asn37 and Arg114) interact with the C6-OH group of unmodified peptidoglycan. The high degree of *O*-acetylation of staphylococcal peptidoglycan inhibits the interaction with lysozyme, thus causing lysozyme resistance. LCS, lysozyme cleavage site. (reproduced from reference 21).

Interestingly, the decrease of *O*-acetylation degree upon treatment with penicillin in *S. aureus* (312), suggested that *O*-acetylation should be related with the cross-linking reaction. Although the mechanism is not clarified, peptidoglycan containing pentapeptides was proposed to be a poor substrate for *O*-acetylation. It was also reported that the presence of teichoic acid and a high degree of peptide cross-linkage in the peptidoglycan may contribute to lysozyme resistance in *S. aureus* (20).

D-Alanylation of teichoic acids. In Gram-positive bacteria, resistance to CAMPs is explained by the increase of the positive surface charge due to D-alanylation of teichoic acids, catalysed by the *dlt* operon gene products and/or incorporation of L-lysine into phosphatidylglycerol, the major membrane lipid, mediated by the *mprF* gene product (364). Deletion of the *dlt* operon leads to the complete absence of D-alanyl esters of TAs in *S. aureus* and confers sensitivity to antimicrobial peptides (266). In *S. aureus*, its high levels of lysozyme resistance are not only based on resistance to its muramidase activity, due to the occurrence of *O*-acetylation, but also to its inherent CAMP resistance, which is related to D-alanylation (129). Several studies in animal models have demonstrated that alanylated teichoic acids contribute to an increased virulence of *S. aureus*, as this modification helps the bacteria to avoid rapid killing by CAMPs from the host immune system (51, 314, 365).

4. Antibiotic resistance in *S. aureus*

S. aureus has always been a challenge for antimicrobial chemotherapy, due to its remarkable capacity to acquire antibiotic resistance mechanisms, overcoming all therapeutic agents that have been developed in the last 70 years.

Before the antibiotic era, the mortality rate of patients with *S. aureus* invasive infections was extremely high, exceeding 80%, and over 70% developed in metastatic infections (315). In the early 1940s, the discovery of penicillin (93), the first cell wall targeting β-lactam antibiotic, significantly improved the prognosis of patients with staphylococcal infections, with over 94% of strains exhibiting susceptibility (223). However, shortly after the introduction of penicillin into clinical use, penicillin-resistant *S. aureus* expressing and secreting a β-lactamase were isolated (1, 284). In the 1960s, more than 80% of hospital and community-acquired staphylococcal

isolates were resistant to penicillin (300) and currently, more than 90% of staphylococcal isolates produce β-lactamases (203). The pattern of resistance, consisting in the emergence of resistance in the hospitals that can then spread into the community, is presently a well-established pattern that occurs with each new antibacterial (42, 203).

In 1960, in order to overcome the problem of penicillin-resistant *S. aureus* strains, the first semi-synthetic penicillin resistant to β-lactamases, methicillin, was introduced in clinical practice (297). However, two years later, *S. aureus* resistant to this antibiotic were isolated (156). These strains, designated as methicillin resistant *S. aureus* (MRSA), have acquired the *mecA* gene, which encodes PBP2A, an extra PBP with low affinity for β-lactam antibiotics (37, 123, 288). In contrast to penicillin resistant *S. aureus*, MRSA show resistance to the entire class of β-lactam antibiotics including penicillins, cephalosporins and carbapenems. Multidrug-resistant MRSA became a serious health problem in hospitals, frequently developing resistance to virtually all antibiotics and spreading worldwide (72).

As a consequence of MRSA spread, vancomycin, a glycopeptide antibiotic that also targets the cell wall and to which MRSA were consistently susceptible, became the remaining most often therapeutic agent against this pathogen. However, the increased use of vancomycin led to the emergence, in 1996, of vancomycin resistant intermediate *S. aureus* strains (VISA) and in the 2000s highly vancomycin resistant *S. aureus* strains (VRSA) also appear in the USA and very recently in Europe as well (41, 84, 134).

In the last decade, a huge effort has been made in order to develop alternative antibiotics to treat MRSA infections (200), including inhibitors of protein synthesis (quinupristin-dalfopristin, tigecycline and linezolid) and

inhibitors of the transmembrane electrical potential gradient (daptomycin); nevertheless, all of them show specific disadvantages and resistance has already been observed for most (122, 125, 131, 339). Currently, the development of compounds that inhibit PBP2A transpeptidase activity constitute a significant progress and seems to represent a good therapeutic choice against MRSA infections (200).

Overall, the remarkable capacity of *S. aureus* to adapt and acquire resistance to the antibiotics that are being introduced into clinical use, turns this microorganism into a major public health concern, as staphylococcal infections may become untreatable in the future.

The mode of action of β-lactam antibiotics will be discussed in detail in the next section, due to its importance for the work developed in this Thesis.

5. β-lactam mode of action and resistance mechanisms

β-lactam antibiotics are the most widely used class of antimicrobial agents, mainly due to their low toxicity to humans, high efficiency, low cost and general broad-spectrum of action. They include several compounds which are divided according to their chemical structure into penicillin derivatives, cephalosporins, monobactams, carbapenems and β-lactamases inhibitors (242).

The β-lactams, which owe their name to the presence of the four member β-lactam ring in their chemical structure, are highly specific inhibitors of the PBP enzymes involved in the polymerization of cell wall, by acting as suicide substrates of the transpeptidase domain of these proteins. In fact, the bactericidal activity of these compounds depends on their structural similarly to the carboxy-terminal D-alanyl-D-alanine of the peptidoglycan precursor, the natural substrate of PBPs. Therefore, the β-lactam molecule

irreversibly binds to the serine residue of the PBPs' active site, through an acylation reaction, which results in the formation of a covalent acyl complex. The resultant acyl enzyme species are irreversibly inactived, preventing the crosslinking (transpeptidation) of the nascent peptidoglycan, and cell wall synthesis (107).

Several mechanisms have evolved in bacteria to resist β-lactam antibiotics, namely: i) degradation of the antibiotic molecule by β-lactamase enzyme (154); ii) production of low-affinity PBPs to β-lactams, which allow the transpeptidation reaction even in the presence of high concentrations of β-lactam antibiotics (104); or iii) decrease of the production of outer membrane proteins, present only in Gram-negative bacteria, which leads to reduction of the outer membrane permeability, and consequently blocks the access of the antibiotic to their cellular target (198, 322).

In *S. aureus*, resistance to β-lactams is known to occur by two mechanisms. One mechanism, conferring resistance only to penicillin, is based on the production of the β-lactamase enzyme encoded by the *bla*Z gene, which cleaves the β-lactam ring of penicillin (245, 292). The second one, conferring resistance to most β-lactams, is due to the acquisition of *mecA* gene, which encodes the low affinity PBP, PBP2A (37, 124, 288).

5.1. β-lactamase resistance mechanism

The β-lactamase resistance mechanism is the most predominant among *S. aureus*, as more than 95% of staphylococcal isolates produce β-lactamases (199, 203). The majority of β-lactamases are related to PBPs, being both considered active-site serine enzymes (98, 213). Therefore, these enzymes inactivate β-lactams by hydrolyzing the amide group of the β-lactam ring, through a mechanism similar to the one described for the inhibition of PBPs by β-lactams (98, 214, 215). However, in this resistance mechanism, β-

lactamases are able to undergo deacylation, by the hydrolysis of the β-lactam ring, inactivating in this way the antibiotic molecule and regenerating their own activity (107).

The β-lactamase BlaZ is predominantly extracellular and its expression is normally induced by the presence of β-lactam antibiotics (45, 291). The gene *blaZ*, which can be plasmid or chromosome located, is under the control of two adjacent regulatory genes, the anti-repressor *blaR1* and the repressor *blaI* (45). The signaling pathway responsible for the induction of β-lactamase synthesis is dependent of the sequential cleavage of the regulatory proteins BlaR1 and BlaI. In the absence of β-lactams, BlaI binds to the *bla* promoter region, which represses the RNA transcription from both *blaZ* and *blaRI-blaI*. In the presence of antibiotic, the β-lactam molecule binds to the extracellular part of transmembrane sensor transducer, BlaRI, which causes a conformational modification that activates its cytoplasmatic domain as a protease, and it cleaves itself. The repressor, BlaI, is then cleaved by the BlaRI protease, so that it no longer binds to the *bla* operator, thus allowing synthesis of *blaZ* mRNA and hence β-lactamase (117, 193, 378).

5.2. *mecA* gene resistance mechanism

5.2.1. *mecA* gene

The MRSA strains show high level of resistance to β-lactams due to the presence of the exogeneous *mecA* gene (2.1kb in length), which encodes PBP2A, a PBP with low affinity to all β-lactam antibiotics, allowing cell wall biosynthesis when the native PBPs have been inactivated by β-lactam antibiotics (37, 124, 288, 343). In fact, a drastic reduction in methicillin resistance was observed in mutant strains with inactivated *mecA*, strongly

suggesting that this gene is crucial for the optimal expression of methicillin resistance in *S. aureus* (217, 236).

Although *mecA* is undoubtedly the main genetic determinant for methicillin resistant in MRSA, very recently, a highly divergent *mecA* gene, designated *mecC*, was identified with relatively low prevalence rates (102, 311).

The *mecA* gene is not native to *S. aureus* but was acquired from another species by an unknown mechanism (16). According to DNA and amino acid sequence alignments, it has been proposed that the *mecA* gene of *S. aureus* may have resulted from a fusion event between a β-lactamase gene and a PBP gene (320). On the other hand, efforts to track the evolutionary origin of *mecA* led to the identification of a close homologue of the *S. aureus mecA* gene, present in all strains of *Staphylococcus sciuri* as the *pbpD* gene*. S. sciuri* is a taxonomically primitive staphylococcal species recovered most frequently from rodents and mammals (54). The purification of the protein product of *pbpD* showed that it shares a number of biochemical properties with *S. aureus* PBP2A (99). In fact, several studies have supported the proposition that *S. sciuri pbpD* may be associated and/or be an evolutionary precursor of *mecA* gene in *S. aureus*: (i) the transpeptidase domain of *S. sciuri pbpD* has 88% similarity, at the amino acid level, with the *mecA* gene of MRSA (370); (ii) introduction of the *pbpD* from a methicillin-resistant strain of *S. sciuri* into a susceptible strain of *S. aureus* resulted in an increased of β-lactam resistance and allowed continued growth and cell wall synthesis of the bacteria in the presence of high concentrations of β-lactams (307, 372) and iii) reconstruction of the methicillin resistant phenotype in an highly homogeneously resistant MRSA (lacking the SCC*mec*), using a plasmid-borne copy of the heterologous upregulated *S. sciuri pbpD* gene (7).

5.2.2. Regulation of *mecA* transcription

Until recently, it was assumed that the expression of *mecA* was regulated by a two component system consisting of a sensor-transducer, MecR1, and a transcriptional repressor, MecI (309), which are located immediately upstream of *mecA* promoter and are transcribed divergently from *mecA*. The regulation of *mecA* expression is similar to the one of *blaZ* gene, since the *mecR1-mecI-mecA* element is homologous to the *S. aureus* β-lactamase *blaR1-blaI-blaZ* genetic system (121, 309, 340). However, while MecI and BlaI repressors are almost identical and seem to be compatible in blocking transcription of both genes (117, 121, 192), MecR1 and BlaRI are specific for their own repressors (221). The MecR1/MecI system consists of two main proteolytic steps, as follows: first, the acylation of MecR1 extracellular sensor domain, through the binding of the β-lactam, leads to a conformational change, which results in the activation of the intracellular proteolytic domain. Then, the active protease induces the cleavage of the MecI repressor that is bound to *mecA* promoter region, allowing for *mecA* to be transcribed (11, 378). This model was recently modified with the identification of a third gene responsible for the induction of *mecA* transcription, designated *mecR2* gene. The cleavage of MecI, which is essential for *mecA* transcription, can be also promoted by MecR2 (12) (Figure 9).

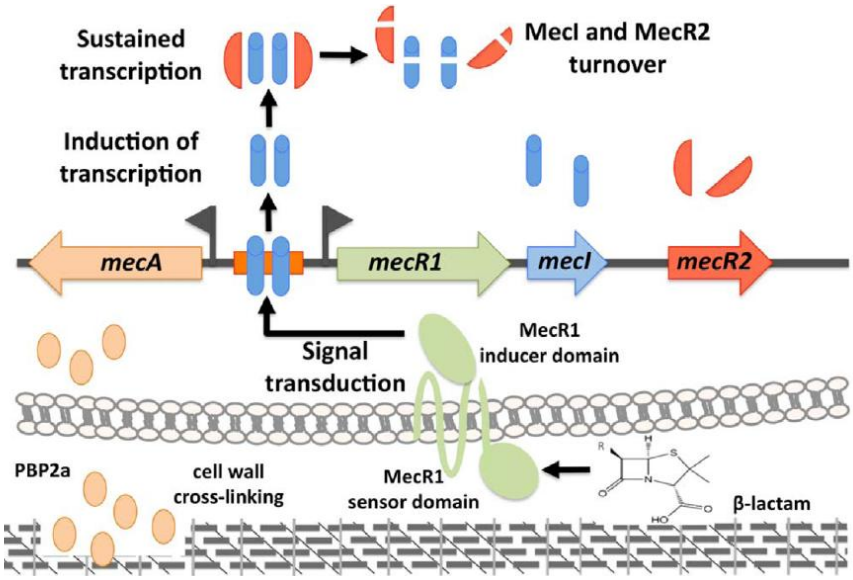

Figure 9. Regulation of *mecA* transcription. In the presence of a β-lactam antibiotic, MecR1 is activated and rapidly induces the expression of *mecA* and *mecR1-mecI-mecR2*. The anti-repressor activity of MecR2 is essential to sustain the *mecA* induction since it promotes the inactivation of MecI by proteolytic cleavage (reproduced from reference 12).

5.2.3. Staphylococcal Cassette Chromosome *mec* (SCC*mec*)

The *mecA* gene is located on a large mobile genetic element (21-67 Kb) named staphylococcal cassette chromosome *mec* (SCC*mec*) (153, 167). This element integrates in the genome of susceptible strains at a specific site at the 3' end of *orfX*, which is located near the origin of replication (167), and encodes a staphylococcal ribosomal methyltransferase (34). The SCC*mec* element is composed of two essential genetic complexes: (i) the *mec* gene complex containing *mecA*, its regulatory elements and insertion sequences upstream or downstream of *mecA*; and (ii) the cassette chromosome recombinase (*ccr*) gene complex, containing *ccrAB* or *ccrC* genes that encode site-specific recombinases, namely invertases/resolvases, responsible for the excision/integration of SCC*mec* from/into the chromosome (145). Also, the basic structure of SCC*mec* element includes three genetic regions called the "joining" (J) regions: i) the

J1 region, located between the chromosomal right junction and the *ccr* genes; ii) the J2 region that comprises the region between *ccr* genes and the *mec* complex; and iii) the J3 region that includes the genetic region between the *mec* complex and *orfX* (132). Some SCC*mec* type variants are defined by differences in the J regions (227, 228).

Up to now, eleven different types of SCC*mec* elements *(*SCC*mec* I to XI) and several variants have been identified and characterized, each type being defined according to the binary combination of the class of *mec* complex and the type of *ccr* gene complex (133, 145).

Currently, five different classes of *mec* complex were described: *mec* complex A, B, C, which is divided in C1 and C2, and *mec* complex E (145). Regarding *ccr* gene complex variability, three distinct *ccr* genes were described: *ccrA*, *ccrB* and *ccrC*, the latter representing a *ccr* complex classified in two main structures: one carrying two adjacent genes, *ccrA* and *ccrB*, and the other carrying only one gene, *ccrC*. So far, seven *ccr* gene complex types were described in *S. aureus*: types 1 to 4, carrying *ccrA* and *ccrB* with four allotypes (*ccrA1B1*, *ccrA2B2*, *ccrA3B3*, *ccrA4B4*); type 5 carrying a single allotype, *ccrC*; type 7 carrying the combination of *ccrA1B6* and type 8 carrying the combination *ccrA1B3* (http://www.sccmec.org).

5.2.4. PBP2A and the mechanism of methicillin resistance in *S. aureus*

PBP2A is a 78 KDa HMW PBP located in the extracellular surface of the cytoplasmic membrane, where it can catalyze the final steps of cell wall assembly (113). The crystal structure of PBP2A revealed the presence of three domains: i) a N-terminal domain, which corresponds to the anchor characteristic of the HMW PBPs; ii) a central non-penicillin binding domain

of unknown function; and iii) a C-terminal penicillin binding domain with the typical folding pattern of the PBP transpeptidases and the serine β-lactamases (196). PBP2A has the particularity of the nucleophilic serine active site being sequestrated within an extended narrow groove, which results in a substantial decrease in the affinity for β-lactams, when compared to the native PBPs (124, 196, 288).

Initially, the model of β-lactam resistance mechanism mediated by PBP2A was based on the fact that, in the presence of β-lactams, the only functional PBP, able to catalyze the final reactions of the cell wall biosynthesis, is the low-affinity PBP2A, as the four native PBPs are fully acylated and consequently inactivated (124, 288). However, it was later described that PBP2A is a poor substitute of the native PBPs, as it shows a weak transpeptidase activity. The peptidoglycan of a MRSA strain, challenged with β-lactams, is weakly cross-linked, being composed mainly by monomeric, dimeric and trimeric structures, in contrast to the highly cross-linked peptidoglycan produced by the same MRSA strain, grown without antibiotic (61). Subsequently, several observations indicated that this model required revision.

5.3. Role of auxiliary genes in β-lactam resistance

Despite being the main player in the mechanism of resistance to methicillin, the production of PBP2A is not sufficient for the optimal expression of methicillin resistance. The first evidence that pointed in that direction came from the study wherein no correlation between the levels of resistance and the respective PBP2A cellular amounts was observed, suggesting that other factors, besides *mecA*, were involved in the expression of resistance to methicillin (123, 236). Additionally, introduction of *mecA* gene, from homogeneous MRSA strains, into MSSA, produced transformants with low

resistance or heterogeneous resistance to methicillin, independently of the *mecA* donor, which indicated that additional genes, belonging to the strains' genetic background, determine the levels of methicillin resistance (236).

The identification of these additional factors was initially addressed by two independent laboratories, that screened Tn*551* mutants of a homogeneous MRSA strain for reduced levels of methicillin resistance (22, 65, 67, 179). The location of the transposon insertion site in those mutants led to the identification of several genes, described as necessary for the full expression of methicillin resistance and designated as factors essential for methicillin resistance (*fem*) or auxiliary (aux) genes.

Initially, only the mutants that showed a drastic decrease in methicillin resistance were selected, allowing the identification of *femAB* (22, 24), *femX*, *femC* and *femD* (23, 66). The *femAB* operon, the first genetic locus identified as an additional player in the methicillin resistance mechanism, encodes two proteins, FemA and FemB (22, 24). This operon is responsible for the formation of the branched-peptide structure characteristic of *S. aureus* peptidoglycan, the pentaglycine bridge, namely FemA is essential for the addition of glycine residues 2 and 3, while FemB is specific for the attachment of glycine residues 4 and 5 (80). The first glycine is added independently of FemA and FemB, through the action of *femX* (178, 296). Characterization of the *femC* mutant revealed that the transposon inserted in the glutamine synthetase repressor (*glnR*), which has a polar effect on the transcription of the glutamine synthetase transcription (*glnA*) (120). The inhibition of the *glnRA* operon produced a decrease in glutamine synthetase activity and therefore, a reduction in the glutamine availability in the cells. Importantly, the analysis of the peptidoglycan composition of the *femC* mutant, by HPLC, revealed a decrease in the proportion of cross-linked peptides and the existence of

new muropeptides structures containing glutamic acid units with free α-carboxyl group instead of the normal isoglutamine residues (253). The *femD* (*glmM*) gene encodes GlmM, a phosphoglucosamine mutase, which catalyses the conversion of phosphoglucosamine-6-phosphate into glucosamine-1-phosphate, leading to the production of UDP-*N*-acetylglucosamine (158). The inhibition of GlmM production results in the decrease of the synthesis of the peptidoglycan precursor, which is revealed by a complete disappearance of the unsubstituted disaccharide monomer from the peptidoglycan (63). Importantly, all these mutants were unaffected regarding *mecA* expression and most of them showed a heterogeneous resistance to methicillin (179) (see section 5.4).

On the other hand, the analysis of the Tn*551* mutants that showed a less severe decrease in methicillin resistance led to the identification of 20 to 30 auxiliary genes, most of them associated with cell wall biosynthesis (65, 67).

The *murE* gene, which encodes the Mur ligase responsible for the addition of the L-lysine residue to the UDP-muramyl-dipeptide cell wall precursor, was also identified as an auxiliary gene by the characterization of a Tn*551* transposon mutant (204). Contrary to previously studied auxiliary genes, the inhibition of *murE* transcription affected the amount of PBP2A, suggesting that the expression of this PBP can be directly or indirectly under the control of the *murE* gene (103).

The analysis of an insertion mutant for *murF* gene, which encodes the UDP-*N*-acetylmuramyl-pentapeptide synthetase, allowed identification of this gene as being important for methicillin resistance. The inactivation of *murF* caused both a decrease in methicillin resistance and a reduction in *mecA* transcription, suggesting that the expression of *mecA* and *murF* seems to be correlated (317). Later, the analysis of a *murF* conditional

mutant demonstrated that MurF is also important for cell division, being essential for cell viability (318).

It was surprising to find that *pbpB* was an auxiliary gene, since it was assumed that PBP2 was inactivated in the presence of β-lactams (270). The inactivation of the transglycosylase domain, but not the transpeptidase domain, of PBP2 results in a decrease of β-lactam resistance, even in the presence of PBP2A (273). This suggested that, in the presence of antibiotic, MRSA peptidoglycan biosynthesis depends on the functional cooperation between the transglycosylase domain of the native PBP2 and the transpeptidase domain of PBP2A (273, 276). Also, in the presence of β-lactams, PBP2A complements the essential peptidoglycan transpeptidase function of PBP2, confirming that PBP2A has transpeptidase activity (276) (Figure 10).

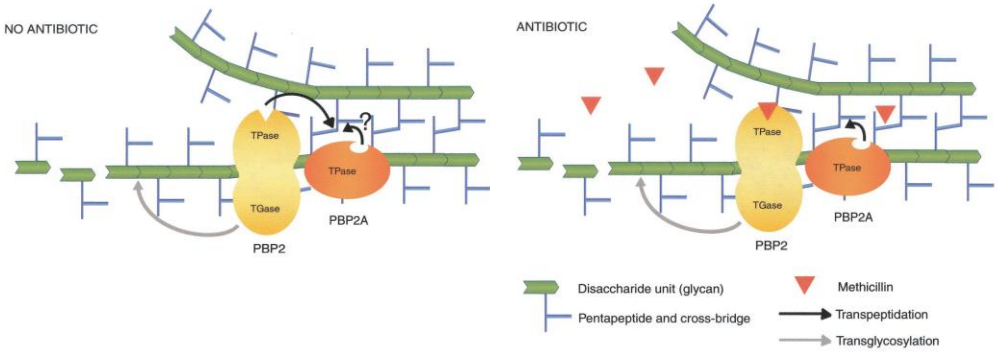

Figure 10. Model for the cooperative functioning of the transglycosylase (TGase) domain of PBP2 and the transpeptidase (TPase) domain of PBP2A in methicillin-resistant *S. aureus*. (left) In the absence of antibiotic, both the TPase and TGase domains of PBP2 participate in the biosynthesis of staphylococcal peptidoglycan. (Right) When antibiotic is present, the TPase domain of PBP2 is acylated and is unable to perform its peptide crosslinking activity. However, the penicillin-insensitive TGase domain of PBP2 remains functional and cooperates with the TPase activity of PBP2A (adapted from reference 273).

Also, while *pbpA* gene, which encodes for PBP1, was involved in methicillin resistance (263), impairment of *pbpD* did not affect the β-lactam resistance

level of MRSA, being not classified as an auxiliary gene (166). Later, *pbpD* was shown to be an auxiliary gene only in the community acquired MRSA (CA-MRSA), playing a key role in the resistance mechanism of these strains (225). Within the four staphylococcal PBPs, only PBP3 is not consider a auxiliary gene for β-lactam resistance, as the inactivation of *pbpC* gene had no significant impact on the methicillin resistance profile (274).

Besides the genes described above, other cell wall related genes were linked to the optimal expression of methicillin resistance (for a review see reference 295): i) *fmtA* gene, which encodes a methionyl- tRNA formyltransferase, affects both methicillin resistance and autolysis in the presence of Triton X-100 (175, 176); ii) *mrp* (or *fmtB*), which protein product was annotated as a sortase A-dependant cell surface protein (371); iii) *tarO* gene, which is involved in the first enzymatic step in WTAs biosynthesis (319), has an impact on both methicillin resistance and autolysis rate (209).

The genetic strategy, based on the screening of Tn*551* insertion mutants, showed some limitations, as genes that are involved in essential reactions for cell survival and that simultaneously contribute to β-lactam resistance are not identified through this approach. Recently, in order to overcome this issue, a genome-wide antisense fragmentation approach was followed to identify essential genes (95). The genes targeted by antisense RNAs were identified in a community acquired and in a hospital acquired MRSA strains (187). The resulting mutants were then screened under inducing conditions to identify additional genes required for β-lactam resistance (187). In this way, more genes were recognized to contribute to β-lactam resistance, including essential factors of cell division (*ftsZ* and *ftsA*), protein secretion (*spsB*), wall teichoic acid biosynthesis (*tarL*) and signal transduction system (*pknB*) (187).

The mechanism behind the contribution of auxiliary genes to the expression of methicillin resistance is far from being completely understood. It is proposed that the auxiliary genes, which are cell wall related, are implicated in β-lactam resistance by providing optimal peptidoglycan precursors substrates for PBPs to complete peptidoglycan synthesis. The inactivation of these genes leads to the production of abnormal muropeptides structures, which are inefficient substrates for PBP2A. Consequently, methicillin molecules may easily bind to the active site of PBP2A due to the lack of correct muropeptide competitors, which results in the decrease of methicillin resistance (65).

Although the mechanism behind the contribution of auxiliary genes to the expression of an optimal methicillin resistance phenotype is not clarified, the identification of their role is a significant step and shows that β-lactam resistance requires the coordinated activity of a large network of interdependent biological processes. In fact, many other genes, not related with cell wall biosynthesis, were recognized as auxiliary genes. The large number of auxiliary genes described until now and the diversity of their cellular functions illustrates the complexity of the β-lactam resistance mechanism in *S. aureus.*

5.4. Heterogeneity

In most MRSA strains the level of antibiotic resistance, minimal inhibitory concentration (MIC), varies widely from one strain to another and also within the progeny of a single MRSA isolate, which reveals a heterogeneous expression of methicillin resistance. Heterogeneous methicillin resistant strains are composed of a majority of cells with low-resistance to methicillin, corresponding to the MIC of the strain, and one or more subpopulations of highly resistant cells present at variable low

frequencies (123, 325). In clinical settings, this heterogeneous phenotype can be easily misinterpreted as sensitive, which consequently, originates treatment failure of infections caused by these strains (123). The level of resistance of the subpopulations and their frequency within the culture, are reproducible and specific features of the MRSA strain (64). In fact, regarding the high resistance level phenotype, once expressed, it is stable and most of these highly resistant subpopulations, designated as "homostar" population, express highly homogeneous resistance and do not revert to the heterogeneous phenotype (88, 123). Therefore, it appears that the heterogeneous composition of MRSA cultures is genetically controlled, i.e., the highly resistant subpopulations of bacteria must carry mutations in some genes, not located in the SCC*mec* element (302). Some genes including *hmrA, hmrB, lytA* and the *dlt* operon were indicated as putative candidates to develop such mutations (101, 177, 240). Moreover, it was recently described that, within a heterogeneous population, the subpopulations that were able to express high-level resistance carried a single mutation in the *relA* gene, causing the accumulation of a small signaling molecule called (p)ppGpp, which in turn leads to persistent activation of the stringent response, an important bacterial stress response (238). Soon after, evidences were provided that support the model in which the ultimate controlling factor of the phenotypic expression of β-lactam resistance in MRSA is a RelA-mediated stringent response (76, 169). Although heterogeneity is mainly due to genomic differences, namely chromosome mutations, methicillin resistance in MRSA is dependent upon the growth medium and other factors such temperature, pH, osmolarity, trace metals, chelating agents, and anaerobiosis (218).

6. Molecular Epidemiology of Methicillin Resistant *S. aureus* (MRSA)

In 1961, two years after the introduction of methicillin in clinical practice, the first MRSA was isolated in the United Kingdom (156). In the following years, MRSA became pandemic, spread worldwide and accumulated resistance to almost all antimicrobial classes (72, 203). During this time, MRSA isolates seemed to be confined mainly to hospitals and other healthcare facilities, being designated as hospital or healthcare acquired MRSA (HA-MRSA). However, in the last two decades, this scenario has changed and the emergence of community-acquired MRSA (CA-MRSA), causing infections among healthy individuals, has been the subject of a growing concern, as MRSA are no longer limited to the hospital environment (41, 42, 130, 235). Nevertheless, as the global epidemic of CA-MRSA continued, CA-MRSA begun to appear as a cause of hospital outbreaks, replacing the classic HA-MRSA clones in some hospitals (35, 171, 255, 256, 261, 278, 308). The emergence of CA-MRSA as a cause of nosocomial infections is a serious threat for public health. First, it puts a wider group of hospitalized patients, healthcare workers and their community contacts potentially at risk of a MRSA infection. Second, the presence of CA-MRSA in hospital environment exposes them to the selective pressure of antibiotic use in hospitals, which can result in the increase of antibiotic resistance. On the other hand, the spread of strains, associated with the healthcare setting, into the community has also been described (137, 212, 219, 329), suggesting that the boundaries between the hospital and community are blurring.

6.1. Origin, evolution and global dissemination of hospital-acquired MRSA (HA-MRSA) clonal lineages

HA-MRSA are the leading cause of the majority of nosocomial infections, including bacteremia, pneumonia, urinary tract, surgical wound infections, and catheter-related infections (116).

Epidemiological studies, conducted by the SENTRY Antimicrobial Surveillance Program, between 1997 and 2003, revealed that the global MRSA prevalence in hospitals was 23% in Australia, 67% in Japan, 40% in South America, 36% in USA and 23% in Europe (18, 72, 119, 334). However, data from the European Antimicrobial Resistance Surveillance Network (EARSS), between 1999 and 2002, revealed that the prevalence of MRSA in Europe is not homogeneous. While in the Northern countries, the percentage of MRSA is approximately 1%, in the Southern countries it is as high as 45% (334).

Another study was based on the genetic analysis of more than 3,000 MRSA isolates from surveillance and outbreak studies in Southern and Eastern Europe, North and Latin America and Asia, through the CEM/NET initiative, between 1994 and 2000 (336), revealed that the majority of the cases of HA-MRSA infections are caused by a small group of epidemic MRSA (EMRSA) clones, which are highly disseminated worldwide (251). Each clonal lineage, designated as Iberian (ST247-IIA), Brazilian (ST239-IIIA), Hungarian (ST239-III), New York/Japan (ST5-II), Pediatric (ST5-VI) and epidemic MRSA-16 (EMRSA-16) (ST36-II) clones, was defined as a result of its specific genetic background and to the geographic site of its first identification. Additionally, two other clones, the EMRSA-15 (ST22-IV) and Berlin (ST45-IV) showed epidemic potential, in the United Kingdom and Germany, respectively (157, 234, 361, 368).

Several studies have addressed the topic related to the origin and evolution of the major contemporary MRSA clones. The most widely accepted theory defends that SCC*mec* was introduced several times into different *S. aureus* lineages that subsequently disseminated (55, 82, 114, 237).

In order to clarify the origin and evolution of MRSA strains, the genetic background of MSSA and early MRSA strains isolated in the 1950/60s, in Denmark and United Kingdom was characterized (55). The comparison of these genetic profiles with the ones of the MRSA contemporary epidemic clones revealed that MSSA strains had gradually acquired resistance traits, in parallel to the introduction of antimicrobials in therapy. Moreover, it was shown that a large group of early MSSA isolates were identical to all early MRSA, in phenotypic and genetic properties, suggesting that these MSSA, with the genotype sequence type 250 (ST250), are the progeny of an *S. aureus* strain*,* that was one of the first recipients of the *mec* element in the MRSA evolutionary history. This early MRSA, with SCC*mec* type I and ST250, designated as Archaic clone, (55, 251) was the sole MRSA clone, in Danish hospitals, for over 17 years, after its emergence in 1960s (114). Furthermore, the Iberian clone (ST247-I) was described as a single locus variant of ST250, indicating that it was a direct descendent of the Archaic clone (55), which was further confirmed by a similar study of *S. aureus,* isolated in the 1960s, from blood infections in Denmark (114).

Soon after, an evolutionary model was proposed through the characterization, (by SCC*mec* typing and MLST), of a large collection of MRSA and MSSA, isolated in 20 countries between 1961 and 1999 (82). The major MRSA clones evolved from five different groups of related genotypes or clonal complexes (CC): CC5, CC8, CC22, CC30 and CC45, each arising from a distinct ancestral genotype. The presence of different SCC*mec* elements within the same sequence type (ST), suggested that

methicillin resistance had emerged on multiple occasions within a specific lineage. In fact, additional high-resolution genetic evolutionary studies revealed that it emerged due to at least 20 SCC*mec* acquisitions, and that the acquisition of this genetic element was four times more common than the replacement of one SCC*mec* between the MRSA clones (294), which emphasizes the role of this genetic element as a driving force for MRSA evolution. Furthermore, a more recent study, proposed that the SCC*mec* element was imported much more frequently than previously suggested, and that MRSA apparently emerged by temporal and geographically independent SCC*mec* acquisitions (246).

Therefore, accordingly to both studies, the CC8 lineage includes the first MRSA, ST250-I (Archaic clone), that was derived through acquisition of SCC*mec* type I, by ST250 MSSA, which itself was derived from the lineage, ST8-MSSA. ST250 MSSA is also the ancestor of other nosocomial MRSA pandemic lineages such as ST247-I (Iberian clone) and ST239-III (Brazilian/Hungarian clone). CC8 also comprises one of the most widespread community acquired clones, the ST8-MRSA-IV (USA300 clone) (82, 294, 331). In the CC5 lineage, ST5-MSSA was the ancestor of three major contemporary clonal lineages: the ST5-MRSA-II (New York/Japan clone), the ST5-MRSA-IV (Pediatric clone) and ST228-MRSA-I (Southern German clone). The CC22 lineage includes ST22-MRSA-IVh, known as EMRSA-15 that originated through the SCC*mec*-IVh acquisition into ST22-MSSA. On the other hand, in CC45, the ancestor ST45-MSSA precedes the ST45-MRSA-IV background of the Berlin clone. In CC30, ST30-MSSA diverged into two distinct clonal types, which are the hospital acquired ST36-MRSA-II (EMRSA-16) and the community acquired ST30-MRSA-IV Southwest Pacific clone (293, 294).

The evolutionary pathways of the five MRSA clonal lineages referred to above showed that worldwide disseminated EMRSA clones have originated through the acquisition of the methicillin resistance determinant, by successful MSSA lineages, frequently associated with disease (55, 82, 114). In this way, the epidemicity of MRSA clones seems to depend on genetic determinants that control the ability to colonize and/or cause disease. The study performed by Katayama *et al* (165) demonstrated that MSSA strains from major MRSA lineages were easier to transform with a plasmid-expressed *mecA* and better able to express resistance, in comparison to MSSA from other lineages, indicating that not all *S. aureus* genetic backgrounds favored the presence of the *mecA* gene in an active form. However, a thorough analysis of selected sequenced genomes did not show evidence that there are specific genetic factors, differentiating epidemic from sporadic *S. aureus* clones (181).

The putative presence in strains of intrinsic properties, that defined the capacity to acquire and maintain the SCC*mec* element is not well understood and is being explored in several laboratories. Such studies should help understand the reasons for the limited number of MRSA clones detected in surveillance studies.

6.2. The emergence and epidemiology of community-acquired MRSA (CA-MRSA)

For a long time MRSA strains were exclusively isolated from nosocomial environment. However, in the mid and late 1990s, a change in the MRSA epidemiology occurred, with the emergence of CA-MRSA strains, which have the capacity to infect otherwise healthy individuals (41, 60, 115, 130).

The epidemiological success of CA-MRSA is due to the combination of methicillin resistance at low fitness cost (59, 73, 188) with a high level of

aggressive virulence, which enables CA-MRSA to spread and cause severe infections in otherwise healthy people (68, 257).

The range of diseases caused by CA-MRSA is similar to the ones caused by MSSA in the community, but are clearly distinct from infections caused by HA-MRSA. Commonly, CA-MRSA causes skin and soft-tissue infections, however, it can be also associated with more severe infections such as folliculitis, cellulites, impetigo, pyomyositis and myositis, septic arthritis, osteomyelitis, sepsis, endocarditis, and necrotizing pneumonia (229, 258).

Regarding the global epidemiology of CA-MRSA, a huge difference in the CA-MRSA prevalence between USA and the rest of the world has been observed. While in the USA the percentage of CA-MRSA infections can reach 59% (235), and is associated with only a single clone (USA300 clone), in Europe, the prevalence of CA-MRSA is lower, ranging from 1 to 29% (185, 211, 212, 367) and these strains are genetically diverse (298). The impact of the fast epidemic spread of CA-MRSA in United States on public health is remarkable, and it contributed for an overall increase of MRSA in this country (164, 171). The annual death rate caused by MRSA the highest among all those caused by an infectious agent, even higher, for example, than the one due to the human immunodeficiency virus, HIV (171).

Studies on the molecular epidemiology of CA-MRSA suggested that CA-MRSA emerged through the acquisition of SCC*mec* elements, mainly SCC*mec* type IV, by community strains of MSSA (70, 250). The existence of MSSA strains in the community sharing the same genetic background as CA-MRSA supports this hypothesis (114). Additionally, it has been described that genetic lineages of CA-MRSA are clearly distinct from the predominant HA-MRSA clones within defined geographic regions,

demonstrating that CA-MRSA did not emerge from local HA-MRSA (346). On the other hand, the larger clonal diversity of CA-MRSA, when compared to HA-MRSA, indicates that more MSSA clones have the ability to become CA-MRSA than HA-MRSA (82, 250).

The first case of CA-MRSA, identified as belonging to the Southwest Pacific (SWP) clone (ST30-IVc) (52), was recovered among Aboriginal patients in remote communities from Western Australia in 1993 (341). However, CA-MRSA were considered a public health threat, only in 1998, when four healthy children died from sepsis and necrotizing pneumonia due to CA-MRSA, belonging to USA400 clone (ST1-IVa), in Minnesota and North Dakota (41).

Since then, several CA-MRSA genetic backgrounds emerged and spread differently in separate geographical areas. Until recently, the five major CA-MRSA clones, not genetically related, could be associated to a continent specific geographic location (346). Therefore, the USA300 clone (ST8-Iva, CC8) and USA400 (ST1-Iva, CC1) were both epidemic in the United States (172, 330), the European clone (ST80-IVc, CC80) was detected in several European countries (87, 186), the Southwest Pacific clone (ST30-IVc, CC30) was prevalent in Asia and Oceania (348), and the ST59 (CC59) clone, circulated mainly in the Asian-Pacific area (140). In recent years, another clone that became important was ST398-V (CC398), which was first considered a livestock-clone associated with colonization in pigs. The first descriptions were reported in France (13) however, nowadays, the ST398 clone is disseminated worldwide, not only in animals but also in humans (86, 168, 342, 344). Currently, CA-MRSA clones are not restricted to a specific geographic region anymore and the five major CA-MRSA clones are disseminated worldwide: the USA 400 clone in Asia, Europe and the USA, the USA300 clone in the USA and Europe, the Southwest Pacific

clone in Australia, Europe and South America, the ST59-V clone in Asia and the USA and the European clone, ST80-IV in Europe, Asia and the Middle-East) (224, 233).

Presently, more than 20 distinct genetic lineages of CA-MRSA are known (60, 68, 224, 366), however, their epidemicity varies. The USA300 is the most epidemic CA-MRSA clone, having been found in as many as 50 different countries (244, 298). Moreover, USA300 is considered the cause of almost all CA-MRSA infections in USA (235), having quickly overcome the USA400 clone. In these last years, the pathogenic success of USA300 in USA has been remarkable as it was able to spread rapidly within the community, to start severe outbreaks in terms of frequency and severity of infection and to emerge as the major cause of hospital associated infections in USA (97, 155, 220, 229, 235).

7. CA-MRSA *versus* HA-MRSA strains - genotypic and phenotypic uniqueness

Soon after its emergence, it was shown that CA-MRSA contain several interestingly features that distinguish them from HA-MRSA (346).

CA-MRSA is generally considered more virulent than HA-MRSA (194, 354). The mechanisms underlying the high virulence of CA-MRSA are based on their efficient evasion of the host immune system (326). The capacity of CA-MRSA to evade or subvert host defenses is mainly due to the presence of mobile genetic elements, such as the phage-encoded Panton-Valentine leukocidin (PVL) (346), the arginine catabolic mobile element (ACME) (75, 81, 112) and to the increased expression of virulence genes, carried by the accessory or adaptative genome, most importantly the ones coding for the phenol-soluble modulins (PSM) and the alpha-hemolysine (60, 194).

PVL is a *S. aureus*-specific exotoxin that forms pores in the cytoplasmic membrane of neutrophils, causing their lysis (197). This bi-component exotoxin is encoded by two co-transcribed genes, *lukF-PV* and *lukS-PV* (369), which are carried by staphylococcal temperate bacteriophages (162, 163). PVL genes have been found in all CA-MRSA strains, namely in the ones associated with severe disease, in contrast to HA-MRSA, in which PVL is usually absent (109, 197, 229, 257, 346, 377). Therefore, PVL was initially considered to be the main cause of the increased virulence and - together with a specific genetic background and the presence of SCC*mec* types IV or V - a genetic marker for CA-MRSA (48, 58, 239, 338, 346).

In addition to the pronounced virulence, common to all CA-MRSA, some particularly strains contain specific additional factors that contribute to their pathogenic success. For instance, the presence of the arginine catabolic mobile element (ACME) in the genome of the successful CA-MRSA USA300 clone is assumed to contribute to its growth, virulence and high capacity to spread. It contains two gene clusters, *arc* and *opp-3,* which encode for an arginine deiminase pathway and an oligopeptide permease system, respectively (75). While *arc* is responsible for the depletion of L-arginine, which is involved in the production of nitric oxide, a molecule used in the innate and adaptive immune responses against bacterial infections (232), *opp-3* belongs to the ABC transporter family, which members have a variety of biological functions such as quorum sensing, resistance to antimicrobial peptides, peptide nutrient uptake and chemotaxis (74).

Regarding methicillin resistance, the structure of the SCC*mec* element differs in CA-MRSA and HA-MRSA strains. Commonly, CA-MRSA carry the smaller and potentially more mobile SCC*mec* types IV, V, VII (59, 151, 152, 338, 346), whereas HA-MRSA are typically associated with the largest chromosomal elements SCC*mec* type I-III (60). Consequently, CA-MRSA

strains are relatively susceptible to other antibiotics, and most are resistant only to β-lactams, since SCC*mec* types IV-VII do not carry ancillary resistance genes.

On the other hand, as antibiotic resistance is associated to a fitness cost (6), which is easily attenuated by compensatory mutations in the bacterial genome (207), the different suitability caused by the diverse SCC*mec* types could influence the virulence of CA and HA-MRSA. In this context, it was recently demonstrated that SCC*mec* type II, but not the SCC*mec* type IV, has a significant cost for the bacteria, which results in a decrease in the growth rate, and in a reduction of the toxin production (49, 50). It appears that the balance between the costs of virulence and antibiotic resistance can explain why MRSA with attenuated virulence and SCC*mec* type II are found mainly in hospital environments where high antibiotic pressure, immunocompromised individuals and vector-mediated transmission are present. The low levels of antimicrobial agents used in the community favors the presence of MRSA harboring SCC*mec* type IV, which does not impose a high biological cost, allowing a more pronounced virulence.

Additionally, the reduced virulence of HA-MRSA containing SCC*mec* type II can be explained by the high levels of *mecA* expression in HA-MRSA, in comparison to CA-MRSA with SCC*mec* type IV (301). It was previously observed that HA-MRSA with SCC*mec* type II tend to be more resistant to oxacillin than CA-MRSA containing SCC*mec* type IV (250), probably because they produce higher levels of PBP2A. In fact, it was later confirmed that the hospital-associated SCC*mec* type II strains are more resistant to oxacillin and produce higher concentrations of PBP2A than CA-MRSA harboring SCC*mec* type IV (301). Consequently, the high levels of PBP2A production, by *mecA* gene on MRSA SCC*mec* type II, induce changes in cell wall that “disable” the strain to respond to the accessory

gene regulator (*agr*) auto-inducing peptide, reducing in this way the secretion of cytolytic toxins. In contrast, the same study showed that the toxicity CA-MRSA SCC*mec* type IV was not affected, probably due to their lower levels of PBP2A production. Therefore, the dual activity of *mecA* on antibiotic resistance and virulence regulation, has allowed CA-MRSA to maintain both virulence and antibiotic resistance and succeed where HA-MRSA could not.

Besides *mecA*, *psm-mec*, a cytolysin gene located in type-II and type-III SCC*mec* but not in type-IV SCC*mec*, connects virulence to methicillin resistance in MRSA (159, 281). It was shown that *psm-mec* RNA specifically binds *agrA* mRNA, a gene belonging to *agr* locus, inhibiting its translation (160). Since AgrA drives the transcription of RNAIII, the inhibition of its translation results in the suppression of virulence. Therefore, absence of *psm-mec* function in CA-MRSA, harboring SCC*mec* type IV, “explains” its high virulence property.

8. References

1. **Abraham, E. P., and E. Chain**. 1940. An enzyme from bacteria able to destroy penicillin. Nature. **146**.
2. **Abrams, A.** 1958. *O*-acetyl groups in the cell wall of *Streptococcus faecalis*. J. Biol. Chem. **230**: 949-959.
3. **Adhikari, R. P. and R. P. Novick**. 2008. Regulatory organization of the staphylococcal *sae* locus. Microbiology. **154**: 949-959.
4. **Akinbi, H. T., R. Epaud, H. Bhatt and T. E. Weaver**. 2000. Bacterial killing is enhanced by expression of lysozyme in the lungs of transgenic mice. J. Immunol. **165**: 5760-5766.
5. **Anderson, M. S., S. S. Eveland, H. R. Onishi and D. L. Pompliano**. 1996. Kinetic mechanism of the *Escherichia coli* UDPMur*N*Ac-tripeptide D-alanyl-D-alanine-adding enzyme: use of a glutathione S-transferase fusion. Biochem. **35**: 16264-16269.
6. **Andersson, D. I., and B. R. Levin**. 1999. The biological cost of antibiotic resistance. Curr. Opin. Microbiol. **2**: 489-493.
7. **Antignac, A., and A. Tomasz**. 2009. Reconstruction of the phenotypes of methicillin-resistant *Staphylococcus aureus* by replacement of the staphylococcal cassette chromosome *mec* with a plasmid-borne copy of *Staphylococcus sciuri pbpD* gene. Antimicrob. Agents Chemother. **53**: 435-441.
8. **Antignac, A., K. Sieradzki and A. Tomasz**. 2007. Perturbation of cell wall synthesis suppresses autolysis in *Staphylococcus aureus*: evidence for coregulation of cell wall synthetic and hydrolytic enzymes. J. Bacteriol. **189**: 7573-7580.
9. **Araki, Y., T. Nakatani, H. Hayashi and E. Ito**. 1971. Occurrence of non-*N*-substituted glucosamine residues in lysozyme-resistant peptidoglycan from *Bacillus cereus* cell walls. Biochem. Biophys. Res. Commun. **42**: 691-697.
10. **Arbeloa, A., H. Segal, J. E. Hugonnet, N. Josseaume, L. Dubost, J. P. Brouard, L. Gutmann, D. Mengin-Lecreulx and M. Arthur**. 2004. Role of class A penicillin-binding proteins in PBP5-mediated beta-lactam resistance in *Enterococcus faecalis*. J. Bacteriol. **186**: 1221-1228.
11. **Archer, G. L., and J. M. Bosilevac**. 2001. Signaling antibiotic resistance in staphylococci. Science. **291**: 1915-1916.
12. **Arede, P., C Milheirico, H. de Lencastre, and D.C. Oliveira**. 2012. The anti-repressor MecR2 promotes the proteolysis of the *mecA* repressor and enables optimal expression of beta-lactam resistance in MRSA. PLoS Pathog. **8**: e1002816.
13. **Armand-Lefevre, L., R. Ruimy, and A. Andremont**. 2005. Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. Emerg. Infect. Dis. **11**: 711-714.
14. **Atilano, M. L., P. M. Pereira, J. Yates, P. Reed, H. Veiga, M. G. Pinho, and S. R. Filipe**. 2010. Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U. S. A. **107**: 18991-18996.

15. **Barreteau, H., A. Kovac, A. Boniface, M. Sova, S. Gobec and D. Blanot**. 2008. Cytoplasmic steps of peptidoglycan biosynthesis. FEMS Microbiol. Rev. **32**: 168-207.
16. **Beck, W. D., B. Berger-Bachi, and F. H. Kayser**. 1986. Additional DNA in methicillin-resistant *Staphylococcus aureus* and molecular cloning of *mec*-specific DNA. J. Bacteriol. **165**: 373-378.
17. **Begg, K. J., A. Takasuga, D. H. Edwards, S. J. Dewar, B. G. Spratt, H. Adachi, T. Ohta, H. Matsuzawa and W. D. Donachie**. 1990. The balance between different peptidoglycan precursors determines whether *Escherichia coli* cells will elongate or divide. J. Bacteriol. **172**: 6697-6703.
18. **Bell, J. M., and J. D. Turnidge**. 2002. High prevalence of oxacillin-resistant *Staphylococcus aureus* isolates from hospitalized patients in Asia-Pacific and South Africa: results from SENTRY antimicrobial surveillance program, 1998-1999. Antimicrob. Agents Chemother. **46**: 879-881.
19. **Bera, A., R. Biswas, S. Herbert and F. Gotz**. 2006. The presence of peptidoglycan *O*-acetyltransferase in various staphylococcal species correlates with lysozyme resistance and pathogenicity. Infect. Immun. **74**: 4598-4604.
20. **Bera, A., R. Biswas, S. Herbert, E. Kulauzovic, C. Weidenmaier, A. Peschel, and F. Gotz**. 2007. Influence of wall teichoic acid on lysozyme resistance in *Staphylococcus aureus*. J. Bacteriol. **189**: 280-283.
21. **Bera, A., S. Herbert, A. Jakob, W. Vollmer, and F. Gotz**. 2005. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. Mol. Microbiol. **55**: 778-787.
22. **Berger-Bächi, B.** 1983. Insertional inactivation of staphylococcal methicillin resistance by *Tn*551. J. Bacteriol. **154**: 479-487.
23. **Berger-Bächi, B., A. Strässle, J. E. Gustafson, and F. H. Kayser**. 1992. Mapping and characterization of multiple chromosomal factors involved in methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents. Chemother. **36**: 1367-1373.
24. **Berger-Bachi, B., L. Barberis-Maino, A. Strassle, and F. H. Kayser**. 1989. FemA, a host-mediated factor essential for methicillin resistance in *Staphylococcus aureus*: molecular cloning and characterization. Mol. Gen. Genet. **219**: 263-269.
25. **Bernard, E., T. Rolain, P. Courtin, A. Guillot, P. Langella, P. Hols, and M. P. Chapot-Chartier**. 2011. Characterization of O-acetylation of *N*-acetylglucosamine: a novel structural variation of bacterial peptidoglycan. J. Biol. Chem. **286**: 23950-23958.
26. **Bertrand, J. A., G. Auger, E. Fanchon, L. Martin, D. Blanot, J. van Heijenoort and O. Dideberg**. 1997. Crystal structure of UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase from *Escherichia coli*. EMBO J. **16**: 3416-3425.
27. **Biswas, R., L. Voggu, U. K. Simon, P. Hentschel, G. Thumm and F. Gotz**. 2006. Activity of the major staphylococcal autolysin Atl. FEMS Microbiol. Lett. **259**: 260-268.
28. **Blake, C. C., D. F. Koenig, G. A. Mair, A. C. North, D. C. Phillips and V. R. Sarma**. 1965. Structure of hen egg-white lysozyme. A three-dimensional Fourier synthesis at 2 Angstrom resolution. Nature. **206**:757-761.

29. **Boman, H. G., I. Faye, G. H. Gudmundsson, J. Y. Lee and D. A. Lidholm**. 1991. Cell-free immunity in Cecropia. A model system for antibacterial proteins. Eur. J. Biochem. **201**: 23-31.
30. **Boneca, I. G., O. Dussurget, D. Cabanes, M. A. Nahori, S. Sousa, M. Lecuit, E. Psylinakis, V. Bouriotis, J. P. Hugot, M. Giovannini, A. Coyle, J. Bertin, A. Namane, J. C. Rousselle, N. Cayet, M. C. Prevost, V. Balloy, M. Chignard, D. J. Philpott, P. Cossart and S. E. Girardin**. 2007. A critical role for peptidoglycan *N*-deacetylation in *Listeria* evasion from the host innate immune system. Proc. Natl. Acad. Sci. U. S. A. **104**: 997-1002.
31. **Bose, J. L., M. K. Lehman, P. D. Fey and K. W. Bayles**. 2012. Contribution of the *Staphylococcus aureus* Atl AM and GL murein hydrolase activities in cell division, autolysis, and biofilm formation. PLoS One. **7**: e42244.
32. **Bouhss, A., D. Mengin-Lecreulx, D. Blanot, J. van Heijenoort and C. Parquet**. 1997. Invariant amino acids in the Mur peptide synthetases of bacterial peptidoglycan synthesis and their modification by site-directed mutagenesis in the UDP-Mur*N*Ac:L-alanine ligase from *Escherichia coli*. Biochem. **36**: 11556-11563.
33. **Bouhss, A., D. Mengin-Lecreulx, D. Le Beller and J. Van Heijenoort**. 1999. Topological analysis of the MraY protein catalysing the first membrane step of peptidoglycan synthesis. Mol. Microbiol. **34**: 576-585.
34. **Boundy, S., M. K. Safo, L. Wang, F. N. Musayev, H. C. O'Farrell, J. P. Rife, and G. L. Archer**. 2013. Characterization of the *Staphylococcus aureus* rRNA methyltransferase encoded by *orfX*, the gene containing the staphylococcal chromosome Cassette *mec* (SCC*mec*) insertion site. J. Biol. Chem. **288**: 132-140.
35. **Boyce, J. M.** 2008. Community-associated methicillin-resistant *Staphylococcus aureus* as a cause of health care-associated infection. Clin. Infect. Dis. **46**: 795-798.
36. **Breukink, E., I. Wiedemann, C. van Kraaij, O. P. Kuipers, H. G. Sahl and B. de Kruijff**. 1999. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science. **286**: 2361-2364.
37. **Brown, D. F., and P. E. Reynolds**. 1980. Intrinsic resistance to beta-lactam antibiotics in *Staphylococcus aureus*. FEBS Lett. **122**: 275-278.
38. **Brumfitt, W., A. C. Wardlaw and J. T. Park**. 1958. Development of lysozyme-resistance in *Micrococcus lysodiekticus* and its association with an increased *O*-acetyl content of the cell wall. Nature. **181**: 1783-1784.
39. **Campbell, J., A. K. Singh, J. P. Santa Maria, Jr., Y. Kim, S. Brown, J. G. Swoboda, E. Mylonakis, B. J. Wilkinson and S. Walker**. 2011. Synthetic lethal compound combinations reveal a fundamental connection between wall teichoic acid and peptidoglycan biosyntheses in *Staphylococcus aureus*. ACS Chem. Biol. **6**: 106-116.
40. **Casewell, M. W., and R. L. Hill**. 1986. The carrier state: methicillin-resistant *Staphylococcus aureus*. J. Antimicrob. Chemother. **18 Suppl A**: 1-12.
41. **Center for Disease Control and Prevention**. 1999. Four pediatric deaths from community acquired methicillin-resistant *Staphylococcus aureus*-Minnesota and North Dakota, 1997–1999. J.A.M.A. **282**: 1123-1125.

42. **Chambers, H. F.** 2001. The changing epidemiology of *Staphylococcus aureus*? Emerg. Infect. Dis. **7**: 178-182.
43. **Cheung, A. L., J. M. Koomey, C. A. Butler, S. J. Projan and V. A. Fischetti**. 1992. Regulation of exoprotein expression in *Staphylococcus aureus* by a locus (*sar*) distinct from *agr*. Proc. Natl. Acad. Sci. U. S. A. **89**: 6462-6466.
44. **Clarke, A. J. and C. Dupont**. 1992. *O*-acetylated peptidoglycan: its occurrence, pathobiological significance, and biosynthesis. Can. J. Microbiol. **38**: 85-91.
45. **Clarke, S. R., and K. G. Dyke**. 2001. The signal transducer (BlaRI) and the repressor (BlaI) of the *Staphylococcus aureus* beta-lactamase operon are inducible. Microbiology. **147**: 803-810.
46. **Cleveland, R. F., A. J. Wicken, L. Daneo-Moore and G. D. Shockman**. 1976. Inhibition of wall autolysis in *Streptococcus faecalis* by lipoteichoic acid and lipids. J. Bacteriol. **126**: 192-197.
47. **Cole, A. M., D. R. Thapa, V. Gabayan, H. I. Liao, L. Liu and T. Ganz**. 2005. Decreased clearance of *Pseudomonas aeruginosa* from airways of mice deficient in lysozyme M. J. Leukoc. Biol. **78**: 1081-1085.
48. **Collignon, P., I. Gosbell, A. Vickery, G. Nimmo, T. Stylianopoulos, and T. Gottlieb**. 1998. Community-acquired methicillin-resistant *Staphylococcus aureus* in Australia. Australian Group on Antimicrobial Resistance. Lancet. **352**: 145-146.
49. **Collins, J., A. Buckling, and R. C. Massey.** 2008. Identification of factors contributing to T-cell toxicity of *Staphylococcus aureus* clinical isolates. J. Clin. Microbiol. **46**: 2112-2114.
50. **Collins, J., J. Rudkin, M. Recker, C. Pozzi, J. P. O'Gara, and R. C. Massey**. 2010 Offsetting virulence and antibiotic resistance costs by MRSA. The I. S. M. E. J. **4**: 577-584.
51. **Collins, L. V., S. A. Kristian, C. Weidenmaier, M. Faigle, K. P. Van Kessel, J. A. Van Strijp, F. Gotz, B. Neumeister and A. Peschel**. 2002. *Staphylococcus aureus* strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. J. Infect. Dis. **186**: 214-219.
52. **Coombs, G. W., G. R. Nimmo, J. M. Bell, F. Huygens, F. G. O'Brien, M. J. Malkowski, J. C. Pearson, A. J. Stephens, and P. M. Giffard**. 2004. Genetic diversity among community methicillin-resistant *Staphylococcus aureus* strains causing outpatient infections in Australia. J. Clin. Microbiol. **42**: 4735-4743.
53. **Coutinho, P. M., E. Deleury, G. J. Davies and B. Henrissat**. 2003. An evolving hierarchical family classification for glycosyltransferases. J. Mol. Biol. **328**: 307-317.
54. **Couto, I., H. de Lencastre, E. Severina, W. Kloos, J. A. Webster, R. J. Hubner, I. S. Sanches, and A. Tomasz**. 1996. Ubiquitous presence of a *mecA* homologue in natural isolates of *Staphylococcus sciuri*. Microb. Drug Resist. **2**: 377-391.
55. **Crisóstomo, M. I., H. Westh, A. Tomasz, M. Chung, D.C. Oliveira, and H. de Lencastre**. 2001. The evolution of methicillin resistance in *Staphylococcus aureus*: similarity of genetic backgrounds in historically

early methicillin-susceptible and -resistant isolates and contemporary epidemic clones. Proc. Natl. Acad. Sci. U. S. A. **98**: 9865-9870.

56. **Crisostomo, M. I., W. Vollmer, A. S. Kharat, S. Inhulsen, F. Gehre, S. Buckenmaier and A. Tomasz**. 2006. Attenuation of penicillin resistance in a peptidoglycan *O*-acetyl transferase mutant of *Streptococcus pneumoniae*. Mol. Microbiol. **61**: 1497-1509.
57. **D'Elia, M. A., K. E. Millar, A. P. Bhavsar, A. M. Tomljenovic, B. Hutter, C. Schaab, G. Moreno-Hagelsieb and E. D. Brown**. 2009. Probing teichoic acid genetics with bioactive molecules reveals new interactions among diverse processes in bacterial cell wall biogenesis. Chem. Biol. **16**: 548-556.
58. **Daum, R. S.** 1998. Community-acquired methicillin-resistant *Staphylococcus aureus* infections. Pediatr. Infect. Dis. J. **17**: 745-746.
59. **Daum, R. S., T. Ito, K. Hiramatsu, F. Hussain, K. Mongkolrattanothai, M. Jamklang, and S. Boyle-Vavra**. 2002. A novel methicillin-resistance cassette in community-acquired methicillin-resistant *Staphylococcus aureus* isolates of diverse genetic backgrounds. J. Infect. Dis. **186**: 1344-1347.
60. **David, M. Z., and R. S. Daum** 2010. Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. Clin. Microbiol. Rev. **23**: 616-687.
61. **de Jonge, B. L., and A. Tomasz**. 1993. Abnormal peptidoglycan produced in a methicillin-resistant strain of *Staphylococcus aureus* grown in the presence of methicillin: functional role for penicillin-binding protein 2A in cell wall synthesis. Antimicrob. Agents Chemother. **37**: 342-346.
62. **de Jonge, B. L., Y. S. Chang, D. Gage and A. Tomasz**. 1992. Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin binding protein 2A. J. Biol. Chem. **267**: 11248-11254.
63. **de Jonge, B. L., Y. S. Chang, D. Gage, and A. Tomasz**. 1992. Peptidoglycan composition in heterogeneous Tn*551* mutants of a methicillin-resistant *Staphylococcus aureus* strain. J. Biol. Chem. **267**: 11255-11259.
64. **de Lencastre, H., A. M. Figueiredo, and A. Tomasz**. 1993. Genetic control of population structure in heterogeneous strains of methicillin resistant *Staphylococcus aureus*. Eur. J. Clin. Microbiol. Infect. Dis. **12**: 13:18.
65. **de Lencastre, H., and A. Tomasz**. 1994. Reassessment of the number of auxiliary genes essential for expression of high-level methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents. Chemother. **38**: 2590-2598.
66. **de Lencastre, H., B. L. de Jonge, P. R. Matthews, and A. Tomasz**. 1994. Molecular aspects of methicillin resistance in *Staphylococcus aureus*. J. Antimicrob. Chemother. **33**: 7-24.
67. **de Lencastre, H., S. W. Wu, M. G. Pinho, A. M. Ludovice, S. Filipe, S. Gardete, R. Sobral, S. Gill, M. Chung, and A. Tomasz**. 1999. Antibiotic resistance as a stress response: complete sequencing of a large number of chromosomal loci in *Staphylococcus aureus* strain COL that impact on the expression of resistance to methicillin. Microb. Drug Resist. **5**: 163-175.

68. **DeLeo, F. R., M. Otto, B. N. Kreiswirth, and H. F. Chambers**. 2010. Community-associated meticillin-resistant *Staphylococcus aureus*. Lancet. **375**: 1557-1568.
69. **den Blaauwen, T., M. A. de Pedro, M. Nguyen-Disteche and J. A. Ayala**. 2008. Morphogenesis of rod-shaped sacculi. FEMS Microbiol. Rev. **32**: 321-344.
70. **Deurenberg, R. H., and E. E. Stobberingh**. 2009. The molecular evolution of hospital- and community-associated methicillin-resistant *Staphylococcus aureus*. Curr. Mol. Med. **9**: 100-115.
71. **Di Berardino, M., A. Dijkstra, D. Stuber, W. Keck and M. Gubler**. 1996. The monofunctional glycosyltransferase of *Escherichia coli* is a member of a new class of peptidoglycan-synthesising enzymes. FEBS Lett. **392**: 184-188.
72. **Diekema, D. J., M. A. Pfaller, F. J. Schmitz, J. Smayevsky, J. Bell, R. N. Jones, M. Beach, and SENTRY Partcipants Group**. 2001. Survey of infections due to *Staphylococcus* species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999. Clin. Infect. Dis. **32 Suppl 2**: S114-S132.
73. **Diep, B. A., G. G. Stone, L. Basuino, C. J. Graber, A. Miller, S. A. des Etages, A. Jones, A. M. Palazzolo-Ballance, F. Perdreau-Remington, G. F. Sensabaugh, F. R. DeLeo, and H. F. Chambers**. 2008. The arginine catabolic mobile element and staphylococcal chromosomal cassette *mec* linkage: convergence of virulence and resistance in the USA300 clone of methicillin-resistant *Staphylococcus aureus*. J. Infect. Dis. **197**: 1523-1530.
74. **Diep, B. A., and M. Otto**. 2008. The role of virulence determinants in community-associated MRSA pathogenesis. Trends Microbiol. **16**: 361-369.
75. **Diep, B. A., S. R. Gill, R. F. Chang, T. H. Phan, J. H. Chen, M. G. Davidson, J. Lin, H. A. Carleton, E. F. Mongodin, G. F. Sensabaugh, and F. Perdreau-Remington**. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. Lancet. **367**: 731-739.
76. **Dordel, J., C. Kim, M. Chung, M. Pardos de la Gandara, M. T. Holden, J. Parkhill, H. de Lencastre, S. D. Bentley, and A. Tomasz**. 2014. Novel Determinants of Antibiotic Resistance: Identification of Mutated Loci in Highly Methicillin-Resistant Subpopulations of Methicillin-Resistant *Staphylococcus aureus*. mBio. **5**: e01000-01013.
77. **Dramsi, S., S. Magnet, S. Davison and M. Arthur**. 2008. Covalent attachment of proteins to peptidoglycan. FEMS Microbiol. Rev. **32**: 307-320.
78. **Dubrac, S., I. G. Boneca, O. Poupel and T. Msadek**. 2007. New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. J. Bacteriol. **189**: 8257-8269.

79. **During, K., P. Porsch, A. Mahn, O. Brinkmann and W. Gieffers**. 1999. The non-enzymatic microbicidal activity of lysozymes. FEBS Lett. **449**: 93-100.
80. **Ehlert, K., W. Schroder, and H. Labischinski**. 1997. Specificities of FemA and FemB for different glycine residues: FemB cannot substitute for FemA in staphylococcal peptidoglycan pentaglycine side chain formation. J. Bacteriol. **179**: 7573-7576.
81. **Ellington, M. J., L. Yearwood, M. Ganner, C. East, and A. M. Kearns**. 2008. Distribution of the ACME-*arcA* gene among methicillin-resistant *Staphylococcus aureus* from England and Wales. J. Antimicrob. Chemother. **61**: 73-77.
82. **Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil , H. Grundmann, and B. G. Spratt**. 2002. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). Proc. Natl. Acad. Sci. U. S. A. **99**: 7687-7692.
83. **Ernst, R. K., T. Guina and S. I. Miller**. 2001. *Salmonella typhimurium* outer membrane remodeling: role in resistance to host innate immunity. Microbes Infect. **3**: 1327-1334.
84. **European Antimicrobial Resistance Surveillance System.** 2009. EARSS annual report 2008, p. 55-58. EARSS, Bilthoven, The Netherlands, http://www.earss.rivm.nl.
85. **Eveland, S. S., D. L. Pompliano and M. S. Anderson**. 1997. Conditionally lethal *Escherichia coli* murein mutants contain point defects that map to regions conserved among murein and folyl poly-gamma-glutamate ligases: identification of a ligase superfamily. Biochem. **36**: 6223-6229.
86. **Fan, J., M. Shu, G. Zhang, W. Zhou, Y. Jiang, Y. Zhu, G. Chen, S. J. Peacock, C. Wan, W. Pan, and E. J. Feil**. 2009. Biogeography and virulence of *Staphylococcus aureus*. PLoS One. **4**: e6216.
87. **Faria, N. A., D. C. Oliveira, H. Westh, D. L. Monnet, A. R. Larsen, R. Skov, and H. de Lencastre**. 2005. Epidemiology of emerging methicillin-resistant *Staphylococcus aureus (*MRSA) in Denmark: a nationwide study in a country with low prevalence of MRSA infection. J. Clin. Microbiol. **43**: 1836-1842.
88. **Figueiredo, A. M., E. Ha, B. N. Kreiswirth, H. de Lencastre, G. L. Noel, L. Senterfit, and A. Tomasz**. 1991. *In vivo* stability of heterogeneous expression classes in clinical isolates of methicillin-resistant staphylococci. J. Infect. Dis. **164**: 883-887.
89. **Fischer, W.** 1988. Physiology of lipoteichoic acids in bacteria. Adv. Microb. Physiol. **29**: 233-302.
90. **Fischer, W., P. Rosel and H. U. Koch**. 1981. Effect of alanine ester substitution and other structural features of lipoteichoic acids on their inhibitory activity against autolysins of *Staphylococcus aureus*. J. Bacteriol. **146**: 467-475.
91. **Fittipaldi, N., M. Gottschalk, G. Vanier, F. Daigle and J. Harel**. 2007. Use of selective capture of transcribed sequences to identify genes preferentially expressed by *Streptococcus suis* upon interaction with porcine brain microvascular endothelial cells. Appl. Environ. Microbiol. **73**: 4359-4364.

92. **Fittipaldi, N., T. Sekizaki, D. Takamatsu, M. de la Cruz Dominguez-Punaro, J. Harel, N. K. Bui, W. Vollmer and M. Gottschalk**. 2008. Significant contribution of the *pgdA* gene to the virulence of *Streptococcus suis*. Mol. Microbiol. **70**: 1120-1135.
93. **Fleming, A.** 1929. On the bacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae* Brit. J. Exp. Pathol. **10**: 226-236.
94. **Fleming, T. J., D. E. Wallsmith and R. S. Rosenthal**. 1986. Arthropathic properties of gonococcal peptidoglycan fragments: implications for the pathogenesis of disseminated gonococcal disease. Infect. Immun. **52**: 600-608.
95. **Forsyth, R. A., R. J. Haselbeck, K. L. Ohlsen, R. T. Yamamoto, H. Xu, J. D. Trawick, D. Wall, L. Wang, V. Brown-Driver, J. M. Froelich, P. King, M. McCarthy, C. Malone, B. Misiner, D. Robbins, Z. Tan, Z. Y. Zhu Zy, G. Carr, D. A. Mosca, C. Zamudio, J. G. Foulkes and J. W. Zyskind**. 2002. A genome-wide strategy for the identification of essential genes in *Staphylococcus aureus*. Mol. Microbiol. **43**: 1387-1400.
96. **Fournier, B. and D. C. Hooper**. 2000. A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic activity of *Staphylococcus aureus*. J. Bacteriol. **182**: 3955-3964.
97. **Francis, J. S., M. C. Doherty, U. Lopatin, C. P. Johnston, G. Sinha, T. Ross, M. Cai, N. N. Hansel, T. Perl, J. R. Ticehurst, K. Carroll, D. L. Thomas, E. Nuermberger, and J. G. Bartlett**. 2005. Severe community-onset pneumonia in healthy adults caused by methicillin-resistant *Staphylococcus aureus* carrying the Panton-Valentine leukocidin genes. Clin. Infect. Dis. **40**: 100-107.
98. **Frere, J. M.** 1995. Beta-lactamases and bacterial resistance to antibiotics. Mol. Microbiol. **16**: 385-395.
99. **Fuda, C., M. Suvorov, Q. Shi, D. Hesek, M. Lee, and S. Mobashery**. 2007. Shared functional attributes between the *mecA* gene product of *Staphylococcus sciuri* and penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. Biochemistry. **46**: 8050-8057.
100. **Fujimoto, D. F. and K. W. Bayles**. 1998. Opposing roles of the *Staphylococcus aureus* virulence regulators, Agr and Sar, in Triton X-100- and penicillin-induced autolysis. J. Bacteriol. **180**: 3724-3726.
101. **Fujimura, T., and K. Murakami.** 1997. Increase of methicillin resistance in *Staphylococcus aureus* caused by deletion of a gene whose product is homologous to lytic enzymes. J. Bacteriol. **179**: 6294-6301.
102. **Garcia-Alvarez, L., M. T. Holden, H. Lindsay, C. R. Webb, D. F. Brown, M. D. Curran, E. Walpole, K. Brooks, D. J. Pickard, C. Teale, J. Parkhill, S. D. Bentley, G. F. Edwards, E. K. Girvan, A. M. Kearns, B. Pichon, R. L. Hill, A. R. Larsen, R. L. Skov, S. J. Peacock, D. J. Maskell, and M. A. Holmes**. 2011. Methicillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect. Dis. **11**: 595-603.
103. **Gardete, S., A. M. Ludovice, R. G. Sobral, S. R. Filipe, H. de Lencastre, and A. Tomasz**. 2004. Role of *murE* in the Expression of beta-lactam antibiotic resistance in *Staphylococcus aureus*. J. Bacteriol. **186**: 1705-1713.

104. **Georgopapadakou, N. H.** 1993. Penicillin-binding proteins and bacterial resistance to beta-lactams. Antimicrob. Agents Chemother. **37**: 2045-2053.
105. **Georgopapadakou, N. H., B. A. Dix and Y. R. Mauriz**. 1986. Possible physiological functions of penicillin-binding proteins in *Staphylococcus aureus*. Antimicrob. Agents. Chemother. **29**: 333-336.
106. **Georgopapadakou, N. H. and F. Y. Liu**. 1980. Penicillin-binding proteins in bacteria. Antimicrob. Agents. Chemother. **18**: 148-157.
107. **Ghuysen, J. M.** 1991. Serine beta-lactamases and penicillin-binding proteins. Annu. Rev. Microbiol. **45**: 37-67.
108. **Giesbrecht, P., T. Kersten, H. Maidhof and J. Wecke**. 1998. Staphylococcal cell wall: morphogenesis and fatal variations in the presence of penicillin. Microbiol. Mol. Biol. Rev. **62**: 1371-1414.
109. **Gillet, Y., B. Issartel, P. Vanhems, J. C. Fournet, G. Lina, M. Bes, F. Vandenesch, Y. Piemont, N. Brousse, D. Floret, and J. Etienne**. 2002. Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. Lancet. **359**: 753-759.
110. **Ginsburg, I.** 2004. Bactericidal cationic peptides can also function as bacteriolysis-inducing agents mimicking beta-lactam antibiotics?; it is enigmatic why this concept is consistently disregarded. Med. Hypotheses. **62**: 367-374.
111. **Ginsburg, I.** 2001. Cationic peptides from leukocytes might kill bacteria by activating their autolytic enzymes causing bacteriolysis: why are publications proposing this concept never acknowledged? Blood. **97**: 2530-2531.
112. **Goering, R. V., L. K. McDougal, G. E. Fosheim, K. K. Bonnstetter, D. J. Wolter, and F. C. Tenover**. 2007. Epidemiologic distribution of the arginine catabolic mobile element among selected methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates. J. Clin. Microbiol. **45**: 1981-1984.
113. **Goffin, C., and J. M. Ghuysen**. 1998. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiol. Mol. Biol. Rev. **62**: 1079-1093.
114. **Gomes, A. R., H. Westh, and H. de Lencastre**. 2006. Origins and Evolution of Methicillin-Resistant *Staphylococcus aureus* Clonal Lineages. Antimicrob. Agents Chemother. **50**: 3237–3244.
115. **Gorak, E. J., S. M. Yamada, and J. D. Brown**. 1999. Community-acquired methicillin-resistant *Staphylococcus aureus* in hospitalized adults and children without known risk factors. Clin. Infect. Dis. **29**: 797-800.
116. **Graffunder, E. M., and R. A. Venezia**. 2002. Risk factors associated with nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) infection including previous use of antimicrobials. J. Antimicrob. Chemother. **49**: 999-1005.
117. **Gregory, P. D., R. A. Lewis, S. P. Curnock, and K. G. Dyke**. 1997. Studies of the repressor (BlaI) of beta-lactamase synthesis in *Staphylococcus aureus*. Mol. Microbiol. **24**: 1025-1037.
118. **Groicher, K. H., B. A. Firek, D. F. Fujimoto and K. W. Bayles**. 2000. The *Staphylococcus aureus lrgAB* operon modulates murein hydrolase activity and penicillin tolerance. J. Bacteriol. **182**: 1794-1801.

119. **Grundmann, H., M. Aires-de-Sousa, J. Boyce, and E. Tiemersma**. 2006. Emergence and resurgence of methicillin-resistant *Staphylococcus aureus* as a public-health threat. Lancet. **368**: 874-885.
120. **Gustafson, J., A. Strassle, H. Hachler, F. H. Kayser, and B. Berger-Bachi**. 1994. The *femC* locus of *Staphylococcus aureus* required for methicillin resistance includes the glutamine synthetase operon. J. Bacteriol. **176**: 1460-1467.
121. **Hackbarth, C. J., and H. F. Chambers**. 1993. *blaI* and *blaR1* regulate beta-lactamase and PBP2a production in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **37**: 1144-1149.
122. **Haroche, J., A. Morvan, M. Davi, J. Allignet, F. Bimet, and N. El Solh**. 2003. Clonal diversity among streptogramin A-resistant *Staphylococcus aureus* isolates collected in French hospitals. J. Clin. Microbiol. **41**: 586-591.
123. **Hartman, B. J., and A. Tomasz**. 1986. Expression of methicillin resistance in heterogeneous strains of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **29**: 85-92.
124. **Hartman, B. J., and A. Tomasz**. 1984. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. J. Bacteriol. **158**: 513-516.
125. **Hayden, M. K., K. Rezai, R. A. Hayes, K. Lolans, J. P. Quinn, and R. A. Weinstein**. 2005. Development of Daptomycin resistance *in vivo* in methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **43**: 5285-5287.
126. **Heilmann, C., J. Hartleib, M. S. Hussain and G. Peters**. 2005. The multifunctional *Staphylococcus aureus* autolysin *aaa* mediates adherence to immobilized fibrinogen and fibronectin. Infect. Immun. **73**: 4793-4802.
127. **Henze, U., T. Sidow, J. Wecke, H. Labischinski and B. Berger-Bachi**. 1993. Influence of *femB* on methicillin resistance and peptidoglycan metabolism in *Staphylococcus aureus*. J. Bacteriol. **175**: 1612-1620.
128. **Henze, U. U. and B. Berger-Bachi**. 1995. *Staphylococcus aureus* penicillin-binding protein 4 and intrinsic beta-lactam resistance. Antimicrob. Agents Chemother. **39**: 2415-2422.
129. **Herbert, S., A. Bera, C. Nerz, D. Kraus, A. Peschel, C. Goerke, M. Meehl, A. Cheung and F. Gotz**. 2007. Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. PLoS Pathog. **3**: e102.
130. **Herold, B. C., L. C. Immergluck, M. C. Maranan, D. S. Lauderdale, R. E. Gaskin, S. Boyle-Vavra, C. D. Leitch, and R. S. Daum**. 1998. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. J.A.M.A. **279**: 593-598.
131. **Hershberger, E., S. Donabedian, K. Konstantinou, and M. J. Zervos**. 2004. Quinupristin-dalfopristin resistance in gram-positive bacteria: mechanism of resistance and epidemiology. Clin. Infect. Dis. **38**: 92-98.
132. **Hiramatsu, K., L. Cui, M. Kuroda and T. Ito**. 2001. The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. Trends Microbiol. **9**: 486-493.
133. **Hiramatsu, K., T. Ito, S. Tsubakishita, T. Sasaki, F. Takeuchi, Y. Morimoto, Y. Katayama, M. Matsuo, K. Kuwahara-Arai, T. Hishinuma**

and T. Baba. 2013. Genomic Basis for Methicillin Resistance in *Staphylococcus aureus*. Infect. Chemother. **45**: 117-136.

134. **Hiramatsu, K., N. Aritaka, H. Hanaki, S. Kawasaki, Y. Hosoda, S. Hori, Y. Fukuchi, and I. Kobayashi**. 1997. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet. **350**: 1670-1673.
135. **Holtje, J. V.** 1998. Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. Microbiol. Mol. Biol. Rev. **62**: 181-203.
136. **Holtje, J. V. and E. I. Tuomanen**. 1991. The murein hydrolases of *Escherichia coli*: properties, functions and impact on the course of infections *in vivo*. J. Gen. Microbiol. **137**: 441-454.
137. **Horner, C., P. Parnell, D. Hall, A. Kearns, J. Heritage, and M. Wilcox.** 2013. Methicillin-resistant *Staphylococcus aureus* in elderly residents of care homes: colonization rates and molecular epidemiology. J. Hosp. Infect. **83**: 212-218.
138. **Hoshino, O., U. Zehavi, P. Sinay and R. W. Jeanloz**. 1972. The isolation and structure identification of a discaccharide containing manno-muramic acid from *Micrococcus lysodeikticus* cell wall. J. Biol. Chem. **247**: 381-390.
139. **Hoskins, J., P. Matsushima, D. L. Mullen, J. Tang, G. Zhao, T. I. Meier, T. I. Nicas and S. R. Jaskunas**. 1999. Gene disruption studies of penicillin-binding proteins 1a, 1b, and 2a in *Streptococcus pneumoniae*. J. Bacteriol. **181**: 6552-6555.
140. **Huang, Y. C., and C. J. Chen**. 2011. Community-associated methicillin-resistant *Staphylococcus aureus* in children in Taiwan, 2000s. Int. J. Antimicrob. Agents. **38**: 2-8.
141. **Hunter, H. N., W. Jing, D. J. Schibli, T. Trinh, I. Y. Park, S. C. Kim and H. J. Vogel**. 2005. The interactions of antimicrobial peptides derived from lysozyme with model membrane systems. Biochim. Biophys. Acta. **1668**: 175-189.
142. **Ibrahim, H. R., T. Matsuzaki and T. Aoki**. 2001. Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. FEBS Lett. **506**: 27-32.
143. **Ikeda, M., M. Wachi, H. K. Jung, F. Ishino and M. Matsuhashi**. 1991. The *Escherichia coli mraY* gene encoding UDP-*N*-acetylmuramoyl-pentapeptide: undecaprenyl-phosphate phospho-*N*-acetylmuramoyl-pentapeptide transferase. J. Bacteriol. **173**: 1021-1026.
144. **Ingavale, S. S., W. Van Wamel and A. L. Cheung**. 2003. Characterization of RAT, an autolysis regulator in *Staphylococcus aureus*. Mol. Microbiol. **48**: 1451-1466.
145. **International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC).** 2009. Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. Antimicrob Agents Chemother **53:** 4961-4967.
146. **Ito, E., and J. L. Strominger**. 1962. Enzymatic synthesis of peptide in bacterial uridine nucleotides. I. Enzymatic addition of L-alanine, D-glutamic acid and L-lysine. J. Biol. Chem. **237**: 2696-2695.

147. **Ito, E., and J. L. Strominger**. 1962. Enzymatic synthesis of peptide in bacterial uridine nucleotides. II. Enzymatic synthesis and addition of D-alanyl-D-alanine. J. Biol. Chem. **237**: 2696-2703.
148. **Ito, E. and J. L. Strominger**. 1973. Enzymatic synthesis of the peptide in bacterial uridine nucleotides. VII. Comparative biochemistry. J. Biol. Chem. **248**: 3131-3136.
149. **Ito, E. and J. L. Strominger**. 1960. Enzymatic synthesis of the peptide in a uridine nucleotide from *Staphylococcus aureus*. J. Biol. Chem. **235**: PC5-6.
150. **Ito, E. and J. L. Strominger**. 1960. Enzymatic addition of lysine to a uridine nucleotide. J. Biol. Chem. **235**: PC7-8.
151. **Ito, T., K. Okuma, X. X. Ma, H. Yuzawa, and K. Hiramatsu**. 2003. Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC. Drug Resist. Updat. **6**: 41-52.
152. **Ito, T., X. X. Ma, F. Takeuchi, K. Okuma, H. Yuzawa, and K. Hiramatsu**. 2004. Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. Antimicrob. Agents Chemother. **48**: 2637-2651.
153. **Ito, T., Y. Katayama, and K. Hiramatsu**. 1999. Cloning and nucleotide sequence determination of the entire *mec* DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. Antimicrob. Agents Chemother. **43**: 1449-1458.
154. **Jacoby, G. A., and A. A. Medeiros**. 1991. More extended-spectrum beta-lactamases. Antimicrob. Agents Chemother. **35**: 1697-1704.
155. **Jenkins, T. C., B. D. McCollister, R. Sharma, K. K. McFann, N. E. Madinger, M. Barron, M. Bessesen, C. S. Price, and W. J. Burman**. 2009. Epidemiology of healthcare-associated bloodstream infection caused by USA300 strains of methicillin-resistant *Staphylococcus aureus* in 3 affiliated hospitals. Infect. Control Hosp. Epidemiol. **30**: 233-241.
156. **Jevons, M. P.** 1961. Celbenin-resistant *Staphylococci*. Br. Med. J. **1**: 124-125.
157. **Johnson, A. P., A. Pearson, and G. Duckworth.** 2005. Surveillance and epidemiology of MRSA bacteraemia in the UK. J. Antimicrob. Chemother. **56**: 455-462.
158. **Jolly, L., S. Wu, J. van Heijenoort, H. de Lencastre, D. Mengin-Lecreulx, and A. Tomasz**. 1997. The *femR315* gene from *Staphylococcus aureus,* the interruption of which results in reduced methicillin resistance, encodes a phosphoglucosamine mutase. J. Bacteriol. **179**: 5321-5325.
159. **Kaito, C., Y. Saito, G. Nagano, M. Ikuo, Y. Omae, Y. Hanada, X. Han, K. Kuwahara-Arai, T. Hishinuma, T. Baba, T. Ito, K. Hiramatsu, and K. Sekimizu**. 2011. Transcription and translation products of the cytolysin gene *psm-mec* on the mobile genetic element SCC*mec* regulate *Staphylococcus aureus* virulence. PLoS Pathog. **7**: e1001267.
160. **Kaito, C., Y. Saito, M. Ikuo, Y. Omae, H. Mao, G. Nagano, T. Fujiyuki, S. Numata, X. Han, K. Obata, S. Hasegawa, H. Yamaguchi, K. Inokuchi, T. Ito, K. Hiramatsu, and K. Sekimizu**. 2013. Mobile genetic element SCC*mec*-encoded *psm-mec* RNA suppresses translation of *agrA* and attenuates MRSA virulence. PLoS Pathog. **9**: e1003269.
161. **Kajimura, J., T. Fujiwara, S. Yamada, Y. Suzawa, T. Nishida, Y. Oyamada, I. Hayashi, J. Yamagishi, H. Komatsuzawa and M. Sugai**.

2005. Identification and molecular characterization of an *N*-acetylmuramyl-L-alanine amidase Sle1 involved in cell separation of *Staphylococcus aureus*. Mol. Microbiol. **58**: 1087-1101.

162. **Kaneko, J., T. Kimura, S. Narita, T. Tomita, and Y. Kamio**. 1998. Complete nucleotide sequence and molecular characterization of the temperate staphylococcal bacteriophage phiPVL carrying Panton-Valentine leukocidin genes. Gene. **215**: 57-67.
163. **Kaneko, J., and Y. Kamio**. 2004. Bacterial two-component and hetero-heptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes. Biosci. Biotechnol. Biochem. **68**: 981-1003.
164. **Kaplan, S. L., K. G. Hulten, B. E. Gonzalez, W. A. Hammerman, L. Lamberth, J. Versalovic, and E. O. Mason**. 2005. Three-year surveillance of community-acquired *Staphylococcus aureus* infections in children. Clin. Infect. Dis. **40**: 1785-1791.
165. **Katayama, Y., D. A. Robinson, M. C. Enright, and H. F. Chambers**. 2005. Genetic background affects stability of *mecA* in *Staphylococcus aureus*. J. Clin. Microbiol. **43**: 2380-2383.
166. **Katayama, Y., H. Z. Zhang, and H. F. Chambers**. 2003. Effect of disruption of *Staphylococcus aureus pbp4* gene on resistance to beta-lactam antibiotics. Microb. Drug Resist. **9**: 329-336.
167. **Katayama, Y., T. Ito, and K. Hiramatsu**. 2000. A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **44**: 1549-1555.
168. **Khanna, T., R. Friendship, C. Dewey, and J. S. Weese**. 2008. Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. Vet. Microbiol. **128**: 298-303.
169. **Kim, C., M. Mwangi, M. Chung, C. Milheirco, H. de Lencastre, and A. Tomasz**. 2013. The mechanism of heterogeneous beta-lactam resistance in MRSA: key role of the stringent stress response. PLoS One. **8**: e82814.
170. **Kim, S. J., L. Cegelski, M. Preobrazhenskaya and J. Schaefer**. 2006. Structures of *Staphylococcus aureus* cell-wall complexes with vancomycin, eremomycin, and chloroeremomycin derivatives by 13C{19F} and 15N{19F} rotational-echo double resonance. Biochemistry. **45**: 5235-5250.
171. **Klevens, R. M., M. A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B. Carey, and S. K. Fridkin**. 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. J.A.M.A. **298**: 1763-1771.
172. **Klevens, R. M., M. A. Morrison, S. K. Fridkin, A. Reingold, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, G. Fosheim, L. K. McDougal, F. C. Tenover and the Active Bacterial Core Surveillance of the Emerging Infections Program Network.** 2006. Community-associated methicillin-resistant *Staphylococcus aureus* and healthcare risk factors. Emerg. Infect. Dis. **12**: 1991-1993.

173. **Kloos, W.** 1997. Taxonomy and systematics of staphylococci indigenous to humans., p. 113-137 *In* KBCaGLA (ed.), The staphylococci in human disease. Churchill Livingstone, New York, U.S.A.
174. **Kokoshis, P. L., D. L. Williams, J. A. Cook and N. R. Di Luzio**. 1978. Increased resistance to *Staphylococcus aureus* infection and enhancement in serum lysozyme activity by glucan. Science. **199**: 1340-1342.
175. **Komatsuzawa, H., K. Ohta, H. Labischinski, M. Sugai, and H. Suginaka**. 1999. Characterization of *fmtA*, a gene that modulates the expression of methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **43**: 2121-2125.
176. **Komatsuzawa, H., M. Sugai, K. Ohta, T. Fujiwara, S. Nakashima, J. Suzuki, C.Y. Lee, and H. Suginaka**. 1997. Cloning and characterization of the *fmt* gene which affects the methicillin resistance level and autolysis in the presence of triton X-100 in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents. Chemother. **41**: 2355-2361.
177. **Kondo, N., K. Kuwahara-Arai, H. Kuroda-Murakami, E. Tateda-Suzuki, and K. Hiramatsu**. 2001. Eagle-type methicillin resistance: new phenotype of high methicillin resistance under *mec* regulator gene control. Antimicrob. Agents. Chemother. **45**: 815-824.
178. **Kopp, U., M. Roos, J. Wecke, and H. Labischinski**. 1996. Staphylococcal peptidoglycan interpeptide bridge biosynthesis: a novel antistaphylococcal target? Microb. Drug Resist. **2**: 29-41.
179. **Kornblum, J., B. J. Hartman, R. P. Novick, and A. Tomasz**. 1986. Conversion of a homogeneously methicillin-resistant strain of *Staphylococcus aureus* to heterogeneous resistance by Tn*551*-mediated insertional inactivation. Eur. J. Clin. Microbiol. **5**: 714-718.
180. **Kozarich, J. W. and J. L. Strominger**. 1978. A membrane enzyme from *Staphylococcus aureus* which catalyzes transpeptidase, carboxypeptidase, and penicillinase activities. J. Biol. Chem. **253**: 1272-1278.
181. **Kuhn, G., T. Koessler, D. C. Melles, P. Francois, A. Huyghe, P. Dunman, M. C. Vos, G. Zanetti, J. Schrenzel, A. van Belkum, and D. S. Blanc**. 2010. Comparative genomics of epidemic versus sporadic *Staphylococcus aureus* strains does not reveal molecular markers for epidemicity. Infect. Genet. Evol. **10**: 89-96.
182. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin and et al.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature. **390**: 249-256.
183. **Laaberki, M. H., J. Pfeffer, A. J. Clarke and J. Dworkin**. 2011. *O*-Acetylation of peptidoglycan is required for proper cell separation and S-layer anchoring in *Bacillus anthracis*. J. Biol. Chem. **286**: 5278-5288.
184. **Laible, N. J. and G. R. Germaine**. 1985. Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against *Streptococcus sanguis* and *Streptococcus faecalis*: inhibition by chitin oligosaccharides. Infect. Immun. **48**: 720-728.

185. **Larsen, A. R., M. Stegger, S. Bocher, M. Sorum, D. L. Monnet, and R. L. Skov**. 2009. Emergence and characterization of community-associated methicillin-resistant *Staphyloccocus aureus* infections in Denmark, 1999 to 2006. J. Clin. Microbiol. **47**: 73-78.
186. **Larsen, A. R., S. Böcher, M. Stegger, R. Goering, L. V. Pallesen, and R. Skov.** 2008. Epidemiology of European community-associated methicillin-resistant *Staphylococcus aureus* clonal complex 80 type IV strains isolated in Denmark from 1993 to 2004. J. Clin. Microbiol. **46**: 62-68.
187. **Lee, S. H., L. W. Jarantow, H. Wang, S. Sillaots, H. Cheng, T. C. Meredith, J. Thompson, and T. Roemer**. 2011. Antagonism of chemical genetic interaction networks resensitize MRSA to beta-lactam antibiotics. Chem. Biol. **18**: 1379-1389.
188. **Lee, S. M., M. Ender, R. Adhikari, J. M. Smith, B. Berger-Bachi, and G. M. Cook**. 2007. Fitness cost of staphylococcal cassette chromosome *mec* in methicillin-resistant *Staphylococcus aureus* by way of continuous culture. Antimicrob. Agents. Chemother. **51**: 1497-1499.
189. **Lehrer, R. I. and T. Ganz**. 1999. Antimicrobial peptides in mammalian and insect host defence. Curr. Opin. Immunol. **11**: 23-27.
190. **LeMarbre, P., J. J. Rinehart, N. E. Kay, R. Vesella and H. S. Jacob**. 1981. Lysozyme enhances monocyte-mediated tumoricidal activity: a potential amplifying mechanism of tumor killing. Blood. **58**: 994-999.
191. **Levy, O.** 2000. Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. Blood. **96**: 2664-2672.
192. **Lewis, R. A., and K. G. Dyke**. 2000. MecI represses synthesis from the beta-lactamase operon of *Staphylococcus aureus*. J. Antimicrob. Chemother. **45**: 139-144.
193. **Lewis, R. A., S. P. Curnock, and K. G. Dyke**. 1999. Proteolytic cleavage of the repressor (BlaI) of beta-lactamase synthesis in *Staphylococcus aureus*. FEMS Microbiol Lett. **178**: 271-275.
194. **Li, M., B. A. Diep, A. E. Villaruz, K. R. Braughton, X. Jiang, F. R. DeLeo, H. F. Chambers, Y. Lu, and M. Otto**. 2009. Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U. S. A. **106**: 5883-5888.
195. **Liang, X., L. Zheng, C. Landwehr, D. Lunsford, D. Holmes and Y. Ji**. 2005. Global regulation of gene expression by ArlRS, a two-component signal transduction regulatory system of *Staphylococcus aureus*. J. Bacteriol. **187**: 5486-5492.
196. **Lim, D., and N. C. Strynadka**. 2002. Structural basis for the beta lactam resistance of PBP2a from methicillin-resistant *Staphylococcus aureus*. Nat. Struct. Biol. **9**: 870-876.
197. **Lina, G., Y. Piemont, F. Godail-Gamot, M. Bes, M. O. Peter, V. Gauduchon, F. Vandenesch, and J. Etienne**. 1999. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin. Infect. Dis. **29**: 1128-1132.
198. **Livermore, D. M.** 2001. Of Pseudomonas, porins, pumps and carbapenems. J. Antimicrob. Chemother. **47**: 247-250.
199. **Livermore, D. M.** 1995. beta-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8**: 557-584.

200. **Llarrull, L. I., J. F. Fisher, and S. Mobashery**. 2009. Molecular basis and phenotype of methicillin resistance in *Staphylococcus aureus* and insights into new beta-lactams that meet the challenge. Antimicrob. Agents Chemother. **53**: 4051-4063.
201. **Logardt, I. M. and H. Y. Neujahr**. 1975. Lysis of modified walls from *Lactobacillus fermentum*. J. Bacteriol. **124**: 73-77.
202. **Lovering, A. L., L. H. de Castro, D. Lim and N. C. Strynadka**. 2007. Structural insight into the transglycosylation step of bacterial cell-wall biosynthesis. Science. **315**: 1402-1405.
203. **Lowy, F. D.** 2003. Antimicrobial resistance: the example of *Staphylococcus aureus*. J. Clin. Invest. **111**: 1265-1273.
204. **Ludovice, A. M., S. W. Wu, and H. de Lencastre**. 1998. Molecular cloning and DNA sequencing of the *Staphylococcus aureus* UDP-*N*-acetylmuramyl tripeptide synthetase (*murE*) gene, essential for the optimal expression of methicillin resistance. Microb. Drug Resist. **4**: 85-90.
205. **Luong, T. T., S. W. Newell and C. Y. Lee**. 2003. Mgr, a novel global regulator in *Staphylococcus aureus*. J. Bacteriol. **185**: 3703-3710.
206. **Maidhof, H., B. Reinicke, P. Blumel, B. Berger-Bachi and H. Labischinski**. 1991. *femA*, which encodes a factor essential for expression of methicillin resistance, affects glycine content of peptidoglycan in methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. J. Bacteriol. **173**: 3507-3513.
207. **Maisnier-Patin, S., and D. I. Andersson**. 2004. Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. Res. Microbiol. **155**: 360-369.
208. **Majerczyk, C. D., M. R. Sadykov, T. T. Luong, C. Lee, G. A. Somerville and A. L. Sonenshein**. 2008. *Staphylococcus aureus* CodY negatively regulates virulence gene expression. J. Bacteriol. **190**: 2257-2265.
209. **Maki, H., T. Yamaguchi and K. Murakami**. 1994. Cloning and characterization of a gene affecting the methicillin resistance level and the autolysis rate in *Staphylococcus aureus*. J. Bacteriol. **176**: 4993-5000.
210. **Manna, A. C., S. S. Ingavale, M. Maloney, W. van Wamel and A. L. Cheung**. 2004. Identification of sarV (SA2062), a new transcriptional regulator, is repressed by SarA and MgrA (SA0641) and involved in the regulation of autolysis in *Staphylococcus aureus*. J. Bacteriol. **186**: 5267-5280.
211. **Manzur, A., A. M. Dominguez, M. Pujol, M. P. Gonzalez, E. Limon, A. Hornero, R. Martin, F. Gudiol, and J. Ariza**. 2008. Community-acquired methicillin-resistant *Staphylococcus aureus* infections: an emerging threat in Spain. Clin. Microbiol. Infect. **14**: 377-380.
212. **Marchese, A., L. Gualco, E. Maioli, and E. E. Debbia.** 2009. Molecular analysis and susceptibility patterns of methicillin-resistant *Staphylococcus aureus* (MRSA) strains circulating in the community in the Ligurian area, a northern region of Italy: emergence of USA300 and EMRSA-15 clones. Int. J. Antimicrob. Agents **34**: 424-428.
213. **Massova, I., and S. Mobashery**. 1998. Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. Antimicrob. Agents Chemother. **42**: 1-17.

214. **Matagne, A., A. M. Misselyn-Bauduin, B. Joris, T. Erpicum, B. Granier, and J. M. Frere**. 1990. The diversity of the catalytic properties of class A beta-lactamases. Biochem. J. **265**: 131-146.
215. **Matagne, A., and J. M. Frere**. 1995. Contribution of mutant analysis to the understanding of enzyme catalysis: the case of class A beta-lactamases. Biochim. Biophys. Acta. **1246**: 109-127.
216. **Matsuhashi, M., C. P. Dietrich and J. L. Strominger**. 1965. Incorporation of glycine into the cell wall glycopeptide in *Staphylococcus aureus*: role of sRNA and lipid intermediates. Proc. Natl. Acad. Sci. U. S. A. **54**: 587-594.
217. **Matthews, P., and A. Tomasz**. 1990. Insertional inactivation of the *mec* gene in a transposon mutant of a methicillin-resistant clinical isolate of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **34**: 1777-1779.
218. **Matthews, P. R., and P. R. Stewart**. 1984. Resistance heterogeneity in methicillin-resistant *Staphylococcus aureus*. FEMS Microbiol. Lett. **22**:161-164.
219. **Maugat, S., A. de Rougemont, H. Aubry-Damon H, M. E. Reverdy, S. Georges, F. Vandenesch, J. Etienne, and B. Coignard.** 2009. Methicillin-resistant *Staphylococcus aureus* among a network of French private-sector community-based-medical laboratories. Med. Mal. Infect. **39**: 311-318.
220. **McCaskill, M. L., E. O. Mason, S. L. Kaplan, W. Hammerman, L. B. Lamberth, and K. G. Hulten**. 2007. Increase of the USA300 clone among community-acquired methicillin-susceptible *Staphylococcus aureus* causing invasive infections. Pediatr. Infect. Dis. J. **26**: 1122-1127.
221. **McKinney, T. K., V. K. Sharma, W. A. Craig, and G. L. Archer**. 2001. Transcription of the gene mediating methicillin resistance in *Staphylococcus aureus* (*mecA*) is corepressed but not coinduced by cognate *mecA* and beta-lactamase regulators. J. Bacteriol. **183**: 6862-6868.
222. **McPherson, D. C. and D. L. Popham**. 2003. Peptidoglycan synthesis in the absence of class A penicillin-binding proteins in *Bacillus subtilis*. J. Bacteriol. **185**: 1423-1431.
223. **Medeiros, A. A.** 1997. Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. Clin. Infect. Dis. **24 Suppl 1**: S19-45.
224. **Mediavilla, J. R., L. Chen, B. Mathema, and B. N. Kreiswirth**. 2012. Global epidemiology of community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA). Curr. Opin. Microbiol. **15**: 588-595.
225. **Memmi, G., S. R. Filipe, M. G. Pinho, Z. Fu, and A. Cheung**. 2008. *Staphylococcus aureus* PBP4 is essential for beta-lactam resistance in community-acquired methicillin-resistant strains. Antimicrob. Agents Chemother. **52**: 3955-3966.
226. **Mengin-Lecreulx, D. and B. Lemaitre**. 2005. Structure and metabolism of peptidoglycan and molecular requirements allowing its detection by the Drosophila innate immune system. J. Endotoxin. Res. **11**: 105-111.
227. **Milheirico, C., D. C. Oliveira, and H. de Lencastre**. 2007. Multiplex PCR strategy for subtyping the staphylococcal cassette chromosome *mec* type IV in methicillin-resistant *Staphylococcus aureus*: 'SCC*mec* IV multiplex'. J. Antimicrob. Chemother. **60**: 42-48.

228. **Milheiriço, C., Oliveira, D. C., and de Lencastre H.** 2007. Update to the multiplex PCR strategy for assignment of *mec* element types in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **51**: 3374-3377.
229. **Miller, L. G., F. Perdreau-Remington, G. Rieg, S. Mehdi, J. Perlroth, A. S. Bayer, A. W. Tang, T. O. Phung, and B. Spellberg**. 2005. Necrotizing fasciitis caused by community-associated methicillin-resistant *Staphylococcus aureus* in Los Angeles. N. Engl. J. Med. **352**: 1445-1453.
230. **Mohammadi, T., R. Sijbrandi, M. Lutters, J. Verheul, N. Martin, T. den Blaauwen, B. de Kruijff and E. Breukink**. 2014. Specificity of the transport of Lipid II by FtsW in *Escherichia coli*. J. Biol. Chem. **289**: 14707-14718.
231. **Mohammadi, T., V. van Dam, R. Sijbrandi, T. Vernet, A. Zapun, A. Bouhss, M. Diepeveen-de Bruin, M. Nguyen-Disteche, B. de Kruijff and E. Breukink**. 2011. Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane. EMBO J. **30**: 1425-1432.
232. **Moncada, S., and A. Higgs**. 1993. The L-arginine-nitric oxide pathway. N. Engl. J. Med. **329**: 2002-2012.
233. **Monecke, S., G. Coombs, A. C. Shore, D. C. Coleman, P. Akpaka, M. Borg, H. Chow, M. Ip, L. Latzwauk, D. Jonas, K. Kadlec, A. Kearns, F. Laurent, F. G. O'Brien, J. Pearson, A. Ruppelt, S. Schwarz, E. Scicluna, P. Slickers, H. L. Tan, S. Weber, and R. Ehricht**. 2011. A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. PLoS One. **6**: e17936.
234. **Moore, P. C., and J. A. Lindsay.** 2002. Molecular characterisation of the dominant UK methicillin-resistant *Staphylococcus aureus* strains, EMRSA-15 and EMRSA-16. J. Med. Microbiol. **51**: 516-521.
235. **Moran, G. J., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, L. K. McDougal, R. B. Carey, D. A. Talan, and Emergency ID Net Study Group.** 2006. Methicillin-resistant *S. aureus* infections among patients in the emergency department. N. Engl. J. Med. **355**: 666-674.
236. **Murakami, K., and A. Tomasz**. 1989. Involvement of multiple genetic determinants in high-level methicillin resistance in *Staphylococcus aureus*. J. Bacteriol. **171**: 874-879.
237. **Musser, J. M., and V. Kapur**. 1992. Clonal analysis of methicillin-resistant *Staphylococcus aureus* strains from intercontinental sources: association of the *mec* gene with divergent phylogenetic lineages implies dissemination by horizontal transfer and recombination. J. Clin. Microbiol. **30**: 2058-2063.
238. **Mwangi, M. M., C. Kim, M. Chung, J. Tsai, G. Vijayadamodar, M. Benitez, T. P. Jarvie, L. Du, and A. Tomasz**. 2013. Whole-genome sequencing reveals a link between beta-lactam resistance and synthetases of the alarmone (p)ppGpp in *Staphylococcus aureus*. Microb. Drug Resist. **19**: 153-159.
239. **Naimi, T. S., K. H. LeDell, D. J. Boxrud, A. V. Groom, C. D. Steward, S. K. Johnson, J. M. Besser, C. O'Boyle, R. N. Danila, J. E. Cheek, M. T. Osterholm, K. A. Moore, and K. E. Smith**. 2001. Epidemiology and clonality of community-acquired methicillin-resistant *Staphylococcus aureus* in Minnesota, 1996-1998. Clin. Infect. Dis. **33**: 990-996.
240. **Nakao, A., S. Imai, and T. Takano**. 2000. Transposon-mediated insertional mutagenesis of the D-alanyl-lipoteichoic acid (*dlt)* operon raises

methicillin resistance in *Staphylococcus aureus*. Res. Microbiol. **151**: 823-829.

241. **Nanninga, N.** 1998. Morphogenesis of *Escherichia coli*. Microbiol. Mol. Biol. Rev. **62**: 110-129.
242. **Neu, H. C.** 1986. Beta-Lactam antibiotics: structural relationships affecting *in vitro* activity and pharmacologic properties. Rev. Infect. Dis. **8 Suppl 3**: S237-259.
243. **Neuhaus, F. C. and J. Baddiley**. 2003. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. Microbiol. Mol. Biol. Rev. **67**: 686-723.
244. **Nimmo, G. R.** 2012. USA300 abroad: global spread of a virulent strain of community-associated methicillin-resistant *Staphylococcus aureus*. Clin Microbiol Infect. **18**: 725-734.
245. **Novick, R. P.** 1962. Staphylococcal penicillinase and the new penicillins. Biochem. J. **83**: 229-235.
246. **Nübel, U., P. Roumagnac, M. Feldkamp, J. H. Song, K. S. Ko, Y. C. Huang, G. Coombs, H. Westh, R. Skov, M. J. Struelens, R. V. Goering, B. Strommenger, A. Weller, W. Witte, and M. Achtman**. 2008. Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U.S.A. **105**: 14130-14135.
247. **Ogston, A.** 1881. Report upon Micro-Organisms in Surgical Diseases. Br. Med. J. **1**: 369-375.
248. **Ohno, N., T. Yadomae and T. Miyazaki**. 1982. Identification of 2-amino-2-deoxyglucose residues in the peptidoglycan of *Streptococcus pneumoniae*. Carbohydr. Res. **107**: 152-155.
249. **Okonogi, K., Y. Noji, M. Nakao, and A. Imada** 1995. The possible physiological roles of penicillin-binding proteins of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. J. Infec. Chem. **1**: 50-58.
250. **Okuma, K., K. Iwakawa, J. D. Turnidge, W. B. Grubb, J. M. Bell, F. G. O'Brien, G. W. Coombs, J. W. Pearman, F. C. Tenover, M. Kapi, C. Tiensasitorn, T. Ito, and K. Hiramatsu**. 2002. Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community. J. Clin. Microbiol. **40**: 4289-4294.
251. **Oliveira, D. C., A. Tomasz, and H. de Lencastre**. 2002. Secrets of success of a human pathogen: molecular evolution of pandemic clones of meticillin-resistant *Staphylococcus aureus*. Lancet Infect. Dis. **2**: 180-189.
252. **Ornelas-Soares, A., H. de Lencastre, B. de Jonge, D. Gage, Y. S. Chang, and A. Tomasz**. 1993. The peptidoglycan composition of a *Staphylococcus aureus* mutant selected for reduced methicillin resistance. J. Biol. Chem. **268**: 26268-26272.
253. **Ornelas-Soares, A., H. de Lencastre, B. L. de Jonge, and A. Tomasz**. 1994. Reduced methicillin resistance in a new *Staphylococcus aureus* transposon mutant that incorporates muramyl dipeptides into the cell wall peptidoglycan. J. Biol. Chem. **44**: 27246-27250.
254. **Oshida, T., M. Sugai, H. Komatsuzawa, Y. M. Hong, H. Suginaka and A. Tomasz**. 1995. A *Staphylococcus aureus* autolysin that has an *N*-acetylmuramoyl-L-alanine amidase domain and an endo-beta-*N*-acetylglucosaminidase domain: cloning, sequence analysis, and characterization. Proc. Natl. Acad. Sci. U. S. A. **92**: 285-289.

255. **Otter, J. A., and G. L. French.** 2011. Community-associated methicillin-resistant *Staphylococcus aureus* strains as a cause of healthcare-associated infection. J. Hosp. Infect. **79**: 189-193.
256. **Otter, J. A., and G. L. French**. 2006. Nosocomial transmission of community-associated methicillin-resistant *Staphylococcus aureus*: an emerging threat. Lancet Infect. Dis. **6**: 753-755.
257. **Otto, M.** 2010. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. Annu. Rev. Microbiol. **64**: 143-162.
258. **Pannaraj, P. S., K. G. Hulten, B. E. Gonzalez, E. O. Mason, and S. L. Kaplan**. 2006. Infective pyomyositis and myositis in children in the era of community-acquired, methicillin-resistant *Staphylococcus aureus* infection. Clin. Infect. Dis. **43**: 953-960.
259. **Park, J. T. and M. J. Johnson**. 1949. Accumulation of labile phosphate in *Staphylococcus aureus* grown in the presence of penicillin. J. Biol. Chem. **179**: 585-592.
260. **Pasquina, L. W., J. P. Santa Maria and S. Walker**. 2013. Teichoic acid biosynthesis as an antibiotic target. Curr. Opin. Microbiol. **16**: 531-537.
261. **Patel, M., K. B. Waites, C. J. Hoesley, A. M. Stamm, K. C. Canupp, and S. A. Moser**. 2008. Emergence of USA300 MRSA in a tertiary medical centre: implications for epidemiological studies. J. Hosp. Infect. **68**: 208-213.
262. **Peacock, S. J., I. de Silva and F. D. Lowy**. 2001. What determines nasal carriage of *Staphylococcus aureus*? Trends Microbiol. **9**: 605-610.
263. **Pereira, S. F., A. O. Henriques, M. G. Pinho, H. de Lencastre, and A. Tomasz**. 2007. Role of PBP1 in cell division of *Staphylococcus aureus*. J. Bacteriol. **189**: 3525-3531.
264. **Pereira, S. F., A. O. Henriques, M. G. Pinho, H. de Lencastre and A. Tomasz**. 2009. Evidence for a dual role of PBP1 in the cell division and cell separation of *Staphylococcus aureus*. Mol. Microbiol. **72**:895-904.
265. **Peschel, A.** 2002. How do bacteria resist human antimicrobial peptides? Trends Microbiol. **10**: 179-186.
266. **Peschel, A., M. Otto, R. W. Jack, H. Kalbacher, G. Jung and F. Gotz**. 1999. Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J. Biol. Chem. **274**: 8405-8410.
267. **Peschel, A. and H. G. Sahl**. 2006. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat. Rev. Microbiol. **4**: 529-536.
268. **Peschel, A., C. Vuong, M. Otto and F. Gotz**. 2000. The D-alanine residues of *Staphylococcus aureus* teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes. Antimicrob. Agents Chemother. **44**: 2845-2847.
269. **Phillips, D. C.** 1966. The three-dimensional structure of an enzyme molecule. Sci. Am. **215**: 78-90.
270. **Pinho, M. G., A. M. Ludovice, S. Wu, and H. de Lencastre**. 1997. Massive reduction in methicillin resistance by transposon inactivation of the normal PBP2 in a methicillin-resistant strain of *Staphylococcus aureus*. Microb. Drug Resist. **3**: 409-413.

271. **Pinho, M. G. and J. Errington**. 2003. Dispersed mode of *Staphylococcus aureus* cell wall synthesis in the absence of the division machinery. Mol. Microbiol. **50**: 871-881.
272. **Pinho, M. G. and J. Errington**. 2005. Recruitment of penicillin-binding protein PBP2 to the division site of *Staphylococcus aureus* is dependent on its transpeptidation substrates. Mol. Microbiol. **55**: 799-807.
273. **Pinho, M. G., H. de Lencastre, and A. Tomasz**. 2001. An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. Proc. Natl. Acad. Sci. U. S. A. **98**: 10886-10891.
274. **Pinho, M. G., H. de Lencastre, and A. Tomasz**. 2000. Cloning, characterization, and inactivation of the gene *pbpC*, encoding penicillin-binding protein 3 of *Staphylococcus aureus*. J. Bacteriol. **182**: 1074-1079.
275. **Pinho, M. G., M. Kjos and J. W. Veening**. 2013. How to get (a)round: mechanisms controlling growth and division of coccoid bacteria. Nat. Rev. Microbiol. **11**: 601-614.
276. **Pinho, M. G., S. R. Filipe, H. de Lencastre, and A. Tomasz**. 2001. Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in *Staphylococcus aureus*. J. Bacteriol. **183**: 6525-6531.
277. **Pooley, H. M., and D. Karamata** 1994. Teichoic acid synthesis in *Bacillus subtilis*: genetic organization and biological roles. Bacterial Cell Wall. Elsevier Science Publishers. 187-197.
278. **Popovich, K. J., R. A. Weinstein, and B. Hota**. 2008. Are community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) strains replacing traditional nosocomial MRSA strains? Clin. Infect. Dis. **46**: 787-794.
279. **Popowska, M., M. Kusio, P. Szymanska and Z. Markiewicz**. 2009. Inactivation of the wall-associated de-*N*-acetylase (PgdA) of *Listeria monocytogenes* results in greater susceptibility of the cells to induced autolysis. J. Microbiol. Biotechnol. **19**: 932-945.
280. **Projan, S. J., and R. P. Novick**. 1997. The molecular basis of pathogenicity., p. 55-81 *In* D. B. Archer (ed.), The staphylococci in human disease. Churchill Livingstone Inc., New York, U.S.A.
281. **Queck, S. Y., B. A. Khan, R. Wang, T. H. Bach, D. Kretschmer, L. Chen, B. N. Kreiswirth, A. Peschel, F. R. Deleo, and M. Otto**. 2009. Mobile genetic element-encoded cytolysin connects virulence to methicillin resistance in MRSA. PLoS Pathog. **5**: e1000533.
282. **Ramadurai, L. and R. K. Jayaswal**. 1997. Molecular cloning, sequencing, and expression of lytM, a unique autolytic gene of *Staphylococcus aureus*. J. Bacteriol. **179**: 3625-3631.
283. **Ramadurai, L., K. J. Lockwood, M. J. Nadakavukaren and R. K. Jayaswal**. 1999. Characterization of a chromosomally encoded glycylglycine endopeptidase of *Staphylococcus aureus*. Microbiology. **145 (Pt 4)**: 801-808.
284. **Rammelkamp, C. H., and T. Maxon** 1942. Resistance of *Staphylococcus aureus* to the action of penicillin Proc. Royal Soc. Expert. Biol. Med. **51**: 386-389.

285. **Recsei, P., B. Kreiswirth, M. O'Reilly, P. Schlievert, A. Gruss and R. P. Novick**. 1986. Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agar*. Mol. Gen. Genet. **202**: 58-61.
286. **Reed, P., H. Veiga, A. M. Jorge, M. Terrak and M. G. Pinho**. 2011. Monofunctional transglycosylases are not essential for *Staphylococcus aureus* cell wall synthesis. J. Bacteriol. **193**: 2549-2556.
287. **Reichmann, N. T. and A. Grundling**. 2011. Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum Firmicutes. FEMS Microbiol. Lett. **319**: 97-105.
288. **Reynolds, P. E., and D. F. Brown**. 1985. Penicillin-binding proteins of beta-lactam-resistant strains of *Staphylococcus aureus*. Effect of growth conditions. FEBS Lett. **192**: 28-32.
289. **Rice, K. C., B. A. Firek, J. B. Nelson, S. J. Yang, T. G. Patton and K. W. Bayles**. 2003. The *Staphylococcus aureus cidAB* operon: evaluation of its role in regulation of murein hydrolase activity and penicillin tolerance. J. Bacteriol. **185**: 2635-2643.
290. **Rice, L. B., L. L. Carias, S. Rudin, R. Hutton, S. Marshall, M. Hassan, N. Josseaume, L. Dubost, A. Marie and M. Arthur**. 2009. Role of class A penicillin-binding proteins in the expression of beta-lactam resistance in *Enterococcus faecium*. J. Bacteriol. **191**: 3649-3656.
291. **Richmond, M. H.** 1965. Dominance of the Inducible State in Strains of *Staphylococcus Aureus* Containing Two Distinct Penicillinase Plasmids. J Bacteriol. **90**: 370-374.
292. **Richmond, M. H.** 1963. Purification and Properties of the Exopenicillinase from *Staphylococcus Aureus*. Biochem. J. **88**: 452-459.
293. **Robinson, D. A., A. M. Kearns, A. Holmes, D. Morrison, H. Grundmann, G. and F. G. O. B. Edwards, F. C. Tenover, L. K. McDougal, A. B. Monk, and M. C. Enright**. 2005. Re-emergence of early pandemic *Staphylococcus aureus* as a community-acquired meticillin-resistant clone. Lancet. **365**: 1256-1258.
294. **Robinson, D. A., and M. C. Enright**. 2003. Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **47**: 3926-3934.
295. **Roemer, T., T. Schneider and M. G. Pinho**. 2013. Auxiliary factors: a chink in the armor of MRSA resistance to beta-lactam antibiotics. Curr. Opin. Microbiol. **16**: 538-548.
296. **Rohrer, S., K. Ehlert, M. Tschierske, H. Labischinski and B. Berger-Bachi**. 1999. The essential *Staphylococcus aureus* gene *fmhB* is involved in the first step of peptidoglycan pentaglycine interpeptide formation. Proc. Natl. Acad. Sci. U. S. A. **96**: 9351-9356.
297. **Rolinson, G. N., S. Stevens, F. R. Batchelor, J. C. Wood, and E. B. Chain**. 1960. Bacteriological studies on a new penicillin-BRL. 1241. Lancet. **2**: 564-567.
298. **Rolo, J., M. Miragaia, A. Turlej-Rogacka, J. Empel, O. Bouchami, N. A. Faria, A. Tavares, W. Hryniewicz, A. C. Fluit, H. de Lencastre, and CONCORD Working Group**. 2012. High genetic diversity among community-associated *Staphylococcus aureus* in Europe: results from a multicenter study. PLoS One. **7**: e34768.

299. **Rosenthal, R. S., J. K. Blundell and H. R. Perkins**. 1982. Strain-related differences in lysozyme sensitivity and extent of O-acetylation of gonococcal peptidoglycan. Infect. Immun. **37**: 826-829.
300. **Roundtree, P. M., and B. M. Freeman** 1956. Infections caused by a particular phage type of *Staphylococcus aureus*. Med. J. Aust. **42**: 157-161.
301. **Rudkin, J. K., A. M. Edwards, M. G. Bowden, M. G. Brown, C. Pozzi, E. M. Waters, W. C. Chan, P. Williams, J. P. O'Gara, and R. C. Massey**. 2012. Methicillin resistance reduces the virulence of healthcare-associated methicillin-resistant *Staphylococcus aureus* by interfering with the *agr* quorum sensing system. J. Infect. Dis. **205**: 798-806.
302. **Ryffel, C., A. Strassle, F. H. Kayser, and B. Berger-Bachi**. 1994. Mechanisms of heteroresistance in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents. Chemother. **38**: 724-728.
303. **Said-Salim, B., P. M. Dunman, F. M. McAleese, D. Macapagal, E. Murphy, P. J. McNamara, S. Arvidson, T. J. Foster, S. J. Projan and B. N. Kreiswirth**. 2003. Global regulation of *Staphylococcus aureus* genes by Rot. J. Bacteriol. **185**: 610-619.
304. **Schindler, M., Y. Assaf, N. Sharon and D. M. Chipman**. 1977. Mechanism of lysozyme catalysis: role of ground-state strain in subsite D in hen egg-white and human lysozymes. Biochemistry. **16**: 423-431.
305. **Schlag, M., R. Biswas, B. Krismer, T. Kohler, S. Zoll, W. Yu, H. Schwarz, A. Peschel and F. Gotz**. 2010. Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl. Mol. Microbiol. **75**: 864-873.
306. **Schneider, T., M. M. Senn, B. Berger-Bachi, A. Tossi, H. G. Sahl, and I. Wiedemann**. 2004. *In vitro* assembly of a complete, pentaglycine interpeptide bridge containing cell wall precursor (lipid II-Gly5) of *Staphylococcus aureus*. Mol. Microbiol. **53**: 675-685.
307. **Severin, A., S. W. Wu, K. Tabei, and A. Tomasz**. 2005. High-level (beta)-lactam resistance and cell wall synthesis catalyzed by the *mecA* homologue of *Staphylococcus sciuri* introduced into *Staphylococcus aureus*. J. Bacteriol. **187**: 6651-6658.
308. **Seybold, U., E. V. Kourbatova, J. G. Johnson, S. J. Halvosa, Y. F. Wang, M. D. King, S. M. Ray, and H. M. Blumberg**. 2006. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of health care-associated blood stream infections. Clin. Infect. Dis. **42**: 647-656.
309. **Sharma, V. K., C. J. Hackbarth, T. M. Dickinson, and G. L. Archer**. 1998. Interaction of native and mutant MecI repressors with sequences that regulate *mecA*, the gene encoding penicillin binding protein 2a in methicillin-resistant staphylococci. J. Bacteriol. **180**: 2160-2166.
310. **Sheng, Y., X. Sun, Y. Shen, A. L. Bognar, E. N. Baker and C. A. Smith**. 2000. Structural and functional similarities in the ADP-forming amide bond ligase superfamily: implications for a substrate-induced conformational change in folylpolyglutamate synthetase. J. Mol. Biol. **302**: 427-440.
311. **Shore, A. C., E. C. Deasy, P. Slickers, G. Brennan, B. O'Connell, S. Monecke, R. Ehricht, and D. C. Coleman**. 2011. Detection of staphylococcal cassette chromosome *mec* type XI carrying highly divergent

mecA, *mecI*, *mecR1*, *blaZ*, and *ccr* genes in human clinical isolates of clonal complex 130 methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **55**: 3765-3773.

312. **Sidow, T., L. Johannsen and H. Labischinski**. 1990. Penicillin-induced changes in the cell wall composition of *Staphylococcus aureus* before the onset of bacteriolysis. Arch. Microbiol. **154**: 73-81.
313. **Siewert, G., and J. L. Strominger**. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XI. Formation of the isoglutamine amide group in the cell walls of *Staphylococcus aureus*. J. Biol. Chem. **243**: 783-790.
314. **Simanski, M., R. Glaser, B. Koten, U. Meyer-Hoffert, S. Wanner, C. Weidenmaier, A. Peschel and J. Harder**. 2013. *Staphylococcus aureus* subverts cutaneous defense by D-alanylation of teichoic acids. Exp. Dermatol. **22**: 294-296.
315. **Skinner, D., and C. S. Keefer**. 1941. Significance of bacter caused by *Staphylococcus aureus* Arch. Intern. Med. **68**: 851-875.
316. **Smith, C. A.** 2006. Structure, function and dynamics in the mur family of bacterial cell wall ligases. J. Mol. Biol. **362**: 640-655.
317. **Sobral, R. G., A. M. Ludovice, S. Gardete, K. Tabei, H. De Lencastre, and A. Tomasz**. 2003. Normally functioning *murF* is essential for the optimal expression of methicillin resistance in *Staphylococcus aureus*. Microb. Drug Resist. **9**: 231-241.
318. **Sobral, R. G., A. M. Ludovice, H. de Lencastre and A. Tomasz**. 2006. Role of *murF* in cell wall biosynthesis: isolation and characterization of a *murF* conditional mutant of *Staphylococcus aureus*. J. Bacteriol. **188**: 2543-2553.
319. **Soldo, B., V. Lazarevic and D. Karamata**. 2002. *tagO* is involved in the synthesis of all anionic cell-wall polymers in *Bacillus subtilis* 168. Microbiology. **148**: 2079-2087.
320. **Song, M. D., M. Wachi, M. Doi, F. Ishino, and M. Matsuhashi**. 1987. Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion. FEBS Lett. **221**: 167-171.
321. **Spratt, B. G., J. Zhou, M. Taylor and M. J. Merrick**. 1996. Monofunctional biosynthetic peptidoglycan transglycosylases. Mol. Microbiol. **19**: 639-640.
322. **Srikumar, R., E. Tsang, and K. Poole**. 1999. Contribution of the MexAB-OprM multidrug efflux system to the beta-lactam resistance of penicillin-binding protein and beta-lactamase-derepressed mutants of *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **44**: 537-540.
323. **Stranden, A. M., K. Ehlert, H. Labischinski and B. Berger-Bachi**. 1997. Cell wall monoglycine cross-bridges and methicillin hypersusceptibility in a *femAB* null mutant of methicillin-resistant *Staphylococcus aureus*. J. Bacteriol. **179**: 9-16.
324. **Sugai, M., T. Akiyama, H. Komatsuzawa, Y. Miyake and H. Suginaka**. 1990. Characterization of sodium dodecyl sulfate-stable *Staphylococcus aureus* bacteriolytic enzymes by polyacrylamide gel electrophoresis. J. Bacteriol. **172**: 6494-6498.
325. **Sutherland, R., and G. N. Rolinson**. 1964. Characteristics of Methicillin-Resistant staphylococci. J. Bacteriol. **87**: 887-899.

326. **Szmigielski, S., G. Prevost, H. Monteil, D. A. Colin, and J. Jeljaszewicz**. 1999. Leukocidal toxins of staphylococci. Zentralbl. Bakteriol. **289**: 185-201.
327. **Szweda, P., M. Schielmann, R. Kotlowski, G. Gorczyca, M. Zalewska and S. Milewski**. 2012. Peptidoglycan hydrolases-potential weapons against *Staphylococcus aureus*. Appl. Microbiol. Biotechnol. **96**: 1157-1174.
328. **Takahashi, J., H. Komatsuzawa, S. Yamada, T. Nishida, H. Labischinski, T. Fujiwara, M. Ohara, J. Yamagishi and M. Sugai**. 2002. Molecular characterization of an *atl* null mutant of *Staphylococcus aureus*. Microbiol. Immunol. **46**: 601-612.
329. **Tavares, A., M. Miragaia, J. Rolo, C. Coelho, H. de Lencastre, and CA-MRSA/MSSA working group.** 2013 High prevalence of hospital-associated methicillin-resistant *Staphylococcus aureus* in the community in Portugal: evidence for the blurring of community-hospital boundaries. Eur. J. Clin. Microbiol. Infect. Dis. **32**: 1269-1283.
330. **Tenover, F. C., L. K. McDougal, R. V. Goering, G. Killgore, S. J. Projan, J. B. Patel, and P. M. Dunman.** 2006. Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. J. Clin. Microbiol. **44**: 108-118.
331. **Tenover, F. C., and R. V. Goering**. 2009. Methicillin-resistant *Staphylococcus aureus* strain USA300: origin and epidemiology. J. Antimicrob. Chemother. **64**: 441-446.
332. **Terrak, M. and M. Nguyen-Disteche**. 2006. Kinetic characterization of the monofunctional glycosyltransferase from *Staphylococcus aureus*. J. Bacteriol. **188**: 2528-2532.
333. **Thakur, A., A. Chauhan and M. D. Willcox**. 1999. Effect of lysozyme on adhesion and toxin release by *Staphylococcus aureus*. Aust. N. Z. J. Ophthalmol. **27**: 224-227.
334. **Tiemersma, E. W., S. L. Bronzwaer, O. Lyytikäinen, J. E. Degener, P. Schrijnemakers, N. Bruinsma, J. Monen, W. Witte, H. Grundman and E. A. R. S. S. Participants**. 2004. Methicillin-resistant *Staphylococcus aureus* in Europe, 1999-2002. Emerg. Infect. Dis. **10**: 1627-1634.
335. **Tomasz, A.** 2000. The Staphylococcal Cell Wall., p. 351-360. *In* V. A. Fischetti, R. P. Novick, J. J. Ferretti, D. A. Portnoy, and J. I. Rood (eds.), Gram-Positive Pathogens. ASM Press, Washington, DC, U.S.A.
336. **Tomasz, A., and H. de Lencastre**. 1997. Molecular microbiology and epidemiology: coexistance or alliance?., p. 309-321. *In* R. P. Wenzel (ed.), Prevention and control of nosocomial infections. Williams & Wilkins, Baltimore, MD, U.S.A.
337. **Traber, K. E., E. Lee, S. Benson, R. Corrigan, M. Cantera, B. Shopsin and R. P. Novick**. 2008. *agr* function in clinical *Staphylococcus aureus* isolates. Microbiology. **154**: 2265-2274.
338. **Tristan, A., M. Bes, H. Meugnier, G. Lina, B. Bozdogan, P. Courvalin, M. E. Reverdy, M. C. Enright, F. Vandenesch, and J. Etienne**. 2007. Global distribution of Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus,* 2006. Emerg. Infect. Dis. **13**: 594-600.
339. **Tsiodras, S., H. S. Gold, G. Sakoulas, G. M. Eliopoulos, C. Wennersten, L. Venkataraman, R. C. Moellering, and M. J. Ferraro**.

2001. Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. Lancet. **358**: 207-208.

340. **Ubukata, K., R. Nonoguchi, M. Matsuhashi, and M. Konno**. 1989. Expression and inducibility in *Staphylococcus aureus* of the *mecA* gene, which encodes a methicillin-resistant *S. aureus*-specific penicillin-binding protein. J. Bacteriol. **171**: 2882-2885.
341. **Udo, E. E., J. W. Pearman, and W. B. Grubb**. 1993. Genetic analysis of community isolates of methicillin-resistant *Staphylococcus aureus* in Western Australia. J. Hosp. Infect. **25**: 97-108.
342. **Uhlemann, A. C., S. F. Porcella, S. Trivedi, S. B. Sullivan, C. Hafer, A. D. Kennedy, K. D. Barbian, A. J. McCarthy, C. Street, D. L. Hirschberg, W. I. Lipkin, J. A. Lindsay, F. R. DeLeo, and F. D. Lowy**. 2012. Identification of a highly transmissible animal-independent *Staphylococcus aureus* ST398 clone with distinct genomic and cell adhesion properties. M. Bio. **3**: e00027.
343. **Utsui, Y., and T. Yokota**. 1985. Role of an altered penicillin-binding protein in methicillin- and cephem-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **28**: 397-403.
344. **van Cleef, B. A., D. L. Monnet, A. Voss, K. Krziwanek, F. Allerberger, M. Struelens, H. Zemlickova, R. L. Skov, J. Vuopio-Varkila, C. Cuny, A. W. Friedrich, I. Spiliopoulou, J. Paszti, H. Hardardottir, and A. P. A. Rossney, A. Pantosti, M. Borg, H. Grundmann, M. Mueller-Premru, B. Olsson-Liljequist, A. Widmer, S. Harbarth, A. Schweiger, S. Unal, and J. A. Kluytmans**. 2011. Livestock-associated methicillin-resistant *Staphylococcus aureus* in humans, Europe. Emerg. Infect. Dis. **17**: 502-505.
345. **van Dam, V., R. Sijbrandi, M. Kol, E. Swiezewska, B. de Kruijff and E. Breukink**. 2007. Transmembrane transport of peptidoglycan precursors across model and bacterial membranes. Mol. Microbiol. **64**: 1105-1114.
346. **Vandenesch, F., T. Naimi, M. C. Enright, G. Lina, G. R. Nimmo, H. Heffernan, N. Liassine, M. Bes, T. Greenland, M. E. Reverdy, and J. Etienne**. 2003. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg. Infect. Dis. **9**: 978-984.
347. **Veiga, P., C. Bulbarela-Sampieri, S. Furlan, A. Maisons, M. P. Chapot-Chartier, M. Erkelenz, P. Mervelet, P. Noirot, D. Frees, O. P. Kuipers, J. Kok, A. Gruss, G. Buist and S. Kulakauskas**. 2007. SpxB regulates *O*-acetylation-dependent resistance of *Lactococcus lactis* peptidoglycan to hydrolysis. J. Biol. Chem. **282**: 19342-19354.
348. **Vlack, S., L. Cox, A. Y. Peleg, C. Canuto, C. Stewart, A. Conlon, A. Stephens, P. Giffard, F. Huygens, A. Mollinger, R. Vohra, and J. S. McCarthy**. 2006. Carriage of methicillin-resistant *Staphylococcus aureus* in a Queensland Indigenous community. Med. J. Aust. **184**: 556-559.
349. **Vocadlo, D. J., G. J. Davies, R. Laine and S. G. Withers**. 2001. Catalysis by hen egg-white lysozyme proceeds via a covalent intermediate. Nature. **412**: 835-838.
350. **Vollmer, W.** 2008. Structural variation in the glycan strands of bacterial peptidoglycan. FEMS Microbiol. Rev. **32**: 287-306.

351. **Vollmer, W., and A. Tomasz**. 2000. The *pgdA* gene encodes for a peptidoglycan N-acetylglucosamine deacetylase in *Streptococcus pneumoniae*. J. Biol. Chem. **275**: 20496-20501.
352. **Vollmer, W., D. Blanot and M. A. de Pedro**. 2008. Peptidoglycan structure and architecture. FEMS Microbiol Rev. **32**: 149-167.
353. **Vollmer, W., B. Joris, P. Charlier and S. Foster**. 2008. Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol. Rev. **32**: 259-286.
354. **Voyich, J. M., K. R. Braughton, D. E. Sturdevant, A. R. Whitney, B. Said-Salim, S. F. Porcella, R. D. Long, D. W. Dorward, D. J. Gardner, B. N. Kreiswirth, J. M. Musser, and F. R. DeLeo** 2005. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. J. Immunol. **175**: 3907-3919.
355. **Wada, A. and H. Watanabe**. 1998. Penicillin-binding protein 1 of *Staphylococcus aureus* is essential for growth. J. Bacteriol. **180**: 2759-2765.
356. **Walsh, T. R. and R. A. Howe**. 2002. The prevalence and mechanisms of vancomycin resistance in *Staphylococcus aureus*. Annu. Rev. Microbiol. **56**: 657-675.
357. **Wang, G., L. F. Lo, L. S. Forsberg and R. J. Maier**. 2012. *Helicobacter pylori* peptidoglycan modifications confer lysozyme resistance and contribute to survival in the host. MBio. **3**: e00409-00412.
358. **Wang, G., A. Olczak, L. S. Forsberg and R. J. Maier**. 2009. Oxidative stress-induced peptidoglycan deacetylase in *Helicobacter pylori*. J. Biol. Chem. **284**: 6790-6800.
359. **Wang, L., M. K. Khattar, W. D. Donachie and J. Lutkenhaus**. 1998. FtsI and FtsW are localized to the septum in *Escherichia coli*. J. Bacteriol. **180**: 2810-2816.
360. **Wang, Q. M., R. B. Peery, R. B. Johnson, W. E. Alborn, W. K. Yeh and P. L. Skatrud**. 2001. Identification and characterization of a monofunctional glycosyltransferase from *Staphylococcus aureus*. J. Bacteriol. **183**: 4779-4785.
361. **Wannet, W. J., E. Spalburg, M. E. Heck, G. N. Pluister, R. J. Willems, and A. J. De Neeling**. 2004. Widespread dissemination in The Netherlands of the epidemic berlin methicillin-resistant *Staphylococcus aureus* clone with low-level resistance to oxacillin. J. Clin. Microbiol. **42**: 3077-3082.
362. **Wecke, J., M. Lahav, I. Ginsburg and P. Giesbrecht**. 1982. Cell wall degradation of *Staphylococcus aureus* by lysozyme. Arch. Microbiol. **131**: 116-123.
363. **Weidenmaier, C., J. F. Kokai-Kun, S. A. Kristian, T. Chanturiya, H. Kalbacher, M. Gross, G. Nicholson, B. Neumeister, J. J. Mond and A. Peschel**. 2004. Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. Nat. Med. **10**: 243-245.
364. **Weidenmaier, C. and A. Peschel**. 2008. Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. Nat. Rev. Microbiol. **6**: 276-287.
365. **Weidenmaier, C., A. Peschel, V. A. Kempf, N. Lucindo, M. R. Yeaman and A. S. Bayer**. 2005. DltABCD- and MprF-mediated cell envelope

modifications of *Staphylococcus aureus* confer resistance to platelet microbicidal proteins and contribute to virulence in a rabbit endocarditis model. Infect. Immun. **73**: 8033-8038.

366. **Witte, W.** 2009. Community-acquired methicillin-resistant *Staphylococcus aureus*: what do we need to know? Clin. Microbiol. Infect. **15 Suppl 7**: 17-25.
367. **Witte, W., B. Strommenger, C. Cuny, D. Heuck, and U. Nuebel**. 2007. Methicillin-resistant *Staphylococcus* aureus containing the Panton-Valentine leucocidin gene in Germany in 2005 and 2006. J. Antimicrob. Chemother. **60**: 1258-1263.
368. **Witte, W., M. Kresken, C. Braulke, and C. Cuny.** 1997. Increasing incidence and widespread dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals in central Europe, with special reference to German hospitals. Clin. Microbiol. Infect. **3**: 414-422.
369. **Woodin, A. M.** 1960. Purification of the two components of leucocidin from *Staphylococcus aureus*. Biochem. J. **75**: 158-165.
370. **Wu, S., C. Piscitelli, H. de Lencastre, and A. Tomasz**. 1996. Tracking the evolutionary origin of the methicillin resistance gene: cloning and sequencing of a homologue of *mecA* from a methicillin susceptible strain of *Staphylococcus sciuri*. Microb. Drug Resist. **2**: 435-441.
371. **Wu, S. W., and De Lencastre, H.** 1999. Mrp--a new auxiliary gene essential for optimal expression of methicillin resistance in *Staphylococcus aureus*. Microb. Drug Resist. **5**: 9-18.
372. **Wu, S. W., H. de Lencastre, and A. Tomasz**. 2001. Recruitment of the *mecA* gene homologue of *Staphylococcus sciuri* into a resistance determinant and expression of the resistant phenotype in *Staphylococcus aureus*. J. Bacteriol. **183**: 2417-2424.
373. **Wyke, A. W., J. B. Ward, M. V. Hayes and N. A. Curtis**. 1981. A role *in vivo* for penicillin-binding protein-4 of *Staphylococcus aureus*. Eur. J. Biochem. **119**: 389-393.
374. **Yamada, S., M. Sugai, H. Komatsuzawa, S. Nakashima, T. Oshida, A. Matsumoto, and H. Suginaka**. 1996. An autolysin ring associated with cell separation of *Staphylococcus aureus*. J. Bacteriol. **178**: 1565-1571.
375. **Yuan, Y., D. Barrett, Y. Zhang, D. Kahne, P. Sliz and S. Walker**. 2007. Crystal structure of a peptidoglycan glycosyltransferase suggests a model for processive glycan chain synthesis. Proc. Natl. Acad. Sci. U. S. A. **104**: 5348-5353.
376. **Zapun, A., T. Vernet and M. G. Pinho**. 2008. The different shapes of cocci. FEMS Microbiol. Rev. **32**: 345-360.
377. **Zetola, N., J. S. Francis, E. L. Nuermberger, and W. R. Bishai**. 2005. Community-acquired meticillin-resistant *Staphylococcus aureus*: an emerging threat. Lancet Infect. Dis. **5**: 275-286.
378. **Zhang, H. Z., C. J. Hackbarth, K. M. Chansky, and H. F. Chambers**. 2001. A proteolytic transmembrane signaling pathway and resistance to beta-lactams in staphylococci. Science. **291**: 1962-1965.
379. **Zipperle, G. F., Jr., J. W. Ezzell, Jr. and R. J. Doyle**. 1984. Glucosamine substitution and muramidase susceptibility in *Bacillus anthracis*. Can. J. Microbiol.**30**:553-559.

Chapter II

Identification of Genetic Determinants and Enzymes Involved with the Amidation of Glutamic Acid Residues in the Peptidoglycan of *Staphylococcus aureus*

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Abstract

The glutamic acid residues of the peptidoglycan of *Staphylococcus aureus* and many other bacteria become amidated by an as yet unknown mechanism. In this communication we describe the identification, in the genome of *S. aureus* strain COL, of two co-transcribed genes, *murT* and *gatD*, which are responsible for peptidoglycan amidation. MurT and GatD have sequence similarity to substrate-binding domains in Mur ligases (MurT) and to the catalytic domain in CobB/CobQ-like glutamine amidotransferases (GatD). The amidation of glutamate residues in the stem peptide of *S. aureus* peptidoglycan takes place in a later step than the cytoplasmic phase – presumably the lipid phase - of the biosynthesis of the *S. aureus* cell wall precursor. Inhibition of amidation caused reduced growth rate, reduced resistance to beta-lactam antibiotics and increased sensitivity to lysozyme which inhibited culture growth and caused degradation of the peptidoglycan.

Introduction

Peptidoglycan forms an essential stress-bearing and shape maintaining layer in the bacterial cell envelope. Its biosynthetic pathway is the target of important classes of antimicrobials such as beta-lactams and glycopeptides, and the polymerized cell wall is targeted by antimicrobial enzymes like lysozyme. The biosynthesis of peptidoglycan is a complex process involving several consecutive enzymatic steps that take place in the cytoplasm and on the inner and outer surface of the cytoplasmic membrane. The cytoplasmic stage of biosynthesis culminates in the formation of the UDP-*N*-acetylmuramic acid (UDP-Mur*N*Ac) covalently linked to a pentapeptide which is composed of L-alanine, D-iso-glutamic acid, L-lysine (or meso-diaminopimelic acid, DAP) and D-alanyl D-alanine. The assembly of this stem peptide moiety involves a superfamily of enzymes, the Mur ligases (22). In the next steps of biosynthesis, the UDP-Mur*N*Ac-pentapeptide is attached to a membrane acceptor undecaprenyl phosphate (C55-P) followed by the addition of Glc*N*Ac to the Mur*N*Ac residues yielding the structure known as lipid II. Lipid II, i.e., the bactoprenol linked disaccharide pentapeptide is then transported to the outer surface of the cytoplasmic membrane where it serves as a substrate for polymerization reactions catalyzed by transpeptidases and transglycosylases to form the polymeric cell wall peptidoglycan.

Chemical analysis of the *S. aureus* peptidoglycan showed that the structure of these polymers differed from the structure of the cytoplasmic disaccharide pentapeptide cell wall precursor: some hydroxyl groups in the glycan chain were acetylated; and the second amino acid residue of the muropeptides was not isoglutamic acid but its amidated version, iso-glutamine.

The mechanisms of these secondary modifications of the cell wall are not well understood. Enzymes and genetic determinants involved with the acetylation of the glycan chain and the role of this structural modification in the resistance of *S. aureus* against host lysozyme - have only been described recently (2).

While amidation of the stem peptide residues at positions 2 or 3 or both is frequent among gram-positive bacteria, the physiological roles of this chemical modification have remained a matter of speculation (26) and the genetic determinants and enzymes responsible for the conversion of iso-glutamic acid to iso-glutamine residues have also remained unknown.

In this communication we describe the identification of a small operon composed of two genes – *murT* and *gatD* – in the genome of the beta-lactam resistant *S. aureus* strain COL. Amino acid sequence of the protein products of these genes show similarity to murein ligases (*murT*) and to CobB/CobQlike glutamine amidotransferases (*gatD*). The properties of a conditional mutant of *murT/gatD* indicate that this operon is responsible for the conversion of isoglutamic acid to iso-glutamine residues in the peptidoglycan of *S. aureus.*

Materials and Methods

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Staphylococcus aureus* strains were grown at 37ºC with aeration in tryptic soy broth (TSB) or tryptic soy agar (TSA) (Difco Laboratories, Detroit, Mich.). Transposition mutant RUSA208 (19) and the conditional mutant strains RN4220*pCadmurT-gatD* and COL*pCadmurT-gatD*, the double mutant RUSA208*pCadmurT-gatD*, the complemented strains COL*pCadmurT-gatD*+pSK*murT* and COL*pCadmurT-gatD*+pSK*gatD* and the control strain COL*pCadmurT-gatD*+pSK were grown in the presence of the respective antibiotics (Table 1). The growth medium was supplemented with 0.2 µM of cadmium chloride ($CdCl_2$; Sigma, St. Louis, MO), unless otherwise described. *Escherichia coli* strains (Table 1) were grown in Luria-Bertani broth (LB; Difco Laboratories) with aeration at 37ºC. Erythromycin (10 µg/ml), neomycin sulphate (50 µg/ml), kanamycin (50 µg/ml), chloramphenicol (10 µg/ml) and ampicillin (100 µg/ml) from Sigma were used for the selection and maintenance of *S. aureus* and *E. coli* mutants.

In silico analysis of the *murT-gatD* gene products. The amino acid sequences of ORFs SACOL1951 (MurT) and SACOL1950 (GatD) were retrieved from the UniProtKB database (25), and their domain architecture was checked using the InterProScan tool (28). The domains were aligned through TCoffee (18). Given the limited similarity between sequences, secondary structure inference was used as an independent benchmark for the alignment. This inference was accomplished through Psipred (4). Position specific annotation other than the one present in the InterPro documentation was collected from references (9, 22).

DNA methods. Restriction enzymes from New England Biolabs (Beverly, MA) were used as recommended by the manufacturer. Routine PCR

amplification was performed with Tth DNA polymerase (HT Biotechnology, Cambridge, United Kingdom) and PCR amplification for cloning purposes was performed using Pfu DNA polymerase (Stratagene, Heidelberg, Germany). For plasmid DNA extraction High pure Plasmid Purification Kit (Roche, Basel, Switzerland) was used. PCR and digestion products were purified using High pure PCR Purification Kit (Roche). Ligation reactions were performed using Rapid DNA Ligation kit (Roche).

Reverse transcription analysis. Reverse transcription (RT)-PCR was performed as described (24) using total RNA from strain COL as the template. The primers used for the reverse transcription reactions are described in Table S1 and the amplification conditions were: 94ºC for 2 min; 40 cycles of 94ºC for 30 s, 53ºC for 30 s, and 72ºC for 2 min; and one final extension step of 72ºC for 5 min.

Construction of pMurT' plasmid. A 918- bp DNA fragment of *murT* gene was amplified by PCR using chromosomal DNA from strain COL as a template and the specific primers PmurT'-R and PmurT'-F (Table S1). The amplification conditions used were as follows: 94ºC for 4 min; 30 cycles, each consisting of 94ºC for 30 s, 50ºC for 30 s, and 72ºC for 1 min 30 s; and one final extension step of 72ºC for 10 min. The amplified fragment and the integrative plasmid pBCB20, carrying a cadmium chloride inducible promoter (R.G. Sobral and M.G. Pinho, unpublished) were both digested with SmaI and ligated, generating plasmid pMurT'.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Strains		
S. aureus		
RN4220	Mc^s; restriction negative	(R. Novick)
COL	Homogeneous Mc^r (MIC, 1600 µg/ml); Em^s	Rockefeller University Collection
RUSA208	COL with Tn*551* insertion in *glnR*, Em^r	(19)
COL*pCadmurT-gatD*	COL with *murT-gatD operon* under *Pcad* control, Kan^r, Neo^r	This study
COL*pCadmurT-gatD*+ pSK*murT*	COL*pCadmurT-gatD* with pSK5632 plasmid with *murT* gene, Kan^r, Neo^r, Cm^r	This study
COL*pCadmurT-gatD*+ pSK*gatD*	COL*pCadmurT-gatD* with pSK5632 plasmid with *gatD* gene, Kan^r, Neo^r, Cm^r	This study
COL*pCadmurT-gatD*+ pSK	COL*pCadmurT-gatD* with pSK5632 plasmid, Kan^r, Neo^r, Cm^r	This study
RUSA208*pCadmurT-gatD*	COL with an insertion of Tn*551* in *glnR*, Ery^r and with *murT-gatD* operon under *Pcad* control, Kan^r, Neo^r	This study
E. coli		
DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE*44 *relA1* Φ80 Δ*lacZ*ΔM15	Invitrogen
Plasmids		
pBCB20	*S. aureus* integrative vector with *Pcad* inducible promotor, Ap^r, Kan^r	R.Sobral and M.Pinho (unpublished)
pMurT'	pBCB20 vector with *murT* rbs and the first 298 codons fused to *Pcad* promotor, Ap^r, Kan^r	This study
pSK5632	*E. coli-S. aureus* shuttle vector, Ap^r, Cm^r	(10)
pSK*murT*	pSK5632 vector with *murT* gene and 300 bps of the immediately upstream region, Ap^r, Cm^r	This study
pSK*gatD*	pSK5632 vector with *gatD* gene, Ap^r, Cm^r	This study

Construction of the conditional mutant. Plasmid pMurT' was electroporated into competent cells of RN4220 with a Gene Pulser apparatus (Bio-Rad, California) under conditions described previously (15). Selection of the transformants was performed using kanamycin (50 µg/ml), neomycin sulphate (50 µg/ml) and 0.2 µM of Cadmium chloride. The correct insertion of pMurT' into RN4220 chromosome was confirmed by PCR, using an internal murT primer chosen outside the region cloned and an internal pBCB20 primer (Table S1). The *murT-gatD* conditional mutation was then transduced, by phage 80α to the background of COL as previously described (20) and mutant COL*pCadmurT-gatD* was obtained.

Construction of complemented strains. A 1673 bp DNA fragment, including the complete *murT* coding sequence and 300 bp of the immediate upstream region was amplified from COL genome using the primers PmurTSalI and PmurTBamHI (Table S1). The amplified *murT* fragment and plasmid pSK5632 (10) were digested with SalI and BamHI and ligated, generating the replicative plasmid pSKmurT**.** The same strategy was used for the construction of the replicative plasmid pSK*gatD***,** in which a 1088 bp DNA fragment including the complete *gatD* gene sequence and 300 bp of the immediately upstream region. Plasmids pSK*murT* and pSK*gatD* were separately introduced into RN4220 by electroporation and subsequently transferred to COL*pCadmurT-gatD* by transduction, generating COL*pCadmurTgatD*+pSK*murT* and COL*pCadmurT-gatD*+pSK*gatD*, respectively. Plasmid pSK5632 was also introduced in the conditional mutant, providing the control strain COL*pCadmurT-gatD*+pSK.

Construction of RUSA208*pCadmurT-gatD* double mutant. The *murT-gatD* conditional mutation was transduced, using phage 80α, to the background of RUSA208. The obtained double mutant

RUSA208*pCadmurT-gatD*, has a transposon insertion in *glnRA* operon and the *murT-gatD* operon under the control of *pCad* promoter.

Northern blotting analysis. Cells were grown in TSB at 37ºC to mid-exponential phase (OD620 nm of 0.7). Prior to harvesting the cells, the RNA protect reagent (QIAGEN, Hilden, Germany) was added to the cultures. Total RNA was isolated as previously described (23). PCR amplified internal fragments of the *murT*, *gatD*, SACOL1949-SACOL1948, SACOL1952, *glnA* and *pta* genes were used as probes for hybridization (the primers used are listed in Table S1). The DNA probes were labeled with [a-32P]dCTP (Perkin Elmer, MA, USA).

Cell wall isolation. Isolation of cell wall was performed as described (2, 6). Briefly, cells were harvested by centrifugation, washed twice with cold 0.9% NaCl, resuspended in 0.9% NaCl and boiled for 20 min. After chilling on ice, the suspension was centrifuged and washed twice with 0.9% NaCl. The cells were disrupted using 106 mm glass beads (Sigma) and FastPrep FP120 apparatus (Bio 101, La Jolla, Calif.), purified, washed, and boiled for 30 min in 5% SDS, diluted in 50 mM Tris/HCl pH 7, to remove non-covalently bound proteins. After centrifugation, the cell wall fragments were diluted in 0.1 M Tris-HCl (pH 6.8) and incubated with 0.5 mg/ml trypsin for 16 h at 37ºC to degrade cell-bound proteins. Purified cell walls were washed with double-distilled water and lyophilized.

Peptidoglycan purification. Lyophylised cell wall was treated with 49% of hydrofluoric acid for 48 hours at 4ºC in order to remove teichoic acids. The teichoic acid free peptidoglycan was washed with water several times to remove all traces of hydrofluoric acid and then lyophylised.

Peptidoglycan analysis by RP-HPLC. Identical amounts of peptidoglycan were digested with mutanolysin (1 mg/ml; Sigma). The resulting

muropeptides were reduced with sodium borohydride and separated by reverse phase-high performance liquid chromatography (RP-HPLC) using a Hypersil ODS (Runcorn Cheshire, UK) column (3 mm particle size, 25064.6 mm, 120 A ° pore size) and a linear gradient from 5% to 30% MeOH in 100 mM sodium phosphate buffer pH 2.5 at a flow rate of 0.5 ml/min as described (7).

Purification of monomeric muropeptides. Highly purified cell wall was prepared as previously described (2) and resuspended to a final concentration of 10 mg/ml. Cell wall material (500 µg) was digested with lysostaphin (300 µg) in 20 mM amonium acetate, pH 4.8, for 24 h at 37ºC with stirring. Subsequently, cellosyl (Hochst AG, Frankfurt, Germany) (15 µg) was added to the reaction mixture which was incubated for 12 h at 37ºC. Finally, additional 15 µg of cellosyl was added and the incubation continued for an additional 12 h. The enzymatic reaction was stopped by boiling the samples for 5 min and insoluble contaminants were removed by centrifugation. The digested cell wall was reduced with sodium borohydride and the resulting monomeric muropeptides were separated by RP-HPLC using a Prontosil (Bischoff, Leonberg, Germany) column (3 mm, particle size, 25064.6 mm, 120 A ° pore size), and a linear gradient from 0% to 30% MeOH in 10 mM sodium phosphate buffer pH 6.0 at a flow rate of 0.5 ml/min.

Mass spectrometry analysis of monomeric muropeptides. The eluted fractions corresponding to the most predominant peaks of the chromatograms were collected after HPLC separation, concentrated to 10–20 µl, and acidified with 1% trifluoroacetic acid (TFA). The samples were then desalted and further concentrated using ZipTips (C- 18, Millipore, UK) according to the standard protocol recommended by the manufacturer. The material was eluted from the ZipTip with 3 µl of 50% acetronitrile, 0.1% TFA

and was sprayed directly into a Finnigan LTQ-FT mass spectrometer (Thermo, Bremen, Germany) operating in positive mode (Pinnacle Proteomics Facility, Newcastle University, UK) (5).

Analysis of UDP-linked precursor pool. The UDP-linked peptidoglycan precursors from the cytoplasmic pool were isolated using a modified protocol (14). Briefly, vancomycin (Sigma) was added (at five times the minimal inhibitory concentration) to mid-exponential grown cultures and incubation proceeded for additional 30 minutes. The cultures were then chilled below 10ºC, cells were harvested, suspended in cold water and slowly stirred into the same volume of boiling water for 15 minutes. After centrifugation the supernatant was collected, lyophilized, dissolved in water and the pH was adjusted to 4.0 using 20% phosphoric acid. The suspension was again centrifuged and the pH of the supernatant adjusted to 2.0. The suspension was centrifuged at 4ºC for 1 h at 200000 g.

The UDP-linked peptidoglycan precursors were separated through the same column used to separate the muropeptides of peptidoglycan – using a linear gradient from 0 to 30% of MeOH in 100 mM sodium phosphate buffer (pH 2.0), with a flow rate of 0.5 ml/min. Compounds to be analyzed by MS were isolated and desalted using the same column as before with a linear gradient from 0 to 30% of MeOH in 10 mM of sodium phosphate (pH 4.3) for 25 min with a flow rate of 0.5 ml/min. Mass spectral data were obtained by MALDI-TOF analysis (Pinnacle Proteomics Facility, Newcastle University, UK).

Growth curves. Overnight grown cultures of strains COL and COL*pCadmurTgatD*, COL*pCadmurT-gatD*+pSK*murT*, COL*pCadmurT-gatD*+pSK*gatD* and COL*pCadmurT-gatD*+pSK were diluted 1:1,000 into fresh TSB supplemented with the respective antibiotics (Table 1). The conditional mutants were grown in media containing $CdCl_2$ concentrations

at 0, 0.01, 0.05 and 0.2 µM. The cultures were incubated at 37ºC with agitation and the OD620 nm was monitored over time.

Determination of beta-lactam resistance. Overnight grown cultures of strains COL and COL*pCadmurTgatD*, COL*pCadmurT-gatD*+pSK*murT* and COL*pCadmurT-gatD*+pSK*gatD* and COL*pCadmurT-gatD*+pSK were plated on TSA supplemented with increasing concentrations of $CdCl_2$ (0, 0.01, 0.05 and 0.2 µM) and incubated at 37ºC for 24 hours. Oxacillin (Sigma) diffusion disks (1 mg) were used to determine inhibition halos.

Turbidometric assay of peptidoglycan hydrolysis. To analyze the susceptibility of peptidoglycan to lysozyme hydrolysis, a turbidometric assay was used as described (2, 12). Briefly, 0.5 mg of purified peptidoglycan from the conditional mutant, grown with and without $CdCl_2$, were sonicated in 1 ml of 100 mM Sodium-Potassium phosphate buffer pH 6.6. Human lysozyme or hen egg white lysozyme (Sigma) was added to a final concentration of 300 µg/ml and the reaction was incubated at 37ºC. The optical density was monitored at 660 nm.

Determination of lysozyme and polymyxin resistance. The impact of lysozyme on exponential growth was determined as described (12). Overnight cultures of the conditional mutant grown with inducer were diluted to an OD620 nm of 0.1 in fresh TSB (with and without inducer). The cultures were incubated at 37ºC until an OD620 nm of 1.0. Then, each culture was diluted 1:10 into fresh TSB medium and lysozyme (300 µg/ml) was added at an OD620 nm of 1.0. The growth was monitored for several hours. The same procedure was done using 20 µg/ml of Polymyxin B (Sigma), a cationic antimicrobial peptide.

Results

The two open reading frames, SACOL1951 and SACOL1950, were automatically annotated in the genome of *S. aureus* strain COL as a putative Mur ligase family-like protein and a CobB/CobQ-like glutamine amidotransferase, respectively. The preliminary annotations of these genes, designated *murT* and *gatD*, respectively, suggested a role for their protein products in cell wall metabolism.

***murT* and *gatD* genes are co-transcribed as a small operon.** DNA sequence analyses of *murT-gatD* region suggested that *murT* and *gatD* are located in the same operon and might be co-transcribed from a common promoter: the *murT* stop codon and the *gatD* methionine codon are separated by 4 bp only; both genes are transcribed in the same direction and no promoter region sequence could be found upstream from gatD (Figure 1A).

Reverse transcription-PCR (RT-PCR) was performed using total cDNA of strain COL with forward primers specifically binding to *murT* and a reverse primer specifically binding to *gatD*. The test yielded products of the expected size (Figure 1B, lanes C and D). No PCR product was obtained from the negative control using primers from the SACOL1949-1948 region, which was found by northern blotting not to be transcribed (Figure 1B, lane A). A PCR product of the expected size was obtained for the positive control, using primers internal to *pta*, a housekeeping gene. The results of the RT-PCR test indicated that both *murT* and *gatD* are co-transcribed from a common promoter.

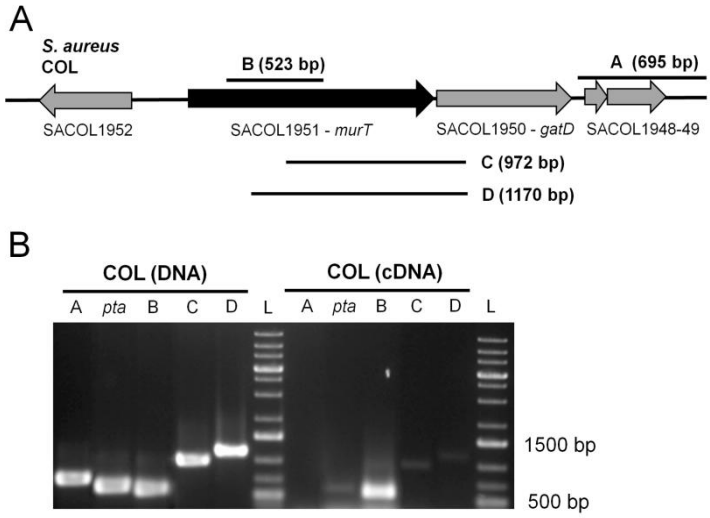

Figure 1. RT-PCR amplification of the *murT-gatD* region. (A) *S. aureus* COL genome region encompassing *murT* and *gatD* genes and the vicinity regions. Fragments A, B, C, and D which were amplified by RT-PCR using primers from Table S1, are shown. (B) Amplification results by PCR using COL DNA and by RT-PCR using COL cDNA produced from a total RNA sample. A fragment from *pta* gene was used as a positive internal control. Fragment A was used as a negative control, as no transcript was detectable for SACOL1948 and SACOL1949 by northern blotting (data not shown).

The *murT-gatD* operon is a syntenic block. Analysis of genome sequences available showed that the *murT* and gatD genes occur, widespread among bacteria, as a syntenic block, although it is not a universal feature. This is in agreement with our RT-PCR results, which identified the two genes as a small operon. The distribution of this syntenic block among the prokaryotes, with emphasis on the *Staphylococcaceae*, is shown in Figure S1.

Construction of a *murT-gatD* conditional mutant. In order to explore the functions of these uncharacterized genes we constructed a mutant strain containing a single chromosomal copy of *murT-gatD* under the control of an inducible promoter (*pCad*). A DNA fragment of *murT* gene which includes the first 298 codons and the ribosome binding site but not the promotor region, was cloned into the integrative plasmid pBCB20 (see Table 1). The recombinant plasmid was electroporated into RN4220 and the

chromosomal construct was transduced into the background of the MRSA strain COL. The only complete functional copies of *murT* and *gatD* genes were located immediately downstream from the *pCad*, generating mutant COL*pCadmurT-gatD* (Figure S2). Hence, this strain expresses the *murT-gatD* genes when grown in the presence of Cd2+, and both genes are depleted when Cd2+ is absent from the growth medium (see below).

Transcriptional analysis of the *murT-gatD* conditional mutant. Northern blotting assays were performed in order to confirm the specificity of transcription of the *murT-gatD* operon controlled by the $CdCl_2$ concentration in the medium. The transcription of *murT*, *gatD*, SACOL1952 and SACOL1948-SACOL1949 genes was analyzed for COL and mutant COL*pCadmurT-gatD* grown with several concentrations of inducer. The level of *murT* and *gatD* transcription was found to increase with the inducer concentration in the medium (data not shown). No alterations were detected under the same conditions in the transcription level of the ORFs located in the immediate vicinity of the *murT-gatD* operon, SACOL1952 and SACOL1949-SACOL1948, which were found to be not transcribed even for strain COL (data not shown). The housekeeping gene *pta* was used as control. For strain COL, a single transcript was visualized for each gene: an mRNA structure of approximately 1780 nt long hybridized with *murT* probe and an mRNA structure of approximately 2300 nt long was obtained for hybridization with *gatD* probe. The size of this last transcript matches the size of both genes, consistent with their co-transcription.

Abnormal peptidoglycan produced upon *murT-gatD* depletion. Cell walls of parental strain COL and of the conditional mutant, grown with and without Cd2+, were purified and digested with cellosyl and lysostaphin. The resulting monomeric muropeptides were reduced and analyzed by RP-HPLC. The muropeptide profiles revealed that, when the transcription of

murT-gatD operon was inhibited, two new muropeptide structures appeared in the RP-HPLC profile (Figure 2A – peaks V and VI). These two muropeptide species showed shorter retention times than peak I, which is common to all the profiles. To identify the structural modifications, all peaks annotated in Figure 2A were isolated and analyzed by MS. The MS results (Table 2) indicate that the two new peaks (V and VI) observed in the profile of the *murT/gatD* depleted cells corresponded to muropeptide structures with D-iso-glutamate in the stem peptide replacing D-iso-glutamine. Peaks I, II, III and IV correspond to muropeptide structures with D-iso-glutamine (Figure 2B). Amidated muropeptides (Peak I) were still present when the transcription of *murT-gatD* operon was inhibited. This could be due to the activity of MurT and GatD expressed by residual transcription from the *pCad* promoter or to the presence of other enzymes with the same activity. These findings identify the protein products of *murT-gatD* as essential for the full amidation of the Dglutamic acid residues in the *S. aureus* peptidoglycan.

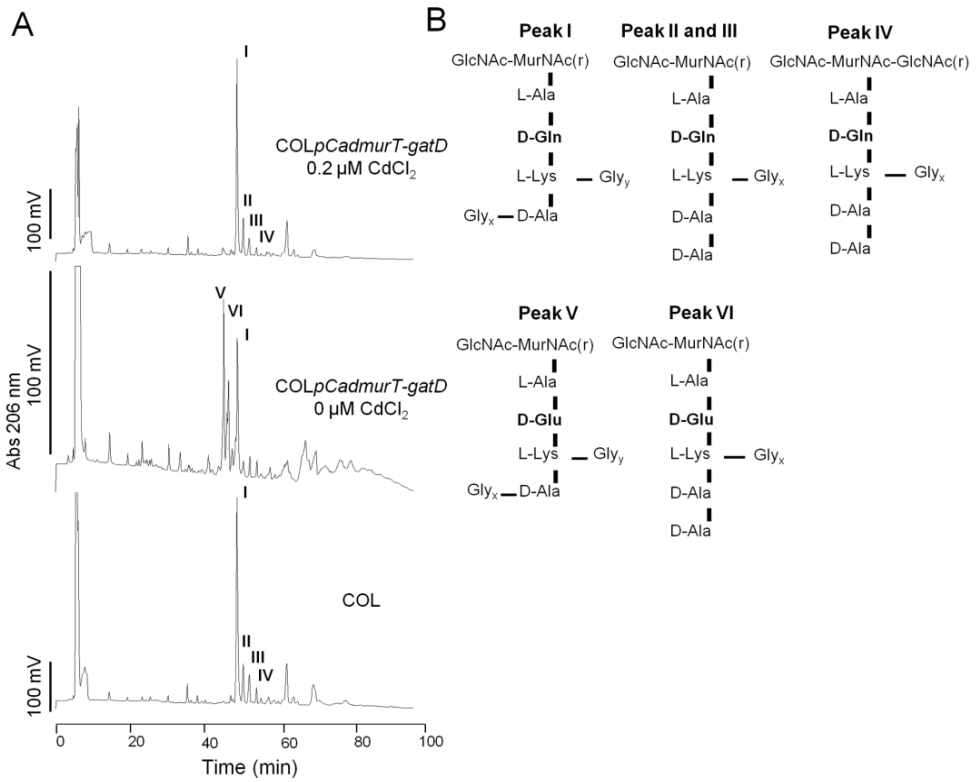

Figure 2. RP-HPLC profiles of cell walls of strains COL and the conditional mutant. (A) RP-HPLC profiles of cell walls prepared from strains COL and COL*pCadmurT-gatD* mutant grown with and without 0.2 µM of $CdCl_2$. The cell walls were purified and digested with cellosyl and lysostaphin and the resulting muropeptides were reduced and analyzed by RP-HPLC. Fractions eluting at 50.73 min (peak I), 52.42 min (peak II), 54.10 min (peak III), 56.13 min (peak IV) in COL strain, and eluting at 47.00 min (peak V) and 48.33 min (peak VI) in COL*pCadmurT-gatD*, grown without the inducer, were collected and analyzed by mass spectrometry. (B) Proposed structures for the muropeptides corresponding to peaks I, II, III, IV, V and VI. Structures with different numbers of glycine residues associated with the D-Ala and L-Lys of the stem peptide, were identified for each peak. The mass of the analyzed compounds are presented in Table 2.

Table 2. Reduced monomeric muropeptides in HPLC fractions analyzed by LTQ-FT mass spectrometry.

Peak Nº	Proposed muropeptide structure(s)[1]	Theoretical neutral mass (Da)	Determined neutral mass(Da)
	Tetra(Gln)Gly_3	1067.4983	1067.5227
	Tetra(Gln)Gly_4	1124.5197	1124.5410
I[a]	Tetra(Gln)Gly_5	1181.5412	1181.5290
	Tetra(Gln)Gly_6	1238.5626	1238.5933
	Tetra(Gln)Gly_7	1295.5841	1295.6296
	Penta(Gln)Gly_2	1081.5139	1081.5242
II[b]	Penta(Gln)Gly_3	1138.5354	1138.5427
	Penta(Gln)Gly_4	1195.5568	1195.5806
III[b]	**Penta(Gln)Gly_3**	1138.5354	1138.5423
	Penta(Gln)Gly_4	1195.5568	1195.5801
	Penta(GlcNAc)(Gln)Gly_2	1284.5933	1284.6128
IV[b]	Penta(GlcNAc)(Gln)Gly_3	1341.6147	1341.6536
	Penta(GlcNAc)(Gln)Gly_4	1398.6362	1398.6787
	Tetra(Glu)Gly_3	1068.4823	1068.4794
V[c]	Tetra(Glu)Gly_4	1125.5037	1125.5191
	Tetra(Glu)Gly_5	1182.5252	1182.4912
VI[c]	**Penta(Glu)Gly_2**	1082.4979	1082.5122
	Penta(Glu)Gly_3	1139.5194	1139.5170

[1] Muropeptides with main MS intensities are in bold.
[a] Structures found in strain COL and in COL*pCadmurT* grown with 0 and 0.2 µM $CdCl_2$.
[b] Structures found in strain COL and in COL*pCadmurT* grown with 0.2 µM $CdCl_2$.
[c] Structures found in COL*pCadmurT* with 0 µM $CdCl_2$.

Comparison of the peptidoglycan composition of the *murT-gatD* mutant and *glnRA* mutant. The cell walls of the parental strain COL and the conditional mutant COL*pCadmurT-gatD* grown with different concentrations of inducer were extracted, the peptidoglycan purified, digested with muramidase and the muropeptides analyzed by RP-HPLC (Figure 3A). The elution profile of the conditional mutant grown in the absence of $CdCl_2$ showed longer retention times for all peaks, when

compared with COL. In addition, the peaks corresponding to muropeptide structures with higher oligomerization level (retention time over 60 min) were split into two or more smaller peaks eluting at very similar retention times. The elution profiles of the mutant grown with 0.01 µM and 0.05 µM of $CdCl_2$ showed gradual re-establishment of the parental muropeptide pattern. For cells grown in 0.2 µM $CdCl_2$ supplemented medium, the optimal inducer concentration, the peptidoglycan HPLC profile was indistinguishable from that of strain COL. The muropeptide elution profile of COL*pCadmurT-gatD*, grown in the absence of inducer, showed similarities to the elution profile of the previously characterized *glnRA* transposition mutant RUSA208 (19) (Figure 3B). In RUSA208, the transposon inserted into the *glnR* gene which codes for the repressor of the glutamine synthetase operon *glnRA*, resulting in the abolishment of *glnA* transcription. The impact of the *glnRA* mutation on the peptidoglycan of RUSA208 has been described as the substitution of the normal D-iso-glutamine residues by D-iso-glutamic acid at position 2 of the stem peptide (19). Substitution of iso-glutamine by iso-glutamic acid residues has been observed among muropeptide monomers (Peak 5A in Figure 3B), among dimeric muropeptides (Peaks 11A & 11B in Figure 3B), among the tripeptide structures (Peak 15B in Figure 3B) and among three of the stem peptides represented by peaks 15A, B & C in Figure 3B. All these structures are also present in the conditional mutant COL*pCadmurT-gatD* grown in the absence or at suboptimal concentrations of the inducer (see Figure 3A).

The *glnA* gene sequence in COL*pCadmurT-gatD* was identical to that in strain COL, excluding the possibility that a mutation in *glnA* causes the deficiency in peptidoglycan amidation as it occurs in RUSA208 strain. Also, the transcription of the *glnA* gene did not vary with the Cd2+ concentration in COL*pCadmurT-gatD* (data not shown) discarding the hypothesis that *murT* and/or *gatD* may indirectly reduce *glnA* transcription.

The peptidoglycan profiles of RUSA208 and COL*pCadmurTgatD* grown with no $CdCl_2$, showed that amidation of the muropeptides still occured partially. This may be due to a leaky expression of *murT-gatD* operon through *pCad* promoter in the absence of $CdCl_2$. In the case of RUSA208, other sources of amino group, besides glutamine, may be used, although less efficiently.

The peptidoglycan HPLC profile of the double mutant RUSA208*pCadmurT-gatD* showed a virtually complete lack of amidated muropeptides (Figure 3A), indicating that the gene products of these two operons are together needed for the amidation of the glutamic acid residue of the peptidoglycan.

Complementation of the *murT-gatD* conditional mutation. The transcriptional analysis showed that the expression of both *murT* and *gatD* genes is being controlled in COL*pCadmurT-gatD* mutant, through the concentration of inducer added to the medium. For this reason we constructed two independent complementation mutants, COL*pCadmurT-gatD*+pSK*murT* and COL*pCadmurT-gatD*+pSK*gatD*, by separately introducing into the COL*pCadmurT-gatD* mutant, the replicative plasmid pSK5632 with either the *murT* or the *gatD* gene. Cloning of the *murT-gatD* operon into pSK5632 was also attempted, but this construct did not yield viable *E. coli* transformants. Strain COL*pCadmurTgatD*+pSK harboring pSK5632 with no cloned gene was constructed and used as control.

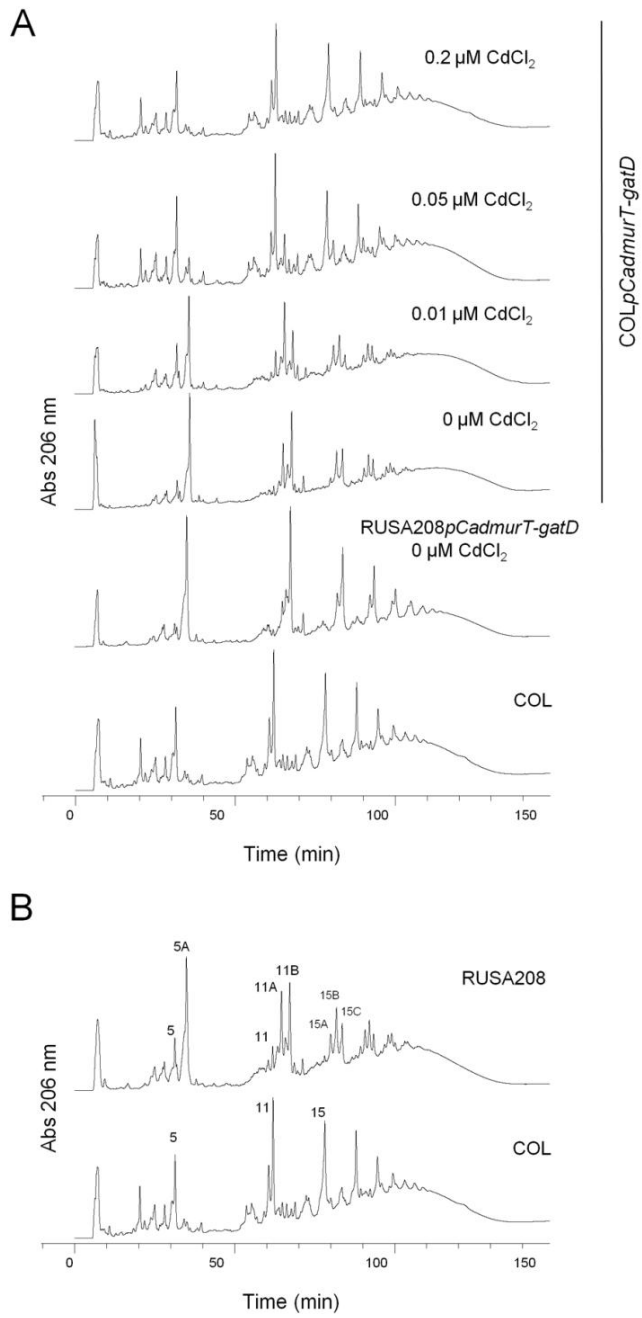

Figure 3. RP-HPLC cell wall profiles. The purified peptidoglycan was digested with mutanolysin, reduced and analyzed by RP-HPLC. (A) muropeptide profiles of strains COL, RUSA208*pCadmurT-gatD*, grown without $CdCl_2$, and COL*pCadmurT-gatD* grown with 0, 0.01, 0.05 and 0.2 µM of $CdCl_2$. (B) muropetide profiles of strains COL, RUSA208. The muropeptide structures corresponding to peaks 5, 5A, 11, 11A, 11B, 15, 15A, 15B and 15C were inferred from mass spectrometric analysis (19).

Re-establishment of the normal peptidoglycan composition in the *murT-gatD* complementation. With the two complementation strains available, we obtained three distinct levels of re-establishment of the normal peptidoglycan: i) the in trans complementation with several copies of the *murT* gene showed a partially restored peptidoglycan with a small amount of muropeptides containing glutamic acid residues (COL*pCadmurT-gatD*+pSK*murT* – 0 µM CdCl2, Figure S3A); ii) the in trans complementation with several copies of *gatD* gene showed no re-establishment of the normal peptidoglycan profile (COL*pCadmurT-gatD*+pSK*gatD* – 0 µM $CdCl_2$, data not shown); iii) the in trans complementation with several copies of the *murT* gene and sub-optimal expression of the chromosomal copy of *murT-gatD* operon showed complete restoration of the peptidoglycan profile (COL*pCadmurT-gatD*+pSK*murT* – 0.01 µM $CdCl_2$, Figure S3B). In the latter case (iii), the 0.01 µM $CdCl_2$ of added inducer is responsible for providing a sub-optimal number of copies of *murT-gatD* transcripts, adding to the already available copies of *murT* transcript provided in trans. The few copies of *gatD* provided in this condition are enough for a complete reestablishment of the normal peptidoglycan composition. Thus, complementation of the *murT-gatD*-depletion phenotype requires the expression of *murT* and at least a basal level of *gatD*.

Composition of cell wall precursor pool of COL*pCadmurT-gatD* mutant. In order to identify the biosynthetic stage at which amidation occurred, the cell wall precursor pool was analyzed by RP-HPLC from strains COL and for the *murT-gatD* conditional mutant grown with and without the inducer. The HPLC profiles were identical for the three conditions analyzed (Figure S4). The major peak, eluting at 38 minutes, was isolated from the cytoplasmic fractions of COL and of the *murT-gatD* conditional mutant grown with and without the inducer. The corresponding

structures were analyzed by MALDI-TOF MS. The results indicated an identical molecular mass of 1149.35 (neutral mass) for each of the three samples, consistent with the structure of the UDP-Mur*N*Ac-L-Ala-D-iGlu-L-Lys-D-Ala-D-Ala, the last cytoplasmic precursor. The presence of D-iso-glutamate in these three structures indicated that the conversion of glutamic acid to iso-glutamine residues must occur at a later stage of cell wall precursor biosynthesis – most likely in the lipid phase – confirming an earlier finding (21).

Properties of the conditional mutant.

Deficit in growth rate. The *murT-gatD* depleted cells had normal morphology as examined by electron microscopy (data not shown) but their growth rate was greatly reduced, indicating that the amidation of peptidoglycan is required for normal growth. COL*pCadmurT-gatD* was unable to grow on solid medium in the absence of Cd2+. In liquid medium the growth rate was significantly reduced in the absence of Cd2+, and it increased with the concentration of inducer added to the medium (Figure 4A and 4B). The growth rate of the COL*pCadmurT-gatD*+pSK*murT* strain in the absence of inducer was higher than the growth rate of the control strain COL*pCadmurT-gatD*+pSK, although a complete restoration could not be obtained. In contrast, the growth rate of the COL*pCadmurT-gatD*+pSK*gatD* strain was lower than that of the control strain COL*pCadmurT-gatD*+pSK (Figure 4C). This behavior was more obvious when the strains were grown in solid medium (Figure 4D).

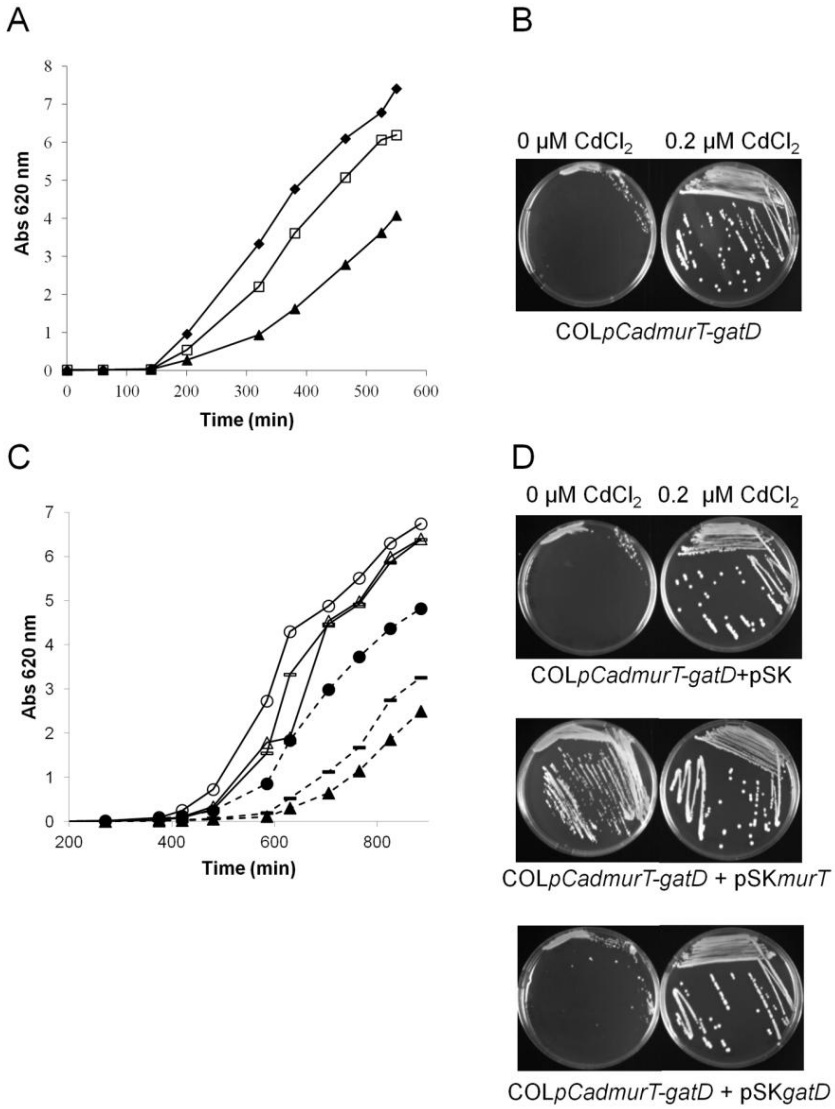

Figure 4. Growth rate of the conditional mutant at different concentrations of $CdCl_2$. (A) Growth curves of strains COL (black diamond), and the COL*pCadmurT-gatD* conditional mutant in TSB supplemented with (black triangle) 0 µM of $CdCl_2$ and (white square) 0.2 µM of $CdCl_2$. (B) Growth on solid medium with or without supplementation of 0.2 µM $CdCl_2$ of COL*pCadmurT-gatD*. (C) Growth curves in liquid medium supplemented with Cm (10 µg/ml) of the complementation mutants COL*pCadmurT-gatD*+pSK*murT* with 0.2 µM $CdCl_2$ (white circle) or without $CdCl_2$ (black circle), COL*pCadmurTgatD*+pSK*gatD* with 0.2 µM $CdCl_2$ (white triangle) or without $CdCl_2$ (black triangle), and the control strain COL*pCadmurTgatD*+pSK with 0.2 µM $CdCl_2$ (white line) or without $CdCl_2$ (black line). (D) Growth on solid medium, with or without supplementation of 0.2 µM $CdCl_2$, of the complementation strains COL*pCadmurT-gatD*+pSK*murT*, COL*pCadmurT-gatD*+pSK*gatD*, and the control strain with the plasmid pSK5632, COL*pCadmurT-gatD*+pSK.

Decrease in beta-lactam resistance. The oxacillin resistance level of COL*pCadmurT-gatD* was found to depend on the inducer concentration: as the $CdCl_2$ concentration was reduced, the size of the growth inhibition halos increased (Figure 5A). In the presence of 0.2 µM of $CdCl_2$, the resistance phenotype was identical to that of COL. COL*pCadmurTgatD*+pSK*murT*, grown in the absence of inducer, completely reestablished the parental phenotype (data not shown).

Increased sensitivity to lysozyme. The *murT-gatD* depleted cells of COL*pCadmurT-gatD* grown in the absence of Cd2+ were sensitive to human lysozyme (Figure 5B), while the same cells grown in the presence of Cd2+ were lysozyme resistant, as was the parental strain COL (data not shown), indicating that peptidoglycan amidation is required to express lysozyme resistance. By contrast, *murT-gatD* depleted cells did not show diminished resistance to the cationic antimicrobial peptide polymyxin B (data not shown). Next, the sensitivity to lysozyme of peptidoglycan of the conditional mutant grown in the absence and presence of inducer were compared. After an incubation period of 60 minutes, lysozyme was able to hydrolyse 46% of peptidoglycan from the conditional mutant grown in the absence of inducer. For the mutant grown with inducer, less than 20% of peptidoglycan was hydrolysed (Figure 5C). *MurT-gatD*-depleted cells and peptidoglycan isolated from them showed also increased susceptibility to hen egg white lysozyme (data not shown).

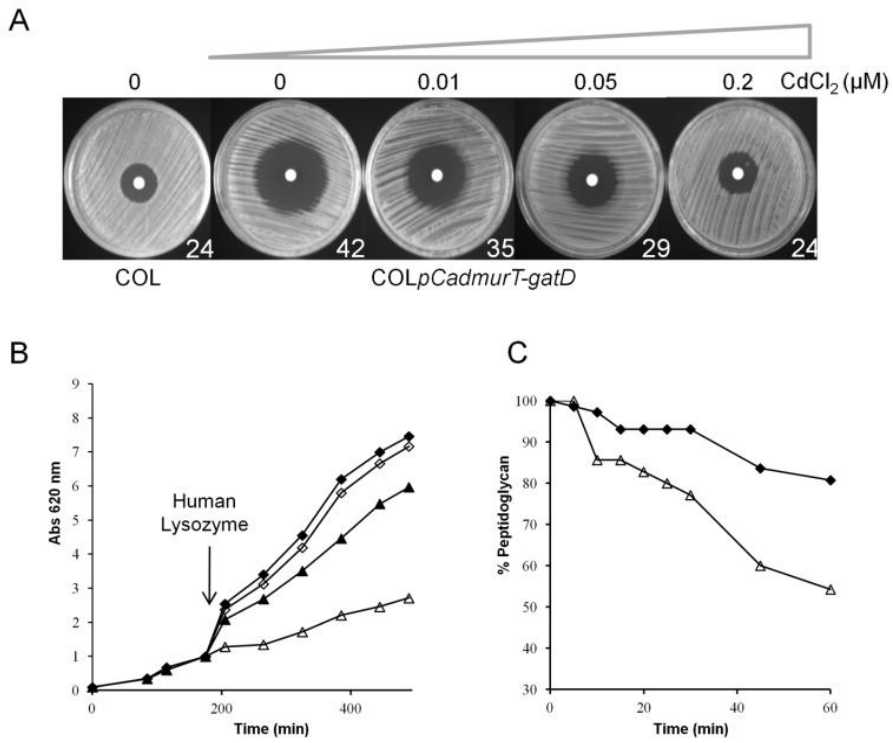

Figure 5. Reduced antibiotic resistance and increased sensitivity to lysozyme in the conditional mutant. (A) Oxacillin inhibition halos (1-mg oxacillin disks) were determined for COL and COL*pCadmurT-gatD* mutant grown with 0, 0.01, 0.05 and 0.2 µM of $CdCl_2$. At the right hand side of each dish is the diameter of the respective inhibition halo in mm. (B) Effect of human lysozyme on the growth rate of: COL*pCadmurT-gatD* grown with 0.2 µM of $CdCl_2$ (black diamond), COL*pCadmurT-gatD* grown with 0.2 µM of $CdCl_2$+lysozyme (white diamond), COL*pCadmurT-gatD* grown with 0 µM of $CdCl_2$ (black triangle), COL*pCadmurT-gatD* grown with 0 µM of $CdCl_2$+lysozyme (white triangle). Human lysozyme was added (300 µg/ml) at an OD620 nm of 1.0 for all strains (arrow). (C) Effect of human lysozyme (300 µg/ml) on peptidoglycan purified from COL*pCadmurT-gatD* grown with 0.2 µM of $CdCl_2$ (black diamond), or COL*pCadmurT-gatD* grown with 0 µM of $CdCl_2$ (white triangle).

Amino acid sequence analysis of MurT and GatD. MurT shares approximately 15% identity and 53% similarity with the sequence of the Mur ligases of *S. aureus*. Interestingly, while MurT shares the characteristic Mur ligase central domain (17, 22) as defined at InterPro (IPR013221), Pfam (PF08245) and Panther (PTHR23135) MurT lacks the flanking N- and C-terminal domains (Figure S5A).

Among the conserved residues were some critical motifs required for ATP and Mg2+ binding and other conserved sites that may not be directly involved in catalysis (Figure 5A). In addition, the MurT protein has a C-terminal domain of unknown function (Pfam: DUF1727, InterPro: IPR013564), which is also found at the C-terminus of more than 900 sequences of prokaryotic proteins at UniProt, and in 5 different domain architectures, all of them sharing the same ORF, or in contiguous ORFs, with Mur central domain (PF08353). GatD shows similarity to one of the two domains of a cobyric acid synthetase protein: a glutamine-dependent amidotransferase (Gn-AT), with glutamine amide transfer (GAT) activity. Its architecture comprises the overlapping domain signatures of CobB/CobQ_GATase (InterPro: IPR017929), and GATase_3 (InterPro: IPR011698) domains. Through multiple sequence alignment of the N-terminal region of three known Gn-ATs, the absence of a large fragment was noted in GatD (Figure S5B). This missing fragment included important residues for the dethiobiotin synthase activity (9) and part of the ATP binding motif. By placing the representation of the secondary structures over the sequence alignment, we can observe considerable agreement between the shared regions, especially near the reactive center of GATase_3 (Figure S5B). This domain harbored the conserved residues directly involved in GAT activity, according to IPR011698. GatD was also found to contain the unusual Triad family glutamine amidotransferase domain with conserved Cys and His residues (Figure S5B), but lacking the Glu residue of the catalytic triad, as the CobB and CobQ proteins (9).

Discussion

The basic structure of *S. aureus* peptidoglycan is known to undergo at least two major secondary modifications, the *O*-acetylation of the free OH groups in the glycan strand and the amidation of the c-carboxyl group of the second residue of the stem peptide, D-iso-glutamate, resulting in the formation of D-isoglutamine. *O*-acetylation of the *S. aureus* peptidoglycan confers lysozyme resistance to the bacteria and its main genetic determinant, the *oatA* gene has been identified and characterized recently (2).

In contrast, the mechanism of the amidation of glutamic acid residues has remained unknown.

In this communication we report the identification of two genetic determinants – *murT* and *gatD* – in the genome of *S. aureus* strain COL - that are required and sufficient for peptidoglycan amidation. A conditional mutant constructed for these two genes, showed abnormal peptidoglycan composition, with decreased amidation of the glutamate residue. The characterization of a double mutant in which not only the expression of *murT-gatD* operon is inhibited but also the operon *glnRA*, responsible for providing glutamine substrate, is impaired, allowed us to infer that *murT* and *gatD* are the key determinants for the amidation of *S. aureus* peptidoglycan.

Furthermore, through the analysis of the precursor pool composition of the mutant strain we showed that this modification step does not occur in the cytoplasm and most probably takes place at the membrane level, confirming previous observations (21).

Other phenotypes associated with *murT-gatD* mutation are decreased growth rate, decreased resistance to beta-lactams and to lysozyme hydrolysis. The strong impact on growth rate suggests that an amidated peptidoglycan may provide better substrates for proteins that catalyze peptidoglycan biosynthesis and cell division. Lack of the amide group may create an unbalance between the synthetic and the hydrolytic machineries of the cell. Electron microscopy pictures of the conditional mutant showed cells with normal size. However, fewer cells showed complete septa, suggesting slower biosynthesis of the septum (data not shown).

The amidation of glutamic residues had already been shown to have a major impact on the expression of beta-lactam resistance, through the *femC* (*glnRA*) mutant of MRSA (11). Consistent with this result, the depletion of *murT-gatD* also shows a major decrease in the oxacillin resistance level. The mechanism of this effect is not well understood (8).

However, similar effects were already described for several other genes (8) many of them related to peptidoglycan biosynthesis. One of the existing theories is that the structurally abnormal lipid II or cell wall peptides are poorer substrates for PBP2A.

More unexpectedly, another feature observed in this mutant was the decrease of resistance to lysozyme action. Lysozyme belongs to the innate immune response and acts on bacteria by hydrolyzing the b-1,4 glycosidic bonds between the two sugar molecules of the glycan strands of peptidoglycan (muramidase activity). Several cell wall modifications have been implicated in the lysozyme resistance mechanism of *S. aureus*, namely the *O*-acetylation in the C-6 position in the Mur*N*ac (2) and the presence of wall teichoic acids (1).

Firstly, we observed in vivo, that the mutant cells grown in the absence of inducer were susceptible to lysozyme action, as the growth was impaired. Besides muramidase activity, lysozyme has also cationic antimicrobial peptide (CAMP) activity (12). The enhanced inhibitory action of lysozyme towards the mutant could be associated with either one of the two activities or both. However, we did not observe any effect of polymyxin B, a CAMP, on the growth rate of the mutant, indicating that glutamate amidation is important to prevent the muramidase activity of lysozyme. This effect could be a direct consequence of glutamate amidation or an indirect effect associated with changes in *O*-acetylation of the Mur*N*ac and/or in wall teichoic acids (WTA). For the mutant grown in the absence of inducer, the purified peptidoglycan, which lacks *O*-acetyl groups and WTA, suffered faster hydrolysis by lysozyme than normally amidated peptidoglycan. These observations allowed us to conclude that glutamate amidation is one of the key factors for lysozyme resistance in *S. aureus*.

The role of glutamate amidation has already been described in the context of pathogenesis. Peptidoglycan is sensed by the human innate immune system via NOD1 and NOD2 (3); NOD1 recognizes as minimal structure the D-Glu-meso-DAP dipeptide, typical of Gram-negative bacteria, and is impaired by D-isoglutamine presence suggesting the involvement of this modification in immune evasion. However, the same was not observed for NOD2, whose binding activity to muropeptides is not affected by the amidation of glutamic acid (27). Also, this modification did not induce cytokine production, indicating that it is not involved in the modulation of pro-inflammatory capacity (16).

Amidation of peptidoglycan glutamic acid residue is common to many bacterial species – not all pathogenic (Figure S1) – suggesting additional physiological roles for this modification. One role of amidation could be to

reduce the number of cell wall carboxylate groups that have recently been implicated together with wall teichoic acid phosphate residues to cooperatively bind divalent cations like Mg2+ or Mn2+ (13).

A model for the cooperative functions of MurT and GatD. The *murT-gatD* operon emerged as a syntenic block that seems to be widespread among bacteria. Interestingly, for the distant taxa of Actinobacteria, in some rare cases, the two ORFs are merged into a single one (Figure S1).

The genome co-localization of the two determinants, together with data from sequence analysis, led us to suggest a model for the coordinated function of MurT and GatD proteins in the peptidoglycan glutamate amidation (Figure 6).

Both proteins together harbor all domain functions required for amidation of peptidoglycan precursor: MurT may be responsible for the recognition of the reaction substrates, the lipid-linked peptidoglycan precursor and ATP, while GatD could be the catalytic subunit involved in the transfer of the amino group from free glutamine to the peptidoglycan precursor. The GatD sequence lacks an ATP binding motif which is common to all members of the Gn-AT family suggesting an activity that depends on the MurT protein which exhibits a typical Mur ligase central domain including the ATP binding motif (Figure 6).

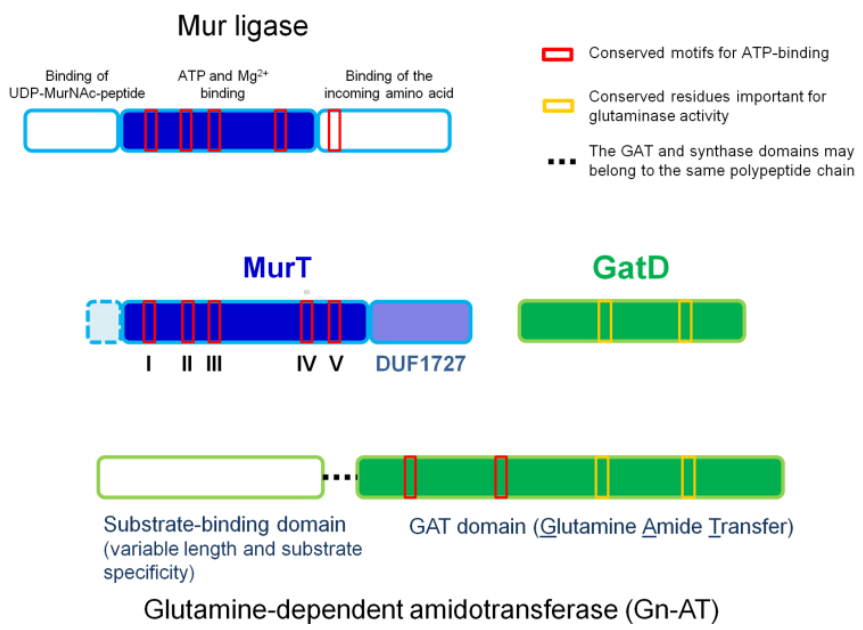

Figure 6. Protein regions necessary for the cooperative function of MurT and GatD proteins. The top panel represents the general topology of Mur ligase family proteins, with three domains. The only domain showing homology with MurT is the central domain involved in nucleotide binding. The conserved motifs for ATP-binding are indicated by red boxes. The lower panel represents the modular structure of Gn-ATs, with a synthase domain and a GAT domain which has glutaminase activity motifs (yellow boxes) and ATP-binding motifs (red boxes). GatD only shares the glutaminase motifs.

Experiments are in progress to better define the roles of MurT and GatD proteins in the mechanism of amidation of *S. aureus* peptidoglycan precursor. Irrespective of mechanistic details, the results with the conditional mutant of *murT/gatD* clearly indicate that the amidation of glutamic acid residues in the *S. aureus* peptidoglycan is catalyzed by the concerted action of these two enzymes. The *murT-gatD* operon appears to be the last missing genetic determinant to account for the structural variation in the *S. aureus* peptidoglycan.

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All the experimental work described in this chapter was performed by T. A. Figueiredo.

References

1. **Bera, A., R. Biswas, S. Herbert, E. Kulauzovic, C. Weidenmaier, A. Peschel, and F. Gotz**. 2007. Influence of wall teichoic acid on lysozyme resistance in *Staphylococcus aureus*. J. Bacteriol. **189**:280-283.
2. **Bera, A., S. Herbert, A. Jakob, W. Vollmer, and F. Gotz**. 2005. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. Mol. Microbiol. **55**: 778-787.
3. **Boneca, I. G.** 2005. The role of peptidoglycan in pathogenesis. Curr. Opin. Microbiol. **8**:46-53.
4. **Bryson, K., L. J. McGuffin, R. L. Marsden, J. J. Ward, J. S. Sodhi and D. T. Jones**. 2005. Protein structure prediction servers at University College London. Nucleic Acids Res. **33**:W36-38.
5. **Bui, N. K., J. Gray, H. Schwarz, P. Schumann, D. Blanot and W. Vollmer**. 2009. The peptidoglycan sacculus of *Myxococcus xanthus* has unusual structural features and is degraded during glycerol-induced myxospore development. J Bacteriol. **191**:494-505.
6. **Crisostomo, M. I., W. Vollmer, A. S. Kharat, S. Inhulsen, F. Gehre, S. Buckenmaier and A. Tomasz**. 2006. Attenuation of penicillin resistance in a peptidoglycan *O*-acetyl transferase mutant of *Streptococcus pneumoniae*. Mol. Microbiol. **61**:1497-1509.
7. **de Jonge, B. L., Y. S. Chang, D. Gage and A. Tomasz**. 1992. Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin binding protein 2A. J. Biol. Chem. **267**:11248-11254.
8. **de Lencastre, H., S. W. Wu, M. G. Pinho, A. M. Ludovice, S. Filipe, S. Gardete, R. Sobral, S. Gill, M. Chung, and A. Tomasz**. 1999. Antibiotic resistance as a stress response: complete sequencing of a large number of chromosomal loci in *Staphylococcus aureus* strain COL that impact on the expression of resistance to methicillin. Microb. Drug Resist. **5**:163-175.
9. **Galperin, M. Y. and N. V. Grishin**. 2000. The synthetase domains of cobalamin biosynthesis amidotransferases *cobB* and *cobQ* belong to a new family of ATP-dependent amidoligases, related to dethiobiotin synthetase. Proteins. **41**:238-247.
10. **Grkovic, S., M. H. Brown, K. M. Hardie, N. Firth and R. A. Skurray**. 2003. Stable low-copy-number *Staphylococcus aureus* shuttle vectors. Microbiology. **149**:785-794.
11. **Gustafson, J., A. Strassle, H. Hachler, F. H. Kayser, and B. Berger-Bachi**. 1994. The *femC* locus of *Staphylococcus aureus* required for methicillin resistance includes the glutamine synthetase operon. J. Bacteriol. **176**:1460-1467.
12. **Herbert, S., A. Bera, C. Nerz, D. Kraus, A. Peschel, C. Goerke, M. Meehl, A. Cheung and F. Gotz**. 2007. Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. PLoS Pathog. **3**:e102.
13. **Kern, T., M. Giffard, S. Hediger, A. Amoroso, C. Giustini, N. K. Bui, B. Joris, C. Bougault, W. Vollmer and J. P. Simorre**. 2010. Dynamics characterization of fully hydrated bacterial cell walls by solid-state NMR:

evidence for cooperative binding of metal ions. J. Am. Chem. Soc. **132**:10911-10919.

14. **Kohlrausch, U. and J. V. Holtje**. 1991. One-step purification procedure for UDP-*N*-acetylmuramyl-peptide murein precursors from *Bacillus cereus*. FEMS Microbiol Lett. **62**:253-257.
15. **Kraemer, G. R., and J. J. Iandolo** 1990. High-frequency transformation of *Staphylococcus aureus* by electroporation. Curr. Microbiol. **21**:373–376.
16. **Kraus, D., H. Kalbacher, J. Buschmann, B. Berger-Bachi, F. Gotz and A. Peschel**. 2007. Muropeptide modification-amidation of peptidoglycan D-glutamate does not affect the proinflammatory activity of *Staphylococcus aureus*. Infect Immun. **75**:2084-2087.
17. **Massiere, F., and M. A. Badet-Denisot**. 1998. The mechanism of glutamine-dependent amidotransferases. Cell Mol. Life Sci. **54**: 205-222.
18. **Notredame, C., D. G. Higgins, and J. Heringa** 2000. T-Coffee: A novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. **302**:205–217.
19. **Ornelas-Soares, A., H. de Lencastre, B. de Jonge, D. Gage, Y. S. Chang, and A. Tomasz**. 1993. The peptidoglycan composition of a *Staphylococcus aureus* mutant selected for reduced methicillin resistance. J. Biol. Chem. **268**: 26268-26272.
20. **Oshida, T., and A. Tomasz**. 1992. Isolation and characterization of a Tn*551*-autolysis mutant of *Staphylococcus aureus*. J Bacteriol. **174**:4952-4959.
21. **Siewert, G., and J. L. Strominger**. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XI. Formation of the isoglutamine amide group in the cell walls of *Staphylococcus aureus*. J. Biol. Chem. **243**: 783-790.
22. **Smith, C. A.** 2006. Structure, function and dynamics in the mur family of bacterial cell wall ligases. J. Mol. Biol. **362**:640-655.
23. **Sobral, R. G., A. M. Ludovice, S. Gardete, K. Tabei, H. De Lencastre, and A. Tomasz**. 2003. Normally functioning *murF* is essential for the optimal expression of methicillin resistance in *Staphylococcus aureus*. Microb. Drug Resist. **9**:231-241.
24. **Sobral, R. G., A. M. Ludovice, H. de Lencastre and A. Tomasz**. 2006. Role of *murF* in cell wall biosynthesis: isolation and characterization of a *murF* conditional mutant of *Staphylococcus aureus*. J. Bacteriol. **188**:2543-2553.
25. **UniProtConsortium**. 2010. Ongoing and future developments at the Universal Protein Resource. Nucleic Acids Res. **39**:D214–219.
26. **Vollmer, W., D. Blanot and M. A. de Pedro**. 2008. Peptidoglycan structure and architecture. FEMS Microbiol Rev. **32**:149-167.
27. **Wolfert, M. A., A. Roychowdhury and G. J. Boons**. 2007. Modification of the structure of peptidoglycan is a strategy to avoid detection by nucleotide-binding oligomerization domain protein 1. Infect. Immun. **75**:706-713.
28. **Zdobnov, E. M., and R. Apweiler.** 2001. InterProScan–an integration platform for the signature-recognition methods in InterPro. Bioinformatics. **17**: 847-848.

Supplementary Information

Supplementary Tables

Table S1. Primers used in this study.

Primer	Sequence (5' - 3')[a]	Source or reference
Amplification of Transcripts (RT-PCR)		
P1949-R1	CTCTGAACATCGCATCAATGG	This study
P1948-R1	CGGGATCCCTAAACTACGGAGGGATGTG	This study
PmurT-D1	CTTCGGTGAAATTGATATTATGG	This study
PmurT-XR1	ATTGATCATCGCTTCTTTTCG	This study
PgatD-R1	GTGGAAGTGATAGAGAACAAGC	This study
PSK50f	ACGCGTCGACGAACAATTAGAAGGCGA	This study
PmurT-D2	TATACATCAGACAATGGTCG	This study
PgatD-XR1	GCGCCTCGAGCGAGATTTCTTCTGTC	This study
Construction of the conditional mutant		
PmurT'F	TCCCCCGGGCGAGTGGAAATTT**GAGG**AGG	This study
PmurT'R	CGAGATCTGACCATTGTCTGATGTATACG	This study
PmurT-R1	GTTCTCTATCACTTCCACCACC	This study
PmurT-R2	GTGTTGATTGCATGATGAATGC	This study
PcadF	GCACTTATTCAAGTGTATTT	Novick
PcadR	GTTCAGACATTGACCTTCAC	Novick
Amplification of DNA probes (Northern blotting)		
PmurT-D	TCCCCCGGGCGAGTGGAAATTTGAGGAGG	This study
PmurT-R	CGAGATCTGACCATTGTCTGATGTATACG	This study
PgatD-D	GTGGAAGTGATAGAGAACAAGC	This study
PgatD-R	GAATACCCTTACGTTCACAAGC	This study
P1952-D	GAATGTACGAGCGCCAAGTTC	This study
P1952-R	CAATGGCAGCATACTGTGATAAAG	This study

Continued in the next page.

Table S1. Primers used in this study (cont.).

Primer	Sequence (5' - 3')[a]	Source or reference
FemC-D	CTGGACAAGGTAAAGTTGCACG	This study
FemC-R	CTAGTCCAGCTTCTAAGATTGC	This study
Construction of the complementation mutants		
PmurTSalI	ACGCGTCGACATATGCGTGTGCTGC	This study
PmurTBamHI	CGGGATCCATATTATGATTGACCTCCTTCAAAC	This study
PgatDSalI	ACGCGTCGACGAACAATTAGAAGGCGA	This study
PgatDBamHI	CGGGATCCAAATCCATTGATGCG	This study

[a] The restriction sites included in the primers are underlined and the putative ribosome binding site is indicated in boldface type**.**

Supplementary Figures

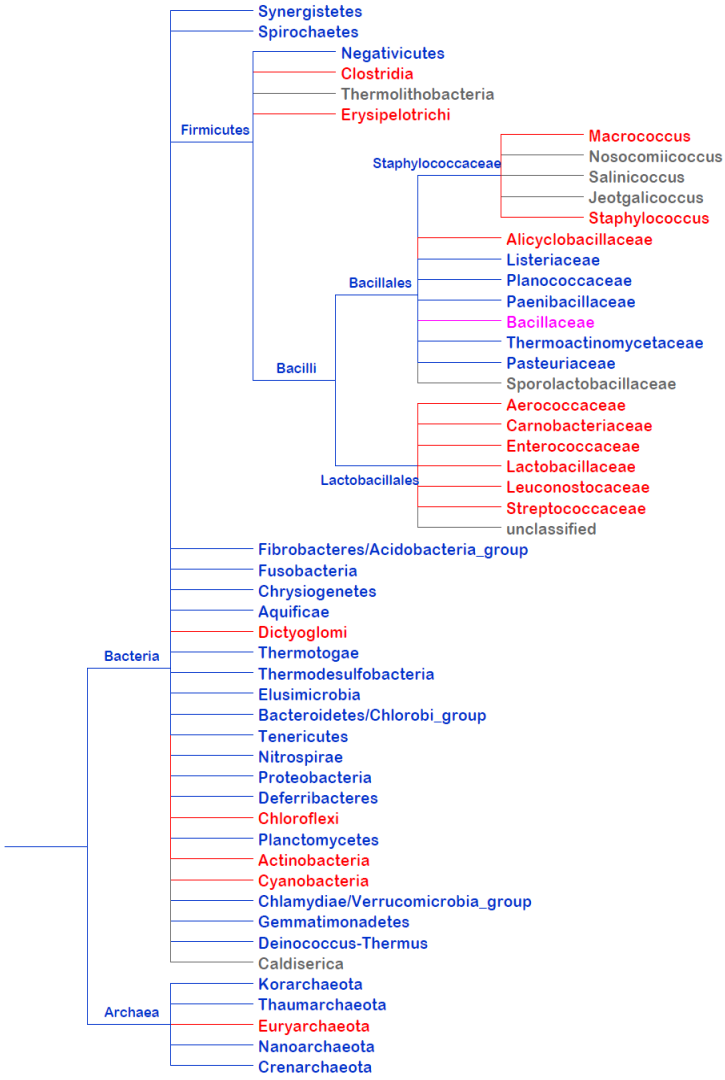

Figure S1. Distribution of *murT/gatD* (red) among prokaryotes. Bacillaceae is depicted in magenta because only one species has the pair. In grey are the taxonomic groups for which no sequence information is available. Actinobacteria present three cases of fused ORFs. The tree representation was built with the help of iTOL (http://itol.embl.de/), and it is based on the structure of the NCBI Taxonomy hierarchy. It should not be considered as a proper phylogenetic tree.

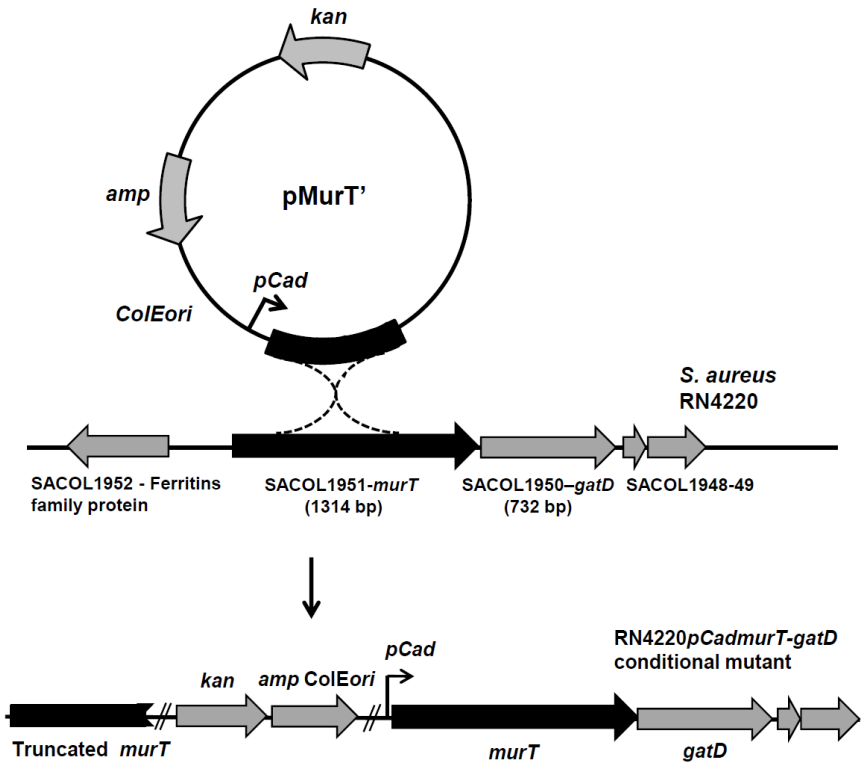

Figure S2. Construction of the *murT-gatD* conditional mutant. A 918 bp DNA fragment containing the ribosome binding site and the 5' sequence of SACOL1951 ORF was cloned downstream from *pCad* promoter. The resulting plasmid, pMurT', was introduced into *S. aureus* RN4220 by electroporation and integrated into the chromosomal SACOL1951-1950 region by Campbell type recombination. The only complete copy of *murT-gatD* operon is under the control of the *pCad* promoter, in the COL*pCadmurT-gatD* conditional mutant.

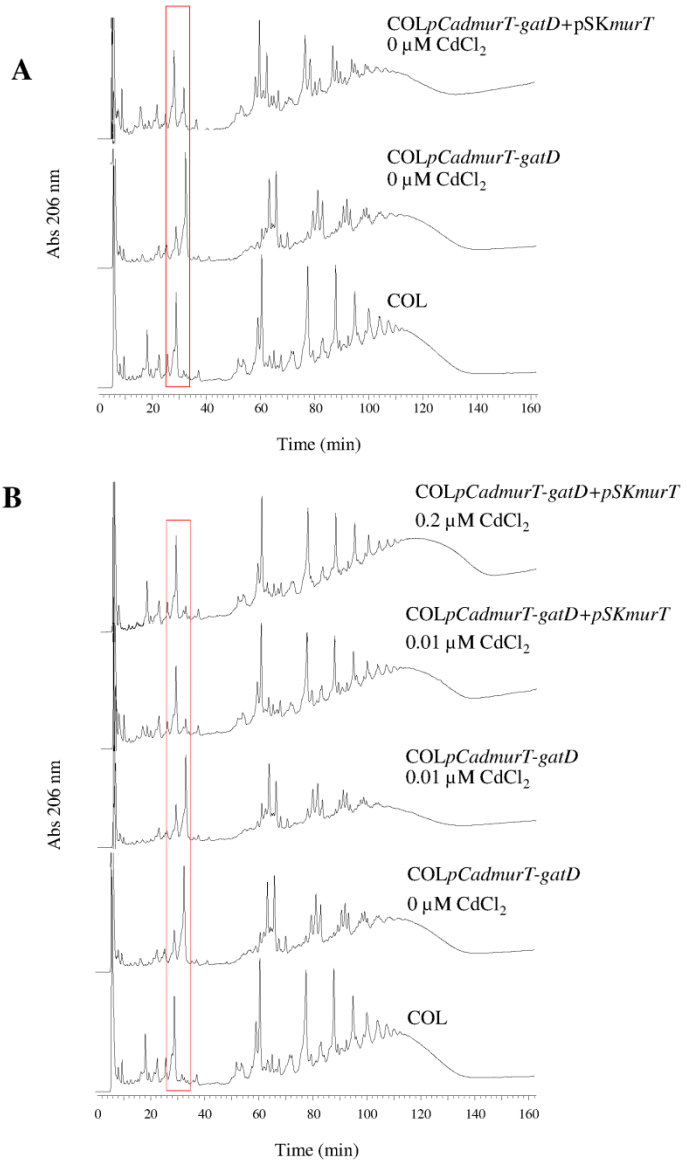

Figure S3. RP-HPLC profiles of purified peptidoglycan digested with mutanolysin. (A) Comparison of peptidoglycan elution profiles of strains COL, mutant COL*pCadmurT-gatD* grown without inducer and the complementation strain COL*pCadmurTgatD*+pSK*murT* grown without inducer. The complementation strain shows partial re-establishment of the abnormal amidation level. (B) Comparison of peptidoglycan elution profiles of strains COL, mutant COL*pCadmurT-gatD* grown without inducer, with sub-optimal inducer concentration (0.01 µM of $CdCl_2$) and the complementation strain COL*pCadmurT-gatD*+pSK*murT* grown with 0.01 µM of $CdCl_2$ and with 0.2 µM of $CdCl_2$. The complementation strain grown with sub-optimal inducer concentration shows complete re-establishment of the abnormal amidation level.

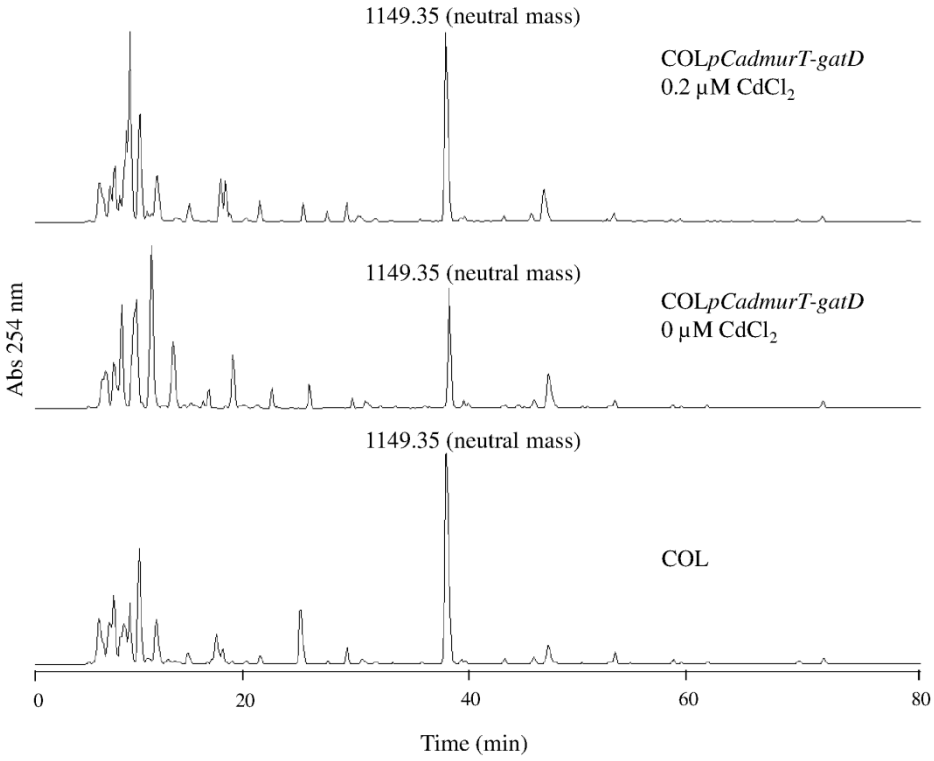

Figure S4. RP-HPLC profiles of UDP-linked precursor pools. The UDP-linked precursor pools of the strain COL and COL*pCadmurT-gatD* grown with or without 0.2 µM of $CdCl_2$. The major precursor structure (elution time of 38.0 min) was identified by mass spectrometry as UDP-Mur*N*Ac-L-Ala-D-iGlu-L-Lys-DAla-D-Ala.

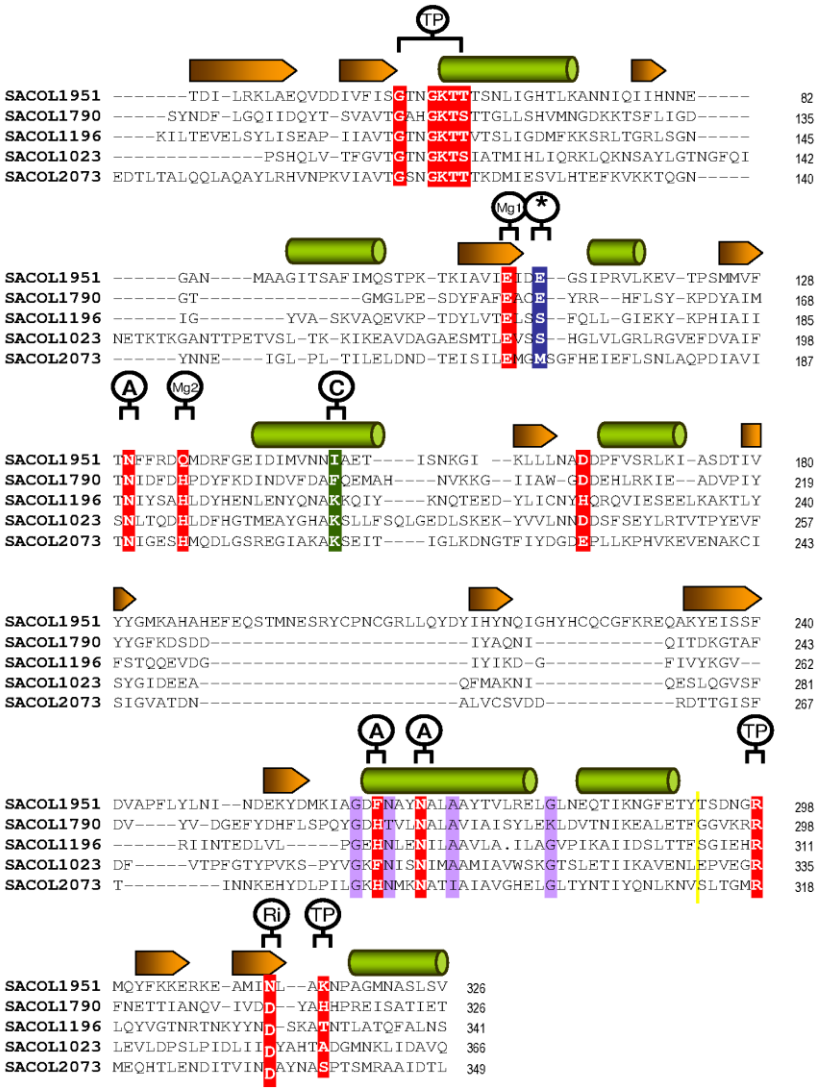

Figure S5A. Structure-informed aminoacid sequence alignments. Sequence alignment of the central domain of Mur ligases. Residues involved with the nucleotide binding of four known *S. aureus* COL Mur ligases and MurT are labelled TP (ATP triphosphate), Mg1 and Mg2 (magnesium), A (adenine), and Ri (ATP ribose). The residue labelled C is the carbamoylated lysine residue observed in all the Mur enzymes except MurC; in this enzyme a glutamate residue, indicated with an asterisk (*) seems to play the same role in Mg2 coordination. The initial alignment was performed by TCoffee (2), the secondary structure was inferred for all sequences through Psipred (1), and the alignment was manually edited according to the latter.SACOL1951-MurT; SACOL1790-MurC; SACOL1196-MurD; SACOL1023-MurE; SACOL2073-MurF. In the top line a-helixes (green cylinders) and b-strands (orange arrows) were inferred for the sequences of the known Mur ligases.

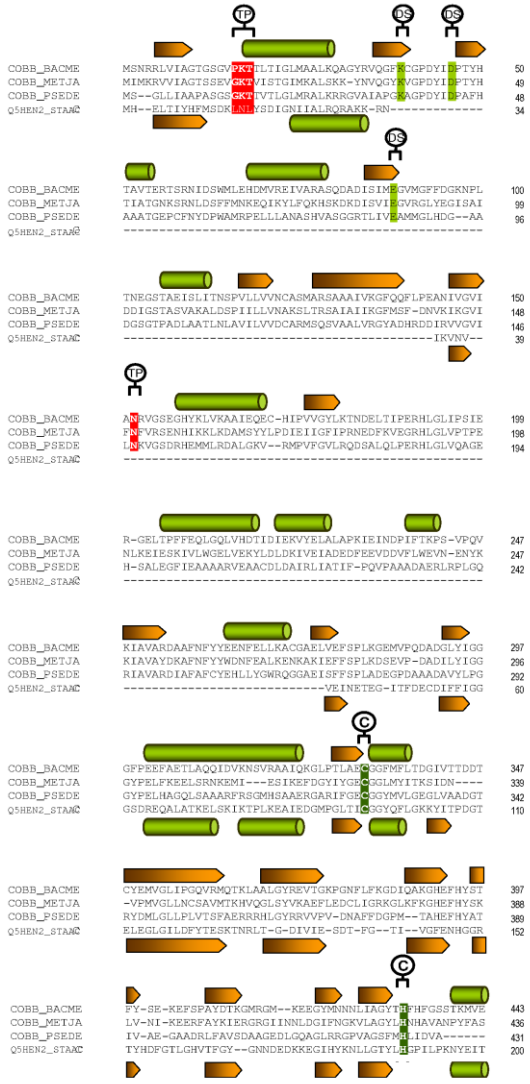

Figure S5B. Structure-informed aminoacid sequence alignments. Sequence alignment of the N-terminal halves of three known GATases. The residues involved in nucleotide binding of *S. aureus* COL GatD and three known GATases are indicated with a filled red box and are labelled TP (ATP triphosphate). Residues in green boxes marked with DS are deemed important for the dethiobiotin synthetase activity. The residues in the filled box labelled C are annotated as being directly involved with reactive center according to the GATase 3 (IPR011698) domain documentation. The initial alignment was performed by TCoffee (2), the secondary structure was inferred for all sequences through Psipred (1), and the alignment was manually edited according to the latter.GatD-Q5HEN2_STAAC (*S. aureus*); COBB_BACME (*Bacillus megaterium*); COBB_METJA (*Methanocaldococcus jannaschii*); COBB_PSEDE (*Pseudomonas denitrificans*). In the top line a-helixes (green cylinders) and b-strands (orange arrows) were inferred for the sequences of these three known GATases. In the bottom line, the same information is shown for GatD.

Supplementary References

1. **Bryson, K., L. J. McGuffin, R. L. Marsden, J. J. Ward, J. S. Sodhi and D. T. Jones**. 2005. Protein structure prediction servers at University College London. Nucleic Acids Res. **33**:W36-38.
2. **Notredame, C., D. G. Higgins, and J. Heringa** 2000. T-Coffee: A novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. **302**:205–217.

Chapter III

MurT-GatD is an enzyme complex responsible for the amidation of glutamic acid residues in the peptidoglycan precursor lipid II of *S. aureus*

Abstract

The peptidoglycan of *Staphylococcus aureus* is virtually free of carboxyl groups due to the amidation of the α-carboxyl group of D-glutamate at position 2 of the stem peptide, resulting in the formation of D-isoglutamine. Recently, the *murT-gatD* operon was identified as the genetic determinant responsible for the amidation of D-glutamate. The MurT and GatD proteins have sequence similarity to the highly conserved central domain of Mur ligases and to the catalytic domain in CobB/CobQ-like glutamine amidotransferases, respectively. In the present study, we describe the co-purification of MurT and GatD recombinant proteins from a vector expressing both *murT* and *gatD* genes and provide evidence indicating that these proteins interact, forming a stable enzymatic complex. *In vitro* analysis of lipid II amidation, using purified GatD and MurT-GatD recombinant proteins, demonstrated that both proteins are required for peptidoglycan amidation; most probably, GatD is responsible for the glutaminase activity and MurT is related to the ATP-dependent synthetase activity and substrate recognition. This second part of my Thesis also provided information essential for the establishment of the structure of the MurT-GatD protein complex. The crystal structure of the GatD protein is already available.

Introduction

In gram-positive bacteria, the thick peptidoglycan multilayer constitutes an essential stress-bearing and shape-maintaining component of the bacterial cell envelope. The biosynthesis of peptidoglycan is a complex process, which requires several enzymatic reactions, and takes place in three distinct cellular compartments: the cytoplasm and the inner and the outer surface of the cytoplasmic membrane. The biosynthesis starts in the cytoplasm with the formation of the UDP-*N*-acetylmuramic acid (UDP-Mur*N*Ac) covalently linked to a pentapeptide, which is composed of L-alanine, D-glutamate, L-lysine and D-alanyl D-alanine. The successive addition of the five aminoacids is catalyzed by a set of enzymes, belonging to the superfamily of Mur ligases (12). The UDP-Mur*N*Ac-pentapeptide is next attached to a membrane acceptor, undecaprenyl phosphate (C55-P), by the translocase MraY, producing a structure known as lipid I. In the subsequent steps of biosynthesis, the enzyme MurG links UDP-Glc*N*Ac to lipid I, resulting in the formation of lipid II (3, 14), which is transported to the outer surface of the cytoplasmic membrane, where it serves as a substrate for polymerization reactions catalyzed by transpeptidases and transglycosylases to form the polymeric cell wall peptidoglycan (9, 15).

Chemical analysis of *S. aureus* peptidoglycan showed that the molecular composition of this polymer differs from the molecular composition of the cytoplasmic disaccharide pentapeptide cell wall precursor, namely some hydroxyl groups of the glycan chain were O-acetylated (1, 2) and the second amino acid residue of the pentapeptide was not glutamic acid but its amidated version, D-iso-glutamine (10).

In search for genes and enzymes responsible for the amidation of glutamic acid carboxyl residues, we identified in *S. aureus* strain COL a small operon composed of two genes, *murT* and *gatD*. The two genes were

encoding enzymes responsible for the conversion of glutamic acid residues to iso-glutamine of peptidoglycan. This secondary modification of the *S. aureus* peptidoglycan was shown to be important for bacterial growth and resistance to beta-lactam antibiotics and lysozyme (5). Furthermore, a substantial reduction of peptidoglycan crosslinking was also observed in a mutant (*femC*), unable to synthesize glutamine, the amino group donor, suggesting that non-amidated cell wall precursors are poor substrates for transpeptidases. Thus, glutamate amidation also plays an important role in proper transpeptidation of neighboring stem peptides (4, 13).

In this study, we document the physical interaction between the MurT and GatD proteins and provide evidence for the importance of this interaction in the amidation of peptidoglycan. In addition, we also demonstrate that the amidation of glutamic acid residues occurs at the lipid II stage of peptidoglycan biosynthesis.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37ºC in Luria-Bertani broth (LB, Difco Laboratories) or in LB agar (LA, Difco Laboratories), in the presence of the respective antibiotics (Table 1), ampicillin 100 μg/ml (Sigma), kanamycin 30 μg/ml (Sigma) and chloranfenicol 50 μg/ml (Sigma). For protein expression, a final concentration of 1mM of IPTG inducer (Sigma) was used. Growth in liquid medium was monitored by measuring the optical density of *E. coli* cultures at 578 nm.

Construction of *S. aureus* GatD as an His_6-tag N-terminal fusion. The *gatD* gene of *S. aureus* strain COL was amplified using primers PGatD-NdeI-d1 and PGatD-NotI-r1 (Table 2) and ligated into pET28a vector using T4 DNA ligase (New England Biolabs) and NdeI (Fermentas) and NotI (Fermentas) restrictions sites to generate a N-terminal His_6-fusion protein. *E. coli* DH5α host was transformed with the ligation mixture and positive transformants were screened by PCR and sequenced. The correct recombinant plasmid was designated pET28a-His_6-*gatD*.

Over-expression and purification of *S. aureus* His_6-GatD. *E. coli* BL21(DE3) CodonPlus RIPL cells transformed with the pET28a-His_6-*gatD* were grown at 37ºC in LB medium supplemented with the appropriate antibiotics. In order to optimize the expression and solubility, several conditions were tested: different induction periods (3h versus overnight), induction temperatures (37ºC, 30ºC, 28ºC) and salt concentrations (100 mM versus 500 mM NaCl). The final induction conditions were the following: at an optical density at 578 nm (OD_{578nm}) of 0.6, 1mM IPTG inducer was added and the culture was grown at 30ºC, to induce expression of the recombinant protein. After 3 h, cells were centrifuged at

6000 g for 15 min at 4ºC, resuspended in extraction buffer (20 mM Tris-HCl pH 8.2, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM PMSF, 0.1 mg/ml of DNaseI (Sigma)) and incubated on ice, for 10 min. The cell suspension was sonicated, with 30% of intensity for 10 min (Branson S450D), and the cell debris were spun down. After addition of Imidazole (Sigma) to a final concentration of 10 mM, the supernatant was mixed with Ni-NTA beads (Qiagen) and incubated at 4ºC for 16 h, with stirring. This mixture was then loaded onto a gravity column. In order to remove the weakly bound material, the column was washed with 10 column bead volumes of extraction buffer containing 10 mM Imidazole. The His-tagged recombinant protein was eluted with extraction buffer containing 200 mM of Imidazole**.** The purity of each elution was controlled by SDS-PAGE. Imidazole was dialyzed out against 20 mM Tris-HCl pH 8.2, 200 mM NaCl, 10 mM $MgCl_2$ and 10% of glycerol. Then, the suspension was diluted to a final concentration of NaCl of 100 mM and purified by anion exchange chromatography using a Q-HP-5 ml column (GE Healthcare) with a gradient of 30 ml from 100 mM to 1 M of NaCl in 20 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2$, 10% of glycerol and at a flow rate of 0.5 ml/min. Elutions of 1 ml were collected and their purity was analyzed by SDS-PAGE. The elution fractions of interest were concentrated using Vivaspin centrifugal concentrators (Sartorius) and purified by size exclusion chromatography using a Superdex 7510/300 Gl column (GE Healthcare) and 20 mM Tris-HCl, pH 8.2, 500 mM NaCl, 10 mM $MgCl_2$ at a flow rate of 0.5 ml/min.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Description[a]	Source or reference
Strains		
S. aureus		
COL	Homogeneous Mc^r	The Rockefeller Univ. Collection
E. coli		
DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE*44 *relA1* F80 D*lac*ZDM15	Invitrogen
BL21-CodonPlus(DE3)-RIPL	B F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal λ* (DE3) *endA* Hte [*argU proL*Cm^r] [*argU ileY leuW* Strep/Spec^r]	Stratagene
BL21-His$_6$-*gatD*	BL21-CodonPlus(DE3)-RIPL strain with pET28a-His$_6$-*gatD*	This study
BL21-*murT-gatD*-His$_6$	BL21-CodonPlus(DE3)-RIPL strain with pET28a-*murT-gatD*-His$_6$	This study
Plasmids		
pET28a	Expression vector with T7/*lac* promoter, N-terminal His tag, thrombin cleavage site, C-terminal His tag; Kan^r	Invitrogen
pET28a-His$_6$-*gatD*	pET28a expressing His$_6$-GatD	This study
pET28a-*murT-gatD*-His$_6$	pET28a expressing MurT-GatD-His$_6$	This study

[a)] Mc^r, methicillin resistant; Cm^r, chloramphenicol resistant; Spec^r, spectinomycin resistant; Kan^r, kanamicin resistant.

Construction of *S. aureus* MurT-GatD as a His$_6$-tag C-terminal fusion. The *murT-gatD* operon of *S. aureus* strain COL was amplified using the primers P1951-NcoI-d1 and P1950-XhoI-r1 (Table 2) and cloned in pET28a vector using NcoI (Fermentas) and XhoI (Fermentas) restrictions sites to generate a C-terminal GatD-His$_6$ fusion protein. *E. coli* DH5α host was transformed with the ligation mixture and positive transformants were

screened by PCR and sequenced. The correct recombinant plasmid was designated pET28a-murT-*gatD*-His_6.

Table 2. Primers used in this study.

Primer	Sequence (5' - 3')a	Reference
GatD-NdeI-d1	GCGCCATATGCATGAATTGACTATTTATC	This study
GatD-NotI-r1	GCGCGCGGCCGCTTAACGAGATTTCTTCTGTC	This study
P1951-NcoI-d1	CATGCCATGGGAAGACAGTGGACGGCAATC	This study
P1950-XhoI-r1	GCGCCTCGAGACGAGATTTCTTCTGTCTATTTG	This study

[a] Restrictions sites are underlined.

Co-expression and purification of MurT-GatD enzyme complex. The pET28a-*murT-gatD*-His_6 plasmid was introduced into *E. coli* BL21(DE3) CodonPlus RIPL and over-expression was performed as described for His_6-GatD purification, with the following modifications. Briefly, after the induction of expression of recombinant proteins, cells were resuspended in extraction buffer (20 mM Tris-HCl, pH 8.2, 1 M NaCl, 10 mM $MgCl_2$, 1 mM PMSF, 0.1 mg/ml of DNaseI), sonicated and the cell debris were spun down. After the addition of Imidazole, to a final concentration of 20 mM, the supernatant was mixed to Ni NTA beads and incubated at 4ºC for 16 h, with stirring. The elution was performed as described for His_6-GatD purification, with modifications. After loading the mixture onto a gravity column, the column was washed with 10 column bead volumes of extraction buffer containing 40 mM Imidazole followed by elution with extraction buffer containing 400 mM of Imidazole. Imidazole was dialyzed out as described above. The suspension was purified by cation exchange chromatography, using a HP-S-5 ml column (GE Healthcare), with a gradient of 30 ml from 100 mM to 1 M of NaCl in 20 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2$, 10% of glycerol, at a flow rate of 0.5 ml/min. Protein elutions of 1 ml were collected

and their purity was analysed by SDS-PAGE. The eluted fractions of interest were concentrated as mentioned before and purified by size exclusion chromatography using a Superdex 7510/300 GI column (GE Healthcare) and 20 mM Tris-HCl, pH 8.2, 1 M NaCl, 10 mM $MgCl_2$ at a flow rate of 0.5 ml/min.

***In vitro* synthesis of amidated lipid II.** The assays for synthesis of amidated lipid II were performed in a total volume of 30 µl containing 2 mg of purified His_6-GatD or MurT-GatD-His_6. Different reaction conditions were tested and the ones that yielded the best results were described in Munch et al (7). Briefly, 0.5 mM of lipid II (http://www2.warwick.ac.uk/efac/sci/lfesci/people/droper/bacwan/) were incubated with 160 mM Tris-HCl, 0.7 % or 0.2 % Triton X-100, 5 mM KCl, 40 mM $MgCl_2$, pH 7.5, 6 mM ATP and 7 mM glutamine, for 4 h at 30ºC. To extract the synthesis products, the reaction mixtures were incubated with the same volume of n-butanol/pyridine acetate, pH 4.2. After centrifugation, the upper phase, containing the lipid products, was analyzed by Thin Layer Chromatography (TLC) using the mobile phase (butanol, acetic acid, water, pyridine, 15:3:12:10) and aluminum foil sheets coated with silica gel adsorbent with a pore size of 60 Å (Sigma), as the stationary phase. Lipid spots were visualized using iodine vapor.

Results

In order to explore the enzymatic mechanism of the two enzymes and their contribution to the amidation reaction, active recombinant proteins were constructed: *i)* a MurT-GatD co-expression system was constructed in order to test the physical interaction between the two proteins; *ii)* MurT and GatD proteins were purified for enzymatic studies and structure determination.

MurT and GatD proteins interact physically. Previously, *murT* and *gatD* genes were shown to be co-transcribed from the same promoter, organized as a small operon. Moreover, analysis of available genome sequences showed that the *murT* and *gatD* genes occur, widespread among Gram-positive bacteria, as a syntenic block, suggesting that the protein products interact functionally. The full *in vivo* complementation of the *murT-gatD* depletion phenotype, confirmed the functional cooperative action of the two proteins (5).

The experimental evidence for a functional interaction between GatD and MurT proteins suggested that the two proteins interact physically and form an enzymatic complex.

To test this hypothesis we designed a co-purification assay for MurT and GatD proteins using a vector expressing both *murT* and *gatD* genes. The *murT-gatD* operon amplified from strain COL was cloned into the *E. coli* expression vector pET28a, fusing the operon sequence with the C-terminal His_6-tag. Such construction allowed the expression of both proteins, MurT and GatD-His_6. The conditions used to over-express both MurT and GatD were the following: *E. coli* expressing MurT-GatD-His_6, was induced with 1 mM of IPTG for 3h at 30ºC. The optimal salt concentration for the elution procedure was 1 M.

Through affinity chromatography, the recombinant GatD-His_6 was recovered from the supernatant and MurT was pulled down along, strongly suggesting that the two proteins are able to form an enzymatic complex (Figure 1). Moreover, the fact that both proteins co-eluted at high ionic conditions (1M NaCl) in the absence of substrate molecules, suggests a strong physical interaction between GatD and MurT.

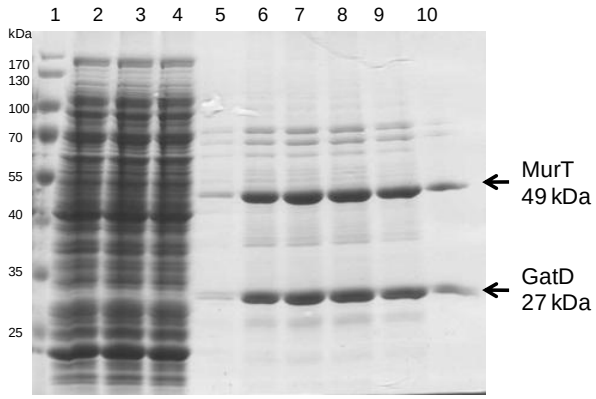

Figure 1. Co-elution of MurT and GatD-His_6 proteins using Ni-NTA beads. Samples of the co-expression and co-purification procedure were resolved by 12%-SDS-PAGE and stained with coomassie blue G-250 (Bio-rad). *Lane 1*: PageRuler prestained protein ladder (Fermentas); *Lane 2*: protein sample before be applied to the column; *Lane 3*: flow through; *Lane 4*: washing step 1; *Lane 5*: washing step 10; *Lane 6*: elution 1; *Lane 7*: elution 2; *Lane 8*: elution 3; *Lane 9*: elution 4; *Lane 10*: elution 5. Numbers on the left side represent the molecular weights of the protein ladder.

Enzymatic activity of MurT and GatD proteins. To determine *in vitro* the amidation activity of MurT and GatD, independent His-tagged proteins were constructed. However, while the His_6-GatD fusion was successfully expressed, the His_6-MurT fusion was not obtained in sufficient amounts for activity assays. The amidation assays were performed with the purified MurT-GatD complex and with GatD protein alone.

Purification of the MurT-GatD-His6 complex. To determine *in vitro* the enzymatic activity of MurT-GatD, the enzymatic complex was purified. Elution fractions 1 to 5 (Figure 1) were purified by cation exchange chromatography, using a salt concentration gradient from 100 mM to 1 M

NaCl (Figure 2). Elutions 14 to 18 (Figure 2) were concentrated and purified by size exclusion chromatography (Figure 3).

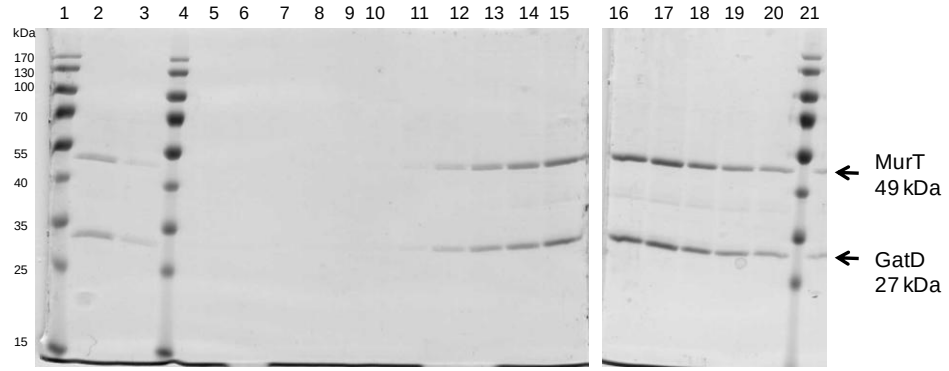

Figure 2. Purification of MurT-GatD-His$_6$ by cation exchange chromatography. Samples of some steps of the purification procedure were resolved by 12% SDS-PAGE and stained with coomassie blue G-250 (Bio-rad). *Lane 1, 4 and 21*: PageRuler prestained protein ladder (Fermentas); *Lane 2*: protein sample before application to the column; *Lane 3*: flow through; *Lane 5*: elution 5; *Lane 6*: elution 6; *Lane 7*: elution 7; *Lane 8*: elution 8; *Lane 9*: elution 9; *Lane 10*: elution 10; *Lane 11*: elution 11; *Lane 12*: elution 12; *Lane 13*: elution 13; *Lane 14*: elution 14; *Lane 15*: elution 15; *Lane 16*: elution 16; *Lane 17*: elution 17; *Lane 18*: elution 18; *Lane 19*: elution 19; *Lane 20*: elution 20. Numbers on the left side represent the molecular weights of the protein ladder.

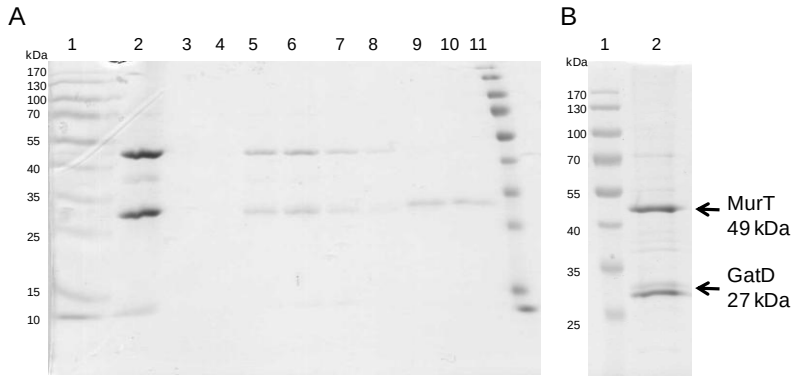

Figure 3. **Purification of MurT-GatD-His$_6$ by size exclusion chromatography**. Samples of some steps of the purification procedure were resolved by 12%-SDS-PAGE and stained with coomassie blue G-250 (Bio-rad)**.** (A) MurT-GatD-His$_6$ elutions purified by size exclusion chromatography. *Lane 1 and 11*: PageRuler prestained protein ladder (Fermentas); *Lane 2*: protein sample before be applied to the column; *Lane 3*: elution 6; *Lane 4*: elution 7*; Lane 5*: elution 8; *Lane 6*: elution 9; *Lane 7*: elution 10; *Lane 8*: elution 11; *Lane 9*: elution 12; *Lane 10*: elution 13. (B) MurT-GatD-His$_6$ protein after concentration, as described in materials and methods, from elutions 9 and 10 from painel A. *Lane 1*: PageRuler prestained protein ladder; *Lane 2*: MurT-GatD-His$_6$ protein. Numbers on the left side represent the molecular weights of the PageRuler prestained protein ladder.

Expression and purification of GatD recombinant protein. To further investigate the role of GatD in glutamate amidation, GatD was purified as a His-tag fusion protein. The conditions used to over express His_6-GatD were the following: induction was performed with 1 mM of IPTG for 3h at 30ºC. The capture of His_6-GatD was performed with Ni-NTA beads and 10 mM imidazole. A step of 200 mM imidazole was used to remove the weakly bound material and to elute His_6-GatD (Figure 4). Elutions 1 to 7 (Figure 4) were further purified by anion exchange chromatography using a concentration gradient from 100 mM to 1 M of NaCl (Figure 5). Due to the concentration ratio between His_6-GatD and contaminant proteins, elutions 11 to 15 (Figure 5) were further purified by size exclusion chromatography (Figure 6).

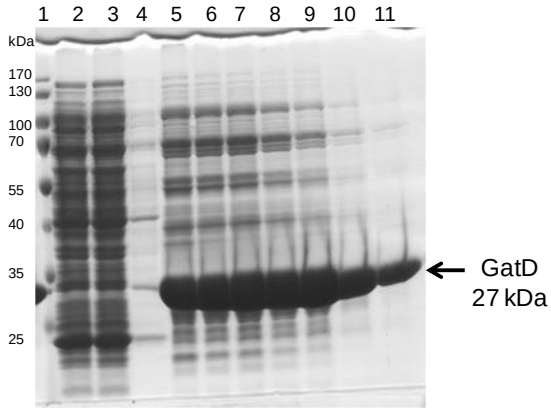

Figure 4. Affinity purification of His_6-GatD protein using Ni-NTA beads. Samples of each phase of the purification procedure were resolved by 12% SDS-PAGE and stained with coomassie blue G-250 (Bio-Rad). Elutions were performed with 200 mM of Imidazole. *Lane 1*: PageRuler prestained protein ladder (Fermentas): *Lane 2*: flow-through; *Lane 3*: washing step 1; *Lane 4:* washing step 10; *Lane 5*: elution 1; *Lane 6*: elution 2; *Lane 7*: elution 3; *Lane 8*: elution 4: *Lane 9*: elution 5; *Lane 10*: elution 6; *Lane 11*: elution 7. Numbers on the left side represent the molecular weights of the protein ladder.

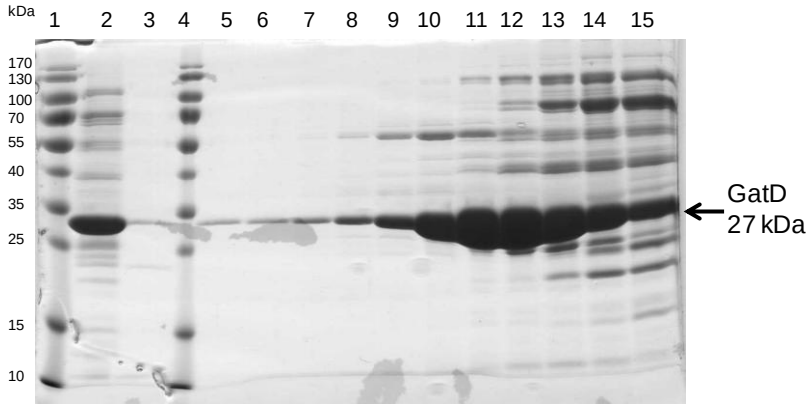

Figure 5. Purification of His_6-GatD protein by anion exchange chromatography. Samples of some steps of the purification procedure were resolved by 12% SDS-PAGE and stained with coomassie blue G-250 (Bio-rad). *Lane 1 and 4*: PageRuler prestained protein ladder (Fermentas); *Lane 2*: Protein sample before be applied to the column; *Lane 3*: flow through; *Lane 5*: elution 7; *Lane 6*: elution 8; *Lane 7*: elution 9; *Lane 8*: elution 10; *Lane 9*: elution 11; *Lane 10*: elution 12; *Lane 11*: elution 13; *Lane 12*; elution 14; *Lane 13*: elution 15; *Lane 14*: elution 16; *Lane 15*: elution 17. Numbers on the left side represent the molecular weights of the protein ladder.

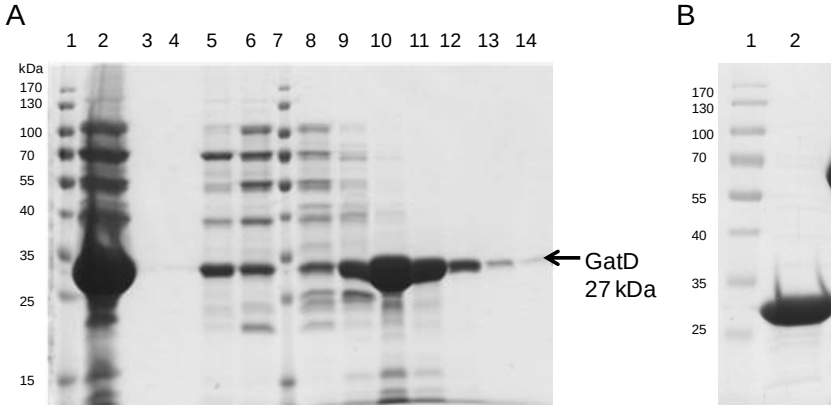

Figure 6. Purification of His_6-GatD protein by size exclusion chromatography. Samples of some steps of the purification procedure were resolved by 12%-SDS-PAGE and stained with coomassie blue G-250 (Bio-rad). (A) His_6-GatD elutions purified by size exclusion chromatography. *Lane 1 and 7*: PageRuler prestained protein ladder (Fermentas); *Lane 2*: Protein sample before be applied to the column; *Lane 3*: elution 7; *Lane 4*: elution 8; *Lane 5*: elution 9; *Lane 6*: elution 10; *Lane 8*: elution 11; *Lane 9*: elution 12; *Lane 10*: elution 13; *Lane 11*: elution 14; *Lane 12*: elution 15; *Lane 13*: elution 16; *Lane 14*: elution 17. (B) His_6-GatD protein after concentration, as described in materials and methods, from elutions 13, 14 and 15 (pannel A). *Lane 1*: PageRuler prestained protein ladder; *Lane 2*: His_6-GatD protein. Numbers on the left side represent the molecular weights of the protein ladder.

Enzymatic activity assays. Siewert and Strominger observed that after addition of ATP and NH_4Cl or glutamine, amidated Lipid I or lipid II could be detected in membranes of *S. aureus* (11). In fact, *S. aureus* cytoplasmic precursors are not amidated, while the cell wall fraction shows prevalence of amidated muropeptides, suggesting that the amidation reaction takes place at the membrane level (5). For these reasons, lipid II was chosen as substrate for the activity assays.

To assess the enzymatic activity of the MurT-GatD complex and GatD alone, lipid II intermediate was incubated with the previously purified recombinant proteins, ATP and glutamine. Different reaction conditions were tested and the ones that yielded the best results were described in Munch *et al* (7).

Separation of the reaction products by TLC showed an additional lipid II band, that migrates slightly faster than unmodified lipid II (Figure 7A, lane 1), which indicates that both lipid structures differ in their polarity. Analysis of the product band was performed by ESI-TOF-Mass Spectrometry, which allowed us to identify the compound as amidated lipid II (7). Therefore, lipid II is a substrate for peptidoglycan amidation, which confirms previous data (11). When the reaction occurs in the presence of purified GatD (Figure 7, lane 2), or in the absence of MurT-GatD (Figure 7A, lane 3), only non-amidated lipid II is observed, indicating that the occurrence of glutamic acid amidation is dependent on the presence of MurT-GatD complex. In addition, this modification is also dependent of glutamine and ATP, since in the absence of these components, only non-amidated lipid II is observed (Figure 7B).

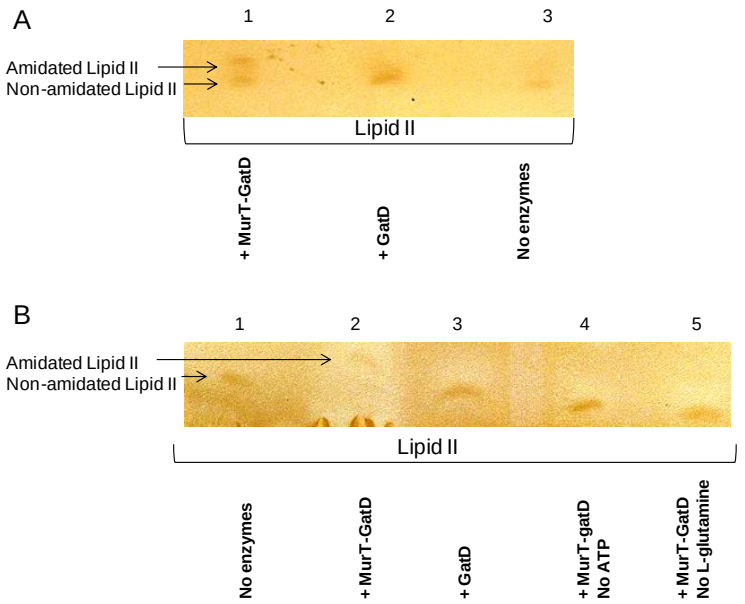

Figure 7. Migration behavior of lipid II and amidated lipid II on TLC. (A) TLC of Lipid II incubated with 0.2% of Triton-X-100. *Lane 1:* Lipid II incubated with MurT-GatD purified complex; *Lane 2*: Lipid II incubated with GatD; *Lane 3*: Lipid II incubated in the absence of MurT-GatD purified complex. (B) TLC of Lipid II incubated with 0.7% of Triton-X-100. *Lane 1*: Lipid II incubated in the absence of MurT-GatD purified complex; *Lane 2*: Lipid II incubated with MurT-GatD purified complex; *Lane 3*: Lipid II incubated with GatD; *Lane 4*: Lipid II incubated in the absence of ATP: *Lane 5*: Lipid II incubated in the absence of L-glutamine.

Structure determination of GatD protein. X-ray structure determination of the enzymatic complex MurT-GatD should provide mechanistic insights into the amidation reaction of peptidoglycan in *S. aureus*. With this aim, the recombinant proteins for GatD and MurT described above were constructed. As previously stated, MurT expression was unsuccessful. Regarding GatD, in collaboration with J. Trincão (Diamond Light Source, Harwell Oxford, Didcot, UK), preliminary crystal growth attempts were unsuccessful as the expression yield was not enough for crystalization purposes. Optimization of the expression and purification strategies were pursued and the native and selenomethionine derivatives of substrate-free GatD were crystallized. For this purpose, the protein was produced using the expression vector pOPINF in *E. Coli* Lemo21pLysS(DE3) expression

strain. These results are described in Appendix I (Vieira, D., T. A. Figueiredo, A. Verma, R. G. Sobral, A. M. Ludovice, H. de Lencastre, and J. Trincao. 2014. Purification, crystallization and preliminary X-ray diffraction analysis of GatD, a glutamine amidotransferase-like protein from *Staphylococcus aureus* peptidoglycan. Acta Crystallogr. F Struct. Biol. Commun. 70: 632-635.)

Discussion

The peptidoglycan of *S. aureus* is a highly dynamic molecule that undergoes several secondary modifications to its primary structure during its biosynthesis. Amidation of the γ-carboxyl group of the D-glutamate residues is one of such modifications and studies described in this Thesis have led to the identification of the *murT-gatD* operon as the genetic determinant involved in this step. MurT shares approximately 15% identity and 53% similarity with the sequence of the Mur ligases of *S. aureus,* while GatD shows similarity to one of the two domains of a cobyric acid synthetase protein: a glutamine amidotransferase, with glutamine amide transfer activity (5).

The glutamine amidotransferases (GATases) are involved in a variety of biological reactions such as biosynthesis of nucleotides, amino acids, aminated sugars, coenzymes and antibiotics. These proteins are modular enzymes, since they are organized in two distinct catalytic domains: the *glutaminase domain* responsible for glutamine hydrolysis and for the amide nitrogen transfer from glutamine to its acceptor substrates; and the *synthetase domain* involved with the recognition and binding of the amino group acceptor substrate. The nitrogen transfer requires synchronization of both reactions and a close interaction between the two catalytic domains. This may be achieved by several – alternative – ways: the catalytic domains may be located on the same polypeptide; on two distinct subunits in heterodimeric GATase; or they can be positioned on subunits from different enzymes, forming an enzymatic complex (6).

In the present study we used co-purification of MurT and GatD to demonstrate that the two proteins interact physically. Furthermore, using *in vitro* activity assays, we showed that lipid II amidation needs the presence of both enzymes, demonstrating that MurT and GatD form a glutamine

amidotransferase bi-enzymatic complex. We infer that GatD is responsible for the glutaminase activity, while the role of MurT is related to the ATP-dependent synthetase activity. Our results demonstrate that lipid II is an efficient amino acceptor molecule and that glutamine is an efficient amino donor.

While obtaining the data described in this Thesis chapter, similar results were obtained by Munch and colleagues (7). In the study of Munch and colleagues, the nature of the nitrogen donor substrate and of the acceptor substrate was further explored. While the majority of GATases seem to be highly specific for using glutamine as an amide nitrogen donor, they can also accept exogenous ammonia as nitrogen source (16). Munch *et al*, (7) demonstrated that at optimal conditions for the ammonia-dependent activity (pH 8.5), the MurT-GatD complex is able to use free ammonia, as nitrogen donor substrate. However, taking into account the neutral pH within the cytoplasm and the fact that the peptidoglycan composition of a *glnRA* transposition mutant (8), unable to synthesize glutamine, is identical to the one of the *murT-gatD* depletion mutant (5), it is most likely that MurT-GatD enzymatic complex, normally, uses glutamine as nitrogen donor. Moreover, the peptidoglycan HPLC profile of a *glnRA* and *murT-gatD* depletion double mutant, showed a complete lack of amidated muropeptides (5), which indicates that the gene products of these two operons are together essential for the lipid II amidation and highlight that this modification is glutamine-dependent.

Regarding the acceptor molecule, Munch *et al* (7) tested the *in vitro* amidation of several structures, namely lipid I, lipid II and lipid II-Gly_5, in the presence of MurT-GatD, glutamine and ATP. The authors concluded that the most efficient substrate was lipid II, although amidation of lipid I and lipid II-Gly_5 also occurred. The presence of a 10 fold excess of Mur*N*Ac-

pentapetide did not interfere with the amidation of lipid II, indicating that the cytoplasmic precursors are not substrates for MurT-GatD dependent amidation reaction.

The glutamine amido transfer domains are highly conserved among the GATases family, whereas the synthetase domains are all different, since they bind to different nitrogen acceptor substrates in different biological reactions (6). Regarding MurT-GatD complex, MurT shares high level of similarity with the sequence of the four Mur ligases of *S. aureus* (5) that catalyze the synthesis of UDP-Mur*N*Ac-pentapeptide through the stepwise addition of five aminoacids. Munch *et al* showed that, despite the sequence similarity, all *S. aureus* Mur Ligases were not able to replace *in vitro* the activity of MurT protein. It has been shown that the amidation reaction itself doesn't require the presence of ATP, however, some GATases are dependent of ATP to activate the nitrogen acceptor substrate (6). Our results indicate that the amidation reaction requires the presence of ATP.

The absence of amidated lipid II when only GatD is present in the *in vitro* reaction, together with the fact that the in trans complementation with several copies of *gatD* gene showed no re-establishment of the normal peptidoglycan profile (5), demonstrate that MurT is essential for peptidoglycan amidation.

Taking into account the frequent presence of MurT-GatD in gram-positive bacterial pathogens; the essentiality of these proteins in *S. aureus;* and their involvement in the resistance to β-lactam antibiotics and to lysozyme – the amidation step in peptidoglycan biosynthesis offers a new target for the development of inhibitors as mono therapeutics and/or as combination agents for existing β-lactam antibiotics.

All the experimental work described in this chapter was performed by T. A. Figueiredo.

References

1. **Bera, A., S. Herbert, A. Jakob, W. Vollmer, and F. Gotz**. 2005. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. Mol. Microbiol. **55**: 778-787.
2. **Bernard, E., T. Rolain, P. Courtin, A. Guillot, P. Langella, P. Hols, and M. P. Chapot-Chartier**. 2011. Characterization of O-acetylation of *N*-acetylglucosamine: a novel structural variation of bacterial peptidoglycan. J. Biol. Chem. **286**: 23950-23958.
3. **Bouhss, A., A. E. Trunkfield, T. D. Bugg, and D. Mengin-Lecreulx**. 2008. The biosynthesis of peptidoglycan lipid-linked intermediates. FEMS Microbiol. Rev. **32**: 208-233.
4. **Boyle-Vavra, S., H. Labischinski, C. C. Ebert, K. Ehlert, and R. S. Daum** 2001. A spectrum of changes occurs in peptidoglycan composition of glycopeptide-intermediate clinical *Staphylococcus aureus* isolates. Antimicrob. Agents Chemother. **45**: 280-287.
5. **Figueiredo, T. A., R. G. Sobral, A. M. Ludovice, J. M. Almeida, N. K. Bui, W. Vollmer, H. de Lencastre, and A. Tomasz**. 2012. Identification of genetic determinants and enzymes involved with the amidation of glutamic acid residues in the peptidoglycan of *Staphylococcus aureus*. PLoS Pathog. **8**: e1002508.
6. **Massiere, F., and M. A. Badet-Denisot**. 1998. The mechanism of glutamine-dependent amidotransferases. Cell Mol. Life Sci. **54**: 205-222.
7. **Munch, D., T. Roemer, S. H. Lee, M. Engeser, S. H. Sahl, and T. Schneider**. 2012. Identification and *in vitro* analysis of the GatD/MurT enzyme-complex catalyzing lipid II amidation in *Staphylococcus aureus*. PLoS Pathog. **8**: e1002509.
8. **Ornelas-Soares, A., H. de Lencastre, B. de Jonge, D. Gage, Y. S. Chang, and A. Tomasz**. 1993. The peptidoglycan composition of a *Staphylococcus aureus* mutant selected for reduced methicillin resistance. J. Biol. Chem. **268**: 26268-26272.
9. **Scheffers, D. J., and M. G. Pinho**. 2005. Bacterial cell wall synthesis: new insights from localization studies. Microbiol. Mol. Biol. Rev. **69**: 585-607.
10. **Schleifer, K. H., and O. Kandler**. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36**: 407-477.
11. **Siewert, G., and J. L. Strominger**. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XI. Formation of the isoglutamine amide group in the cell walls of *Staphylococcus aureus*. J. Biol. Chem. **243**: 783-790.
12. **Smith, C. A**. 2006. Structure, function and dynamics in the mur family of bacterial cell wall ligases. J. Mol. Biol. **362**: 640-655.
13. **Stranden, A. M., K. Ehlert, H. Labischinski and B. Berger-Bachi**. 1997. Cell wall monoglycine cross-bridges and methicillin hypersusceptibility in a

femAB null mutant of methicillin-resistant *Staphylococcus aureus*. J. Bacteriol. **179**: 9-16.

14. **Van Heijenoort, J**. 2007. Lipid intermediates in the biosynthesis of bacterial peptidoglycan. Microbiol. Mol. Biol. Rev. **71**: 620-635.
15. **Van Heijenoort, J**. 2001. Formation of the glycan chains in the synthesis of bacterial peptidoglycan. Glycob. **11**: 25R-36R.
16. **Zalkin, H**. 1993. The amidotransferases. Adv. Enzymol. Relat. Areas. Mol. Biol. **66**: 203-309.

Chapter IV

Contribution of Peptidoglycan Amidation to β-Lactam and Lysozyme Resistance in Different Genetic Lineages of *Staphylococcus aureus*

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Abstract

The enzymes responsible for peptidoglycan amidation in *Staphylococcus aureus*, MurT and GatD, were recently identified and shown to be required for optimal expression of resistance to β-lactams, bacterial growth, and resistance to lysozyme. In this study, we analyzed the impact of peptidoglycan amidation in representative strains of the most widespread clones of methicillin resistant *S. aureus* (MRSA). The inhibition of the expression of *murT-gatD* operon resulted in different phenotypes of resistance to β-lactams and lysozyme according to the different genetic backgrounds. Further, clonal lineages CC1 and CC398 (community-acquired MRSA [CA-MRSA]) showed a stronger dependency on MurT-GatD for resistance to β-lactams, when compared to the impact of the impairment of the cell wall step catalyzed by MurF. In the remaining backgrounds similar phenotypes of β-lactam resistance were observed upon the impairment of both cell-wall-related genes. Therefore, for CA-related backgrounds, the predominant β-lactam resistance mechanism seems to involve genes associated with secondary modifications of peptidoglycan. On the other hand, the lack of glutamic acid amidation had a more substantial impact on lysozyme resistance for cells of CA-MRSA backgrounds, than for hospital-acquired MRSA (HA-MRSA). However, no significant differences were found in the resistance level of the respective peptidoglycan structure, suggesting that the lysozyme resistance mechanism involves other factors. Taken together, these results suggested that the different genetic lineages of MRSA were able to develop different molecular strategies to overcome the selective pressures experienced during evolution.

Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) are a major cause of nosocomial infections worldwide (15, 18, 24, 27) and most cases of hospital-acquired MRSA (HA-MRSA) infections are caused by a few successful multidrug-resistant epidemic clones (38).

In the last two decades, the emergence of community-acquired MRSA (CA-MRSA), causing infections among healthy individuals, has been a subject of growing concern (6, 7, 20, 33). Nevertheless, nowadays, the recent changes in the epidemiology of CA-MRSA suggest that the boundaries between the hospital and community are blurring (21, 29, 30, 50).

Several studies have demonstrated that genetic backgrounds associated with CA-MRSA have a number of features that distinguish them from HA-MRSA. CA-MRSA typically have increased virulence and carry the smaller and easier to transfer staphylococcal chromosomal cassette (SCC*mec*) type IV and V (12). Interestingly, the largest SCC*mec* type II, usually found in HA-MRSA, has a significant fitness cost for the bacteria, resulting in a decrease in the growth rate, and in a reduction of toxin expression levels (9, 10). The balance between the virulence and antibiotic resistance costs may explain why MRSA with SCC*mec* type II are found mainly in hospital environments where high antibiotic pressure, immunocompromised individuals, and vector-mediated transmission are present (10). Moreover, for CA-MRSA strains, in contrast to HA-MRSA, *mecA* was suggested not to be the primary determinant of methicillin resistance, being the expression of *pbp4* the main determinant of resistance (32).

Recently, a small operon, encoding the enzymatic complex MurT-GatD, was identified to be responsible for a secondary modification of peptidoglycan in *S. aureus*, the amidation of glutamic acid in the stem

peptides (17, 34). Inhibition of amidation caused reduced growth rate; reduced resistance to β-lactam antibiotics, shown previously to be affected by auxiliary genes (14); and increased sensitivity to lysozyme in HA-MRSA strain COL (17).

In this communication we report that peptidoglycan amidation has different impacts in the expression of resistance to β-lactams and to lysozyme, depending on the genetic background of the particular strain. These observations suggest that *S. aureus*, from different genetic lineages, include different elements from their core genomes in the strategies of resistance to β-lactam and lysozyme adopted.

Materials and Methods

Bacterial strains and growth conditions. The 11 MRSA strains analyzed in this study are listed in Table 1. The respective mutants with *murT-gatD* and *murF* conditional mutations are listed in Table 2. *S. aureus* strains were grown at 37ºC with aeration in tryptic soy broth (TSB; Difco Laboratories) or tryptic soy agar (TSA; Difco Laboratories). The strains with *murT-gatD* and *murF* conditional mutations were grown in the presence of kanamycin (50 µg/ml; Sigma) and neomycin sulfate (50 µg/ml; Sigma). Growth medium was supplemented with the appropriate concentration of cadmium chloride ($CdCl_2$; Sigma), unless otherwise described.

Construction of *murT-gatD* conditional mutants. The *murT-gatD* conditional mutation (17) was transduced, by phage 80α into the recipient strains (Table 1), as previously described (41) generating the *murT-gatD* conditional mutants in different backgrounds (Table 2).

Construction of *murF* conditional mutants. A 768-bp DNA fragment of the 5' end of *murF* gene, including the ribosome binding site but not the promoter sequence, was amplified using chromosomal DNA from strain COL as template and the specific primers PmurF'-R and PmurF'-F (Table S1). The amplified *murF* fragment and plasmid pBCB20 (R.G. Sobral and M.G. Pinho, unpublished data), carrying a $CdCl_2$ inducible promoter, were digested with SmaI (New England Biolabs) and ligated, generating plasmid pMurF'. Plasmid pMurF' was electroporated into competent cells of RN4220 with a Gene Pulser apparatus (Bio-Rad). The correct insertion of pMurF' into RN4220 chromosome was confirmed by polymerase chain reaction, using an internal *murF* primer chosen downstream of the cloned region (PmurFdn) and an internal primer to *pCad* conditional promoter (Pcad-F) (Table S1). The *murF* conditional mutation was then transduced, by phage 80α into the recipient strains (Table 1), as previously described

(41) generating the *murF* conditional mutants in different backgrounds (Table 2).

Pulsed-field gel electrophoresis. The correct insertion of *murT–gatD* and *murF* conditional mutations into the chromosome of the recipient strains was performed by comparing the pulsed-field gel electrophoresis (PFGE) profiles of the parental strains and the respective transductants. DNA agarose disks of the parental strain and the respective mutant were prepared, digested with SmaI, and separated as described (8).

Southern blot analysis. SmaI chromosomal fragments, from the parental strain and the respective *murF* and *murT-gatD* mutants, were transferred to nylon membranes (Hybond N+; GE Healthcare) that were subsequently hybridized with specific DNA probes labeled with the ECL direct labeling system (GE Healthcare). The DNA probes used for *murT-gatD* and *murF* genes were amplified with primer pairs PmurT-D1+PmurTR1 and PmurF'-R +PmurF'-F, respectively (Table S1).

Population analysis profile. Overnight-grown cultures of the parental strains and the respective *murT-gatD* and *murF* conditional strains were plated at various dilutions on TSA plates, with increasing concentrations of oxacillin (0, 0.75, 1.5, 3, 6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg/ml), and colonies were counted after incubation at 30ºC for 48 hours, as previously described (13).

Table 1. MRSA strains used in this study.

Strain	SCC*mec* Type	HA/CA-MRSA	MLST (ST)	Clone	Clonal Complex	Year of Isolation	Country of Origin	Reference
COL	I	HA-MRSA	ST250	Archaic	CC8	1965	United Kindgon	(39)
HDES57	IV	HA-MRSA	ST22	EMRSA15	CC22	2007	Portugal	(11)
HDE288	VI	HA-MRSA	ST5	Pediatric	CC5	1996	Portugal	(46)
HUR75	III	HA-MRSA	ST239	Brazilian	CC8	1998	Hungary	(38)
HUC599	II	HA-MRSA	ST5	NY/Japan	CC5	2006	Portugal	(1)
DEN2294	IV	CA-MRSA	ST30	Southwest-Pacific	CC30	2001	Denmark	(16)
ST398	V	CA-MRSA	ST398	ST398	CC398	2005	France	(2)
USA400	IV	CA-MRSA	ST1	USA400	CC1	1995-2003	United States	(31)
MW2	IV	CA-MRSA	ST1	USA400	CC1	1998	United States	(6)
WIS	V	CA-MRSA	ST59	Taiwan	CC59	1999	Australia	(36)
C377	IV	CA-MRSA	ST8	USA300	CC8	2005	Spain	(45)

Abbreviations: SCC*mec:* Staphylococcal Chromosomal Cassette; HA-MRSA: Hospital Acquired MRSA; CA-MRSA: Community Acquired MRSA; ST: Sequence Type; CC: Clonal Complex.

Table 2. Mutant Strains and Plasmids used in this study.

Strains	Relevant Characteristics	References
S. aureus		
RN4220	Mc^s; restriction negative	36
M100	Mc^r laboratory step mutant	51
M100*pCadmurT-gatD*	M100 with *murT-gatD* operon under Pcad control, Kan^r, Neo^r	This study
M100*pCadmurF*	M100 with *murF* gene under Pcad control, Kan^r, Neo^r	This study
COL*pCadmurT-gatD*	COL with *murT-gatD* operon under *Pcad* control, Kan^r, Neo^r	17
COL*pCadmurF*	COL with *murF* gene under Pcad control, Kan^r, Neo^r	This study
HUR75*pCadmurT-gatD*	HUR75 with *murT-gatD* operon under *Pcad* control, Kan^r, Neo^r	This study
HUR75*pCadmurF*	HUR75 with *murF* gene under *Pcad* control, Kan^r, Neo^r	This study
HDES57*pCadmurT-gatD*	HDES57 with *murT-gatD* operon under *Pcad* control, Kan^r, Neo^r	This study
HDES57*pCadmurF*	HDES57 with *murF* gene under *Pcad* control, Kan^r, Neo^r	This study
HAR22*pCadmurT-gatD*	HAR22 with *murT-gatD* operon under *Pcad* control, Kan^r, Neo^r	This study
HAR22*pCadmurF*	HAR22 with *murF* gene under *Pcad* control, Kan^r, Neo^r	This study

Continued in the next page.

Table 2. Mutant Strains and Plasmids used in this study (cont.).

Strains	Relevant Characteristics	References
HUC599*pCadmurT-gatD*	HUC599 with *murT-gatD* operon under *Pcad* control, Kan^r, Neo^r	This study
HUC599*pCadmurF*	HUC599 with *murF* gene under *Pcad* control, Kan^r, Neo^r	This study
HDE288*pCadmurT-gatD*	HDE288 with *murT-gatD* operon under *Pcad* control, Kan^r, Neo^r	This study
HDE288*pCadmurF*	HDE288 with *murF* gene under *Pcad* control, Kan^r, Neo^r	This study
MW2*pCadmurT-gatD*	MW2 with *murT-gatD* operon under *Pcad* control, Kan^r, Neo^r	This study
MW2*pCadmurF*	MW2 with *murF* gene under *Pcad* control, Kan^r, Neo^r	This study
USA400*pCadmurT-gatD*	USA400 with *murT-gatD* operon under *Pcad* control, Kan^r, Neo^r	This study
USA400*pCadmurF*	USA400 with *murF* gene under *Pcad* control, Kan^r, Neo^r	This study
ST398*pCadmurT-gatD*	ST398 with *murT-gatD* operon under *Pcad* control, Kan^r, Neo^r	This study
ST398*pCadmurF*	ST398 with *murF gene* under *Pcad* control, Kan^r, Neo^r	This study
WIS*pCadmurT-gatD*	WIS with *murT-gatD* operon under Pcad control, Kan^r, Neo^r	This study
WIS*pCadmurF*	WIS with *murF* gene under *Pcad* control, Kan^r, Neo^r	This study

Continued in the next page.

Table 2. Mutant Strains and Plasmids used in this study (cont.).

Strains	Relevant Characteristics	References
C377*pCadmurT-gatD*	C377 with *murT-gatD* operon under *Pcad* control, Kan^r, Neo^r	This study
C377*pCadmurF*	C377 with *murF* gene under *Pcad* control, Kan^r, Neo^r	This study
DEN2294*pCadmurT-gatD*	DEN2294 with *murT-gatD* operon under *Pcad* control, Kan^r, Neo^r	This study
DEN2294*pCadmurF*	DEN2294 with *murF* gene under *Pcad* control, Kan^r, Neo^r	This study
Escherichia coli		
DH5α	*recA endA1 gyrA96 thi-1 hsdR17 supE44 relA1* F80 *DlacZ*DM15	Invitrogen
Plasmids		
pBCB20	*S. aureus* integrative vector including *Pcad* inducible promotor, Ap^r, Kan^r	R.G. Sobral and M.G. Pinho unpublished
pMurT'	pBCB20 vector with *murT* ribosome-binding site and the first 298 codons fused to *Pcad* promotor; Ap^r, Kan^r	17
pMurF'	pBCB20 vector with *murF* ribosome-binding site and the first 256 codons fused *Pcad* promotor; Ap^r, Kan^r	This Study

Abbreviations: Ap^R, ampicillin resistance; Kan^R: Kanamycin resistance; Neo^R: Neomycin resistant; CC: Clonal Complex.

Peptidoglycan isolation for lysozyme lytic assays. Isolation of cell wall was performed as described previously (3). Briefly, cells were harvested by centrifugation, washed twice with cold 0.9% NaCl, and boiled for 20 minutes. After chilling, the cells were washed twice and disrupted using 106-mm glass beads (Sigma) and FastPrep FP120 apparatus (Bio 101). The suspension was then washed, and boiled for 30 minutes in 5% sodium dodecyl sulfate and 50mM Tris-HCl (pH 7), to remove noncovalently bound proteins. After centrifugation, the cell wall fragments were diluted in 0.1M Tris-HCl (pH 6.8) and incubated with 0.5 mg/ml trypsin for 16 hours at 37ºC to degrade cell bound proteins. Purified cell walls were washed with water and lyophilized and treated with 49% hydrofluoric acid for 48 hours at 4ºC, to remove teichoic acids. The purified peptidoglycan was washed with water and lyophilized.

Turbidometric assay of peptidoglycan hydrolysis. To analyze the susceptibility of peptidoglycan to lysozyme hydrolysis, a turbidometric assay was performed as described previously (3, 17, 19). Briefly, purified peptidoglycan was sonicated in 100mM sodium-potassium phosphate buffer (pH 6.6). Egg white lysozyme (Sigma) was added (300 µg/ml) and the reaction was incubated at 37ºC. The optical density was monitored at 595 nm in 96-well microplates (pure grade, Brand) using a microplate reader (Infinite F200 Pro; Tecan).

Determination of lysozyme resistance of *S. aureus* growing cells. The impact of lysozyme on exponentially growing cultures was determined as described previously (17, 19). Overnight cultures of the conditional mutants, grown with inducer, were inoculated into fresh TSB, with and without inducer. The cultures were incubated at 37ºC to an OD_{620nm} of 1.0. Then, each culture was diluted 1:10 into fresh TSB, with and without inducer, and

lysozyme (300 µg/ml) was added as the OD_{620nm} reached 1.0. The growth was monitored for several hours.

Statistical analysis. A two-tailed Student's t test with Welch correction was used to determine the significance of differences in lysozyme digestion within groups of CA-MRSA and HA-MRSA. Differences were considered statistically significant when p was < 0.005. The Graph Pad Prism 5.0 package was used.

Results

The impact of the impairment of peptidoglycan amidation on oxacillin resistance was previously demonstrated by the construction and characterization of the *murT-gatD* conditional mutation in strain COL. This mutation was shown to impact not only β-lactam resistance, but also growth rate and lysozyme resistance (17). In this study, we first observed that the effects of *murT-gatD* inhibition in the resistance level of strain COL and strain MW2 were clearly distinct, as observed through oxacillin inhibition halos (1-mg disc) (Figure S1). The decrease in the resistance level of the strain was significantly more pronounced for MW2*pCadmurT-gatD*, showing a twofold wider inhibition halo. To test that this behavior was the result of different genetic backgrounds and not a strain-specific trait, the mutation was transferred to another strain of the same clone USA400 and a similar resistance profile was obtained (Figure 1A). These results led to the hypothesis that *murT-gatD* expression and/or the enzymatic step catalyzed by the MurTGatD complex could have different physiological consequences depending on the genetic background.

To address this hypothesis, the *murT-gatD* conditional mutation was transduced to representative strains of the most widespread MRSA clones, among both HA-MRSA and CA-MRSA (Table 1).

The correct transfer of the mutation was determined by comparing the SmaI PFGE profiles of the parental strains and the respective transductants (Figure S2). In addition, Southern blot analysis using specific probes for *murT-gatD* genes confirmed the correct insertion of the conditional mutation.

Impact of *murT-gatD* conditional mutation on resistance to β-lactam antibiotics in different MRSA genetic backgrounds. The impact of

murT-gatD conditional mutation on the β-lactam resistance level of the different MRSA strains was evaluated by performing oxacillin population analysis profiles.

The most striking observation was that MW2/USA400, ST398, and WIS CA-MRSA *murT-gatD* conditional mutants (Figure 1A) grown in the absence of inducer were overall less resistant to oxacillin, when compared with the HA-MRSA mutant strains (Figure 1B). A first early drop in the number of cfu/ml, occurring at 0.75 µg/ml, was common to all analyzed mutant strains, followed by a high frequency of resistant subpopulations, able to grow on higher concentrations of antibiotic. Strikingly, while for the HA-MRSA (COL, HUR75, and HDES57) the subpopulations of the mutants were able to grow on antibiotic concentrations near the MIC (minimal inhibitory concentration) of the parental strain (from 50 to 800 µg/ml, Figure 1B), for the CA-MRSA (MW2, USA400, ST398, and WIS), the mutants' subpopulations only grew at low antibiotic concentrations (from 0.75 to 6.25 µg/ml, Figure. 1A). Consequently, complete growth inhibition occurred at much lower antibiotic concentrations for *murT-gatD* mutants of CA-MRSA backgrounds (Figure 1A).

Within the CA-MRSA strains, C377*pCadmurT-gatD* and DEN2294*pCadmurT-gatD* showed a less striking decrease in resistance to oxacillin (Figure 2A), with subpopulations that were able to grow up to 100 µg/ml. In fact, C377*pCadmurTgatD* showed an overall resistance profile similar to HUR75*pcadmurT-gatD* (Figures 2A and 1B, respectively). The similarities between these resistance profiles were consistent with the fact that HUR75 and C377 are genetically related, belonging to the same clonal complex (CC8).

Likewise, DEN2294 (ST30-IV, Southwest Pacific clone) is genetically related to the HA-MRSA ST36-MRSA-II (EMRSA-16) as they are

descendants from the common ancestral ST30-MSSA (45). A conditional *murT-gatD* mutant was not constructed in the ST36-MRSA-II background, as all the strains available were resistant to the selectable marker of the pMurT' integrative plasmid (kanamycin).

Regarding the Pediatric and New York/Japan clones (HDE288 and HUC599, respectively) the parental strains exhibited, together with a high frequency of resistant subpopulations, lower MIC values (0.75 µg/ml) than the previously analyzed HA-MRSA strains (50–800 µg/ml, Figure 2B). Consistently, for these strains, the *murT-gatD* mutation had a complete inhibitory impact at lower antibiotic concentrations (6.25 and 100 µg/ml for HDE288 and HUC599 mutants, respectively, Figure 2B), than the remaining HA-MRSA mutants.

To explore whether this behavior is associated with distinct steps of peptidoglycan biosynthesis, the impact of a *murF* conditional mutation was also studied in the same genetic backgrounds.

Impact of *murF* conditional mutation on resistance to β-lactam antibiotics in different MRSA genetic backgrounds. A conditional mutation for *murF* gene (*pCadmurF*) was constructed using the same *pCad* inducible promoter, transduced from RN4220*pCadmurF* to the strains listed in Table 1, and oxacillin population analysis profiles were performed (Figures 1 and 2). In the absence of inducer, the *murF* conditional mutants were impaired in the last biosynthetic cytoplasmic step, catalyzed by MurF protein, the addition of the D-alanyl-D-alanine terminus to the stem peptide (49). For most clonal lineages the level of resistance to oxacillin was similar for *murT-gatD* and *murF* mutants (Figures 1B and 2B). However, for MW2/USA400 and ST398 CA-MRSA strains, all CA-MRSA strains, the inhibition of *murT-gatD* transcription caused a more pronounced effect on oxacillin resistance, than inhibition of *murF* transcription (Figure 1A).

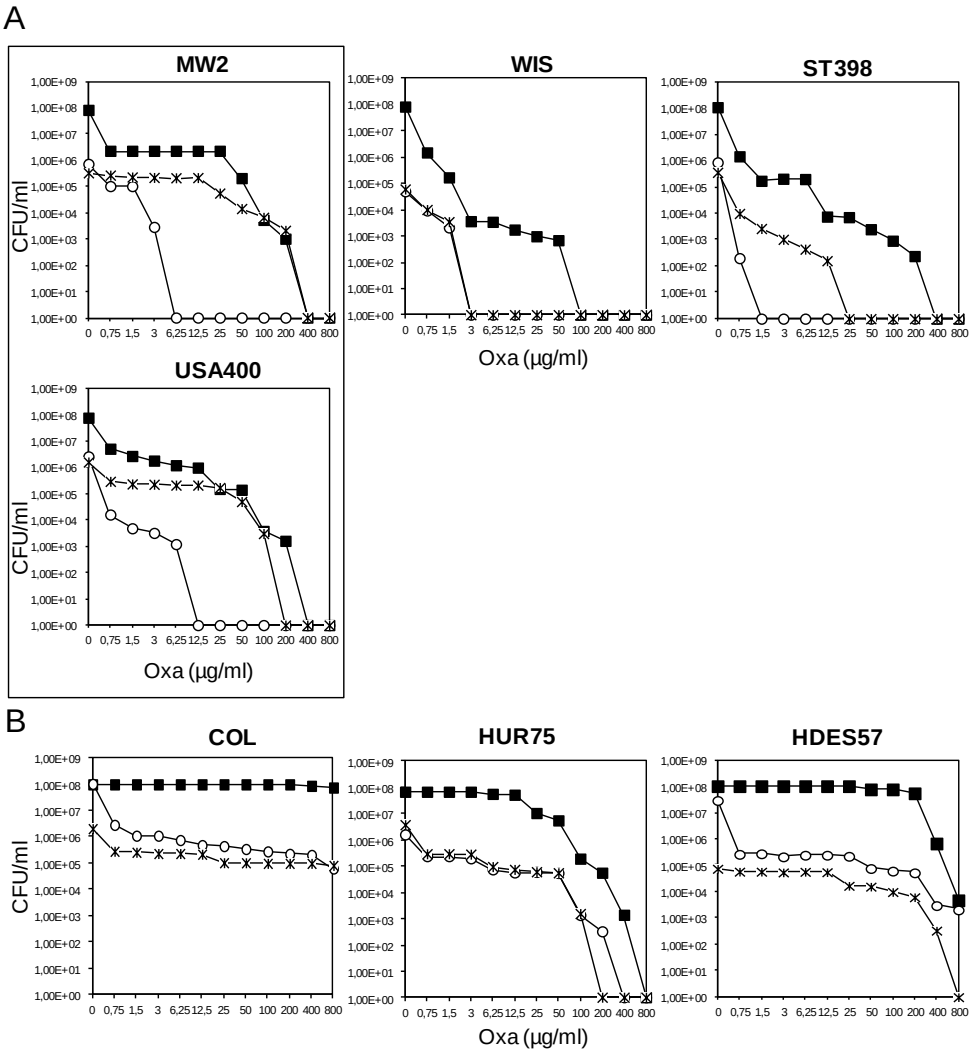

Figure 1. Impact of *murT-gatD* and *murF* conditional mutations on the oxacillin resistance profiles of MW2, USA400, WIS, ST398, COL, HUR75, and HDES57 strains. Overnight cultures of the parental strains and conditional mutants grown with $CdCl_2$ inducer were plated on TSA containing increasing concentrations of oxacillin. Plates were incubated for 48 hours at 30ºC. (■) Oxacillin population analysis profile of parental strains; (○) oxacillin population analysis profile of *murT-gatD* conditional mutants; (∗) oxacillin population analysis profile of *murF* conditional mutants. Oxacillin population analysis profile of (A) CA-MRSA strains and (B) HA-MRSA strains. CA-MRSA, community-acquired methicillin resistant *Staphylococcus aureus*; $CdCl_2$, cadmium chloride; HA-MRSA, hospital-acquired MRSA; TSA, tryptic soy agar.

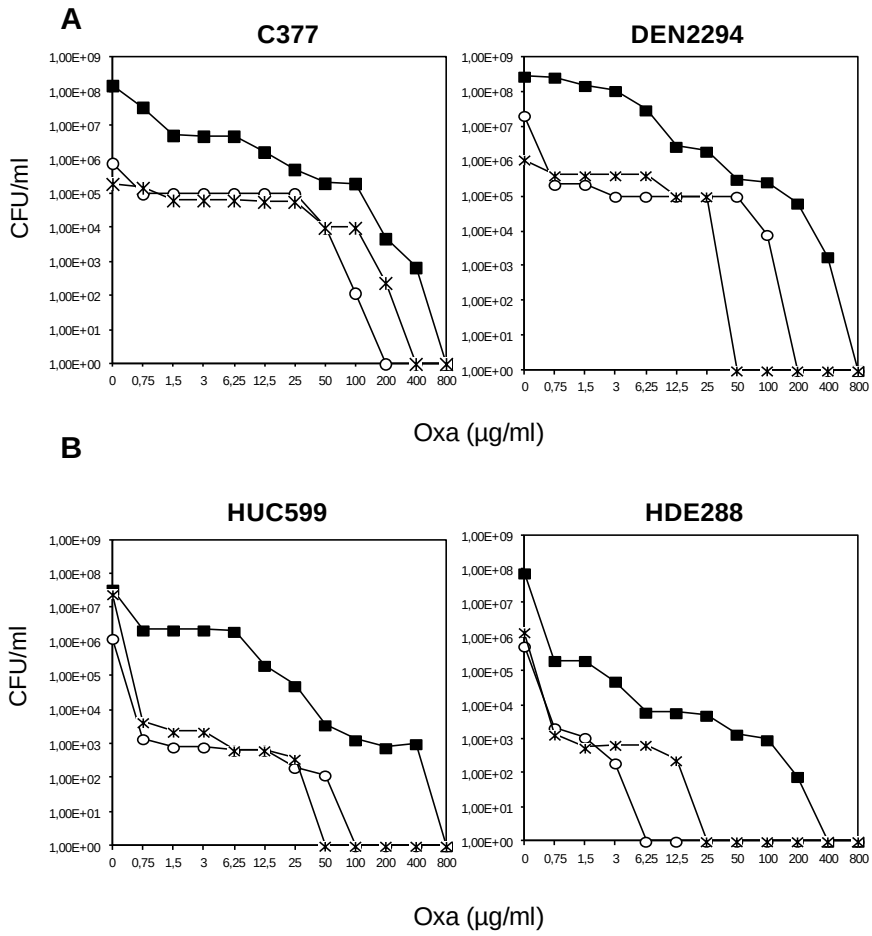

Figure 2. Impact of *murT-gatD* and *murF* conditional mutations on the oxacillin resistance profiles of C377, DEN2294, HUC599, and HDE288 strains. Overnight cultures of the parental strains and conditional mutants, grown with $CdCl_2$ inducer, were plated on TSA containing increasing concentrations of oxacillin. Plates were incubated for 48 hours at 30ºC. (■) Oxacillin population analysis profile of parental strains; (○) oxacillin population analysis profile of murT-gatD conditional mutants; (∗) oxacillin population analysis profile of *murF* conditional mutants. Oxacillin population analysis profile of (A) CA-MRSA strains and (B) HA-MRSA strains.

Impact of *murT-gatD* and *murF* conditional mutations on β-lactam resistance in a *mecA*-negative strain resistant to methicillin. The *pCadmurT-gatD* and *pCadmurF* conditional mutations were transduced to the M100 strain (Table 2), a laboratory step mutant selected for methicillin resistance (51) which encodes a modified PBP3 (43) and does not contain *mecA*. The inhibition of *murF* transcription, in the background of M100

strain, resulted in a decrease in cell viability, shown by a drop in the number of cfu/ml from 108 to 106 (Figure 3). However, no effect was observed in the oxacillin resistance level. In contrast, the impairment of *murTgatD* caused, besides the same decrease in viability, a four fold decrease in oxacillin resistance; the conditional mutant, grown in the absence of inducer, showed complete growth inhibition at 2 µg/ml of oxacillin, in contrast to the parental strain (8 µg/ml) (Figure 3).

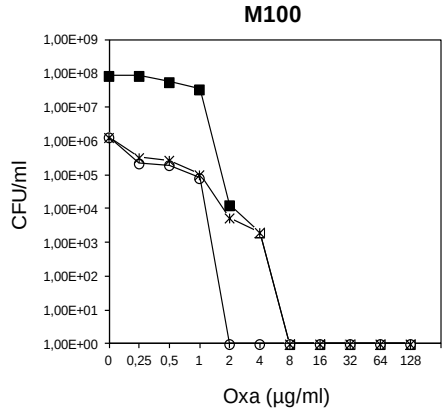

Figure 3. **Impact of *murT-gatD* and *murF* conditional mutations on the oxacillin resistance profile of *mecA*-negative M100 strain**. Overnight cultures of the parental strain and conditional mutants, grown with $CdCl_2$ inducer, were plated on TSA containing increasing concentrations of oxacillin. Plates were incubated for 48 hours at 30ºC. (■) Oxacillin population analysis profile of M100 parental strain; (○) oxacillin population analysis profile of *murT-gatD* conditional mutant; (∗) oxacillin population analysis profile of *murF* conditional mutant.

Impact of *murT-gatD* conditional mutation on lysozyme resistance in different MRSA genetic backgrounds.

Lysozyme resistance assays in living cells. To evaluate the impact of *murT-gatD* conditional mutation on *S. aureus* intrinsic lysozyme resistance, in the several genetic backgrounds, the *murT-gatD* conditional mutants were grown, in the absence and in the presence of inducer, and treated with muramidase during the exponential phase. The cell density of the cultures was then monitored for several hours.

The parental strains, as the conditional mutants grown with inducer, showed no growth alteration upon addition of lysozyme to the medium (shown for HDES57, HDE288, MW2, and C377 strains and for the respective mutants, grown without inducer, Figure 4; data not shown for the remaining strains), confirming that all these strains are resistant.

To address the effects of the impairment of *murT-gatD* transcription on lysozyme resistance level, the cell density values of each mutant culture, grown with and without lysozyme, were compared at 90 minutes after the addition of muramidase (Figure 5A).

Overall, *murT-gatD* mutants constructed in CA-MRSA backgrounds were more sensitive to lysozyme, when compared with mutants constructed in HA-MRSA backgrounds. In fact, while all CA-MRSA mutants showed a decrease in optical density above 70% (mean value of 86.1% – 9.6%) when grown in the presence of lysozyme, in HA-MRSA the decrease was much more variable, ranging between 23.1% and 90.6% (Figure 5A). The difference in the mean lysozyme digestion level, between groups of CA-MRSA and HA-MRSA, was statistically significant ($p < 0.005$, Student's t test). Interestingly, mutants DEN2294*pCadmurT-gatD* and C377*pCadmurT-gatD* were more resistant to lysozyme, than the remaining CA-MRSA strains, showing again a different behavior, as previously observed for the oxacillin resistance profiles (Figures 5A and 2A).

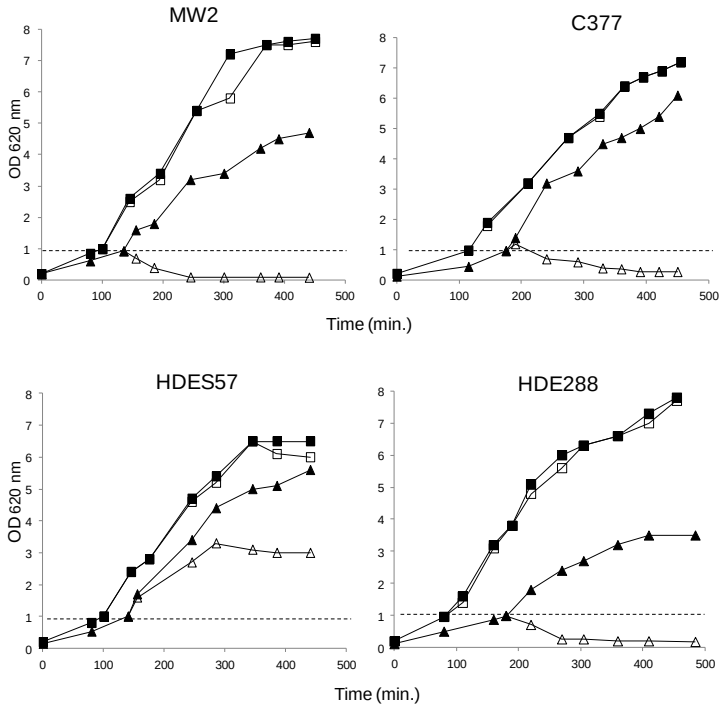

Figure 4. **Impact of *murT-gatD* conditional mutation on lysozyme resistance in HDES57, HDE288, MW2, and C377 strains and the respective mutants, grown without inducer**. Overnight cultures were diluted to OD_{620nm} 0.1 and were incubated at 37ºC to OD_{620nm} of 1.0. Then, each culture was diluted into fresh medium and lysozyme (300 µg/ml) was added at OD_{620nm} 1.0, as indicated by the dashed line. Conditional mutant grown without inducer, in the presence (Δ) and in the absence of lysozyme (▲); parental strain grown in the presence (□) and in the absence of lysozyme (■).

Lysozyme resistance assays with purified peptidoglycan. To determine whether the mutant phenotypes, observed in vivo, were directly associated with the lack of amidation of peptidoglycan, or whether they were associated with other strain specificities, the peptidoglycan of the parental strains and the respective mutant, grown without inducer, was isolated and purified. The peptidoglycan concentration was adjusted and after addition of lysozyme, the optical density was monitored to assess the amount of peptidoglycan digested. The results of the lytic assays showed no statistically significant differences (Student's t test) between the lysozyme resistance of the purified peptidoglycan of the different mutant strains (Figure 5B). The comparison between the susceptibility to lysozyme of

murT-gatD mutants' living cells and their respective purified peptidoglycan (Figure 5A, B) showed no correlation. In fact, while cells from CA-MRSA mutants were more susceptible to lysozyme than cells from HA-MRSA mutants, their respective purified peptidoglycan showed no significant variability between the resistance levels.

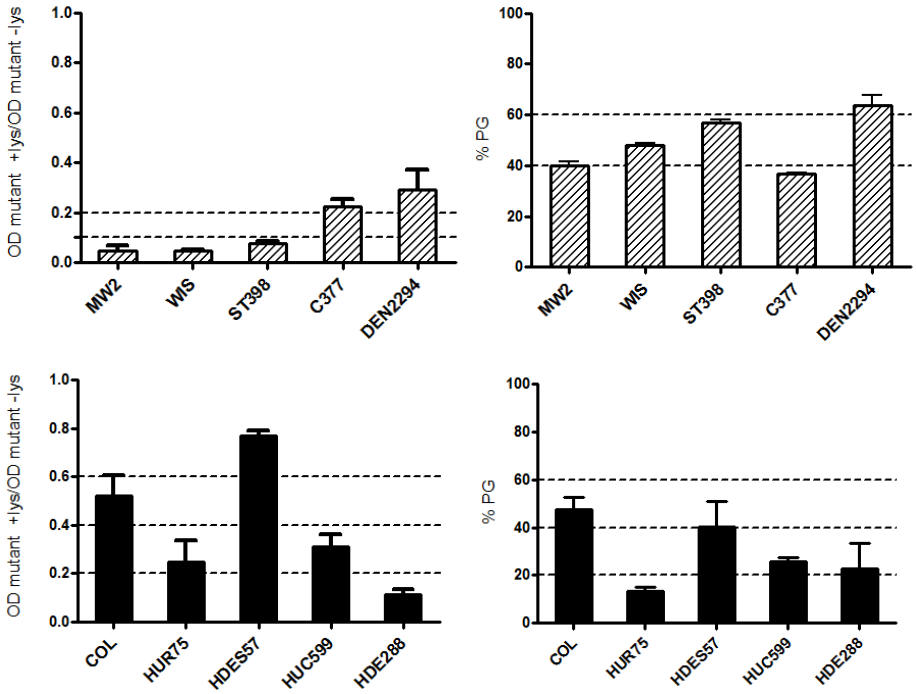

Figure 5. **Impact of *murT-gatD* conditional mutation on lysozyme resistance**. (A) Effect of lysozyme (300 µg/ml) on the growth rate of the conditional mutants grown without inducer. The impact of lysozyme is represented as the ratio between the optical density of the culture of the mutant grown with lysozyme (+ lys) and without lysozyme (- lys), 90 minutes after addition of the muramidase. (B) Effect of lysozyme (300 µg/ml) on purified PG from the conditional mutants grown without inducer. The impact of lysozyme is represented as the percentage of undigested peptidoglycan, 40 minutes after addition of the muramidase. Dashed bars, conditional mutants of CA-MRSA strains; solid bars, conditional mutants of HA-MRSA strains. Also represented are the mean and standard deviation of triplicate experiments. PG, peptidoglycan.

Discussion

In the last half-century, the cell wall biosynthetic pathway has been extensively studied, namely, for its role as an antimicrobial target. However, the enzymes responsible for the amidation of D-glutamic acid of staphylococcal peptidoglycan were described only recently. Figueiredo et al. (17) identified, in MRSA strain COL, the *murT-gatD* operon whose protein products catalyze the amidation of peptidoglycan and showed that this cell wall modification is important for optimal growth, β-lactam resistance, and sensitivity to the host defense factor lysozyme. Munch et al. (34) demonstrated that both enzymes, MurT and GatD, are essential for survival and interact as a glutamine amidotransferase bi-enzymatic complex.

Several genes of the peptidoglycan biosynthetic pathway, among other housekeeping genes, are involved in the β-lactam resistance mechanism. In fact, the mechanism of resistance of MRSA strains is not simply *mecA*-dependent, but also needs the optimal expression of the so-called auxiliary genes (4, 14, 25, 35) among which are essential cell-wall related determinants, such as *murE* (28) *murF* (48); *pbp2* (44); *pbp1* (42); *femABX* (5); and *murT-gatD* operon (17). However, the impact of different steps of peptidoglycan synthesis in β-lactam resistance of MRSA strains from different genetic backgrounds, was only assessed for PBP4 (32). Previously, Katayama et al. (23) showed that impairment of *pbp4* does not affect the β-lactam resistance level of MRSA, being not classified as an auxiliary gene. Later, Memmi et al. (32) showed that *pbp4* is an auxiliary gene in CA-MRSA, being the key player in the resistance mechanism of these specific strains.

In this communication, the *murT-gatD* conditional mutation was studied in the background of the major contemporary MRSA clones. Different impacts

in the oxacillin resistance profile were observed for *murT-gatD* depletion in several MRSA genetic backgrounds, with a more pronounced effect on CA-MRSA-related backgrounds, when compared with HA-MRSA. The conditional mutants of MW2/USA400, ST398, and WIS strains showed complete growth inhibition at antibiotic concentrations *100-fold lower than HA-MRSA mutants. However, this effect was not shared by all CA-MRSA strains; the conditional mutants of C377 and DEN2294, belonging to lineages highly disseminated in community settings (USA300 and Southwest Pacific clones, respectively), showed a less striking decrease in resistance to oxacillin. In the case of C377, this behavior could be explained by the fact that this strain is genetically related with the HA-MRSA HUR75 strain, as they belong to the same clonal complex (CC8). Likewise, DEN2294 CAMRSA (ST30-IV, Southwest Pacific clone) has a genetic background related to the hospital-acquired ST36-MRSA-II (EMRSA-16) as DEN2294 and ST36-MRSA-II have a common ancestral, ST30-MSSA (22, 45).

Although the differences in oxacillin resistance decrease observed between the CA- and HA-related strains are clear, the molecular mechanism behind these different phenotypes is probably directly associated rather with the strains' clonal complexes, and therefore, with their genetic background.

An association between the strains' genetic background and their capacity to acquire and maintain a recombinant plasmid expressing mecA was previously observed (22). Strains from clonal complexes CC1 and CC5 were, among the major MRSA lineages tested, the ones that were less efficiently transformed. However, CC5 lineage has been recently described to be well adapted to the hospital environment through the efficient acquisition of resistance to new antibiotics (26), suggesting that strains belonging to CC5 are able to easily acquire *mecA*, but not able to efficiently

maintain it. Therefore, the CC5 lineage seems to be less dependent on the presence of *mecA* than other major lineages, for the efficient expression of resistance to β-lactams. One hypothesis is that in strains of CC5 β-lactam resistance relies on the presence of specific housekeeping genes, namely, *murT-gatD*, although the presence of *mecA* would still be essential.

Coincidently, strains belonging to CC1 (MW2/USA400) typically associated with the community onset, and CC5 (HDE288 and HUC599), which includes hospital-related strains, were among the ones that showed higher impact from *murT-gatD* impairment. The other strains that showed the same level of impact were WIS and ST398 (CC59 and CC398, respectively), which harbor the small SCC*mec* type V, suggesting that their genetic background would also not favor the stability of *mecA* expression.

In this line of thought, the clonal complexes CC8, CC22, and CC30, which showed higher efficiency of transformation with mecA and stability of mecA expression (22) were, in our study, represented by the strains showing less impact of *murT-gatD* impairment (COL, HUR75, C377, HDES57, and DEN2294). Taken together, these observations suggest that the genetic backgrounds less prone to receiving *mecA* gene recruited preferentially specific housekeeping genes, such as *murT-gatD*, for their β-lactam resistance strategy. To address the importance of *mecA* presence in this alternative resistance strategy, *murT-gatD* mutation was transferred into a *mecA*-independent resistant strain, M100, with a truncated PBP3. The *murT-gatD* impairment resulted in a decrease in the level of resistance of the M100 strain, indicating that peptidoglycan amidation is essential for a *mecA*-independent resistant strategy.

To assess the importance of different steps of peptidoglycan biosynthesis in this alternative strategy of *mecA* associated resistance, *murF* gene was chosen for further testing. On one hand, MurF catalyzes a crucial step of

the primary pathway of peptidoglycan biosynthesis; on the other hand, it is a well-documented auxiliary gene for COL background (48). While for most genetic backgrounds the impact of *murF* impairment in the resistance profile was comparable to the one of *murT-gatD*, for MW2/USA400 and ST398 strains, *murT-gatD* conditional mutation showed a drastic and unique effect. Further, the inhibition of *murF* transcription did not affect the level of resistance in the *mecA*-negative strain M100, suggesting that the contribution of *murF* auxiliary gene for β-lactam resistance is related to the presence of *mecA*.

Therefore, the alternative strategy for β-lactam resistance seems to rely on genes involved in peptidoglycan secondary modifications, as secondary cross-linking (*pbp4*) and amidation (*murT-gatD*) (32). Recently, Zapun et al. (52) showed that in *Streptococcus pneumoniae*, peptidoglycan amidation catalyzed by MurT-GatD complex is necessary for efficient cross-linking by PBP2a, PBP2b, and PBP2X. PBP1a retained some activity for nonamidated lipid precursors. Although the substrate preferences of *S. aureus* PBPs regarding the amidation status of the precursor molecule are not known, it seems reasonable to speculate that PBP4 and/or PBP2 also require amidated precursors to perform transpeptidation, as these two proteins appear to be involved in the alternative mechanism of resistance to β-lactams (32).

Moreover, besides being essential for optimal β-lactam resistance, the *murT-gatD* operon is also needed for optimal lysozyme resistance, evidencing its role in virulence. We also observed that the impairment of *murT-gatD* operon had a strong impact on lysozyme resistance in CA-MRSA backgrounds. For the HA backgrounds, the impact of this mutation is more variable, according to the genetic background. However, the lysozyme resistance levels of purified peptidoglycan were similar for strains

from both CA and HA settings. This observation indicates that specific factors, intrinsic to the strain genetic background, contribute to the final lysozyme resistance level, although dependent on the amidation status of the cell wall.

The results reported in this communication suggest that peptidoglycan amidation is involved through different mechanistic links in the β-lactam resistance strategies of strains from distinct backgrounds, evidencing in this way the existence of more than one physiological approach for survival to antibiotic stress.

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All the experimental work described in this chapter was performed by T. A. Figueiredo.

References

1. **Aires-de-Sousa, M., B. Correia, H. de Lencastre, and Multilaboratory Project Collaborators**. 2008. Changing patterns in frequency of recovery of five methicillin-resistant *Staphylococcus aureus* clones in Portuguese hospitals: surveillance over a 16-year period. J. Clin. Microbiol. **46**: 2912-2917.
2. **Armand-Lefevre, L., R. Ruimy, and A. Andremont.** 2005. Clonal comparison of *Staphylococcus aureus* isolates from healthy pig farmers, human controls, and pigs. Emerg. Infect. Dis. **11**: 711-714.
3. **Bera, A., S. Herbert, A. Jakob, W. Vollmer, and F. Gotz**. 2005. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. Mol. Microbiol. **55**: 778-787.
4. **Berger-Bächi, B**. 1983. Insertional inactivation of staphylococcal methicillin resistance by *Tn*551. J. Bacteriol. **154**: 479-487.
5. **Berger-Bächi, B., A. Strässle, J. E. Gustafson, and F. H. Kayser**. 1992. Mapping and characterization of multiple chromosomal factors involved in methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents. Chemother. **36**:1367-1373.
6. **Center of Disease Control and Prevention**. 1999. Four pediatric deaths from community acquired methicillin-resistant *Staphylococcus aureus*- Minnesota and North Dakota, 1997–1999. J.A.M.A. **282**: 1123-1125.
7. **Chambers, H. F., and F. R. Deleo**. 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat. Rev. Microbiol. **7**: 629-641.
8. **Chung, M., H. de Lencastre, P. Matthews, A. Tomasz, I. Adamsson, M. Aires de Sousa, T. Camou, C. Cocuzza, A. Corso, I. Couto, A. Dominguez, M. Gniadkowski, R. Goering, A. Gomes, K. Kikuchi, A. Marchese, R. Mato, O. Melter, D. Oliveira, R. Palacio, R. Sa-Leao, I. Santos Sanches, J. H. Song, P. T. Tassios and P. Villari**. 2000. Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. Microb. Drug Resist. **6**:189-198.
9. **Collins, J., A. Buckling, and R. C. Massey**. 2008. Identification of factors contributing to T-cell toxicity of *Staphylococcus aureus* clinical isolates. J. Clin. Microbiol. **46**: 2112-2114.
10. **Collins, J., J. Rudkin, M. Recker, C. Pozzi, J. P. O'Gara, and R. C. Massey**. 2010 Offsetting virulence and antibiotic resistance costs by MRSA. The I. S. M. E. J. 4: **577**-584.
11. **Conceição, T., A. Tavares, M. Miragaia, K. Hyde, M. Aires-de-Sousa, and H. de Lencastre**. 2010. Prevalence and clonality of methicillin-resistant *Staphylococcus aureus* (MRSA) in the Atlantic Azores islands: predominance of SCC*mec* types IV, V and VI. Eur. J. Clin. Microbiol. Infect. Dis. **29**:543-550.

12. **David, M. Z., and R. S. Daum.** 2010. Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. Clin. Microbiol. Rev. **23**: 616-687.
13. **de Lencastre, H., A. M. Figueiredo, and A. Tomasz**. 1993. Genetic control of population structure in heterogeneous strains of methicillin resistant *Staphylococcus aureus*. Eur. J. Clin. Microbiol. Infect. Dis. **12**:13-18.
14. **de Lencastre, H., S. W. Wu, M. G. Pinho, A. M. Ludovice, S. Filipe, S. Gardete, R. Sobral, S. Gill, M. Chung, and A. Tomasz**. 1999. Antibiotic resistance as a stress response: complete sequencing of a large number of chromosomal loci in *Staphylococcus aureus* strain COL that impact on the expression of resistance to methicillin. Microb. Drug Resist. **5**:163-175.
15. **DeLeo, F. R., M. Otto, B. N. Kreiswirth, and H. F. Chambers**. 2010. Community-associated meticillin-resistant *Staphylococcus aureus*. Lancet. **375**: 1557-1568.
16. **Faria, N. A., D. C. Oliveira, H. Westh, D. L. Monnet, A. R. Larsen, R. Skov, and H. de Lencastre**. 2005. Epidemiology of emerging methicillin-resistant *Staphylococcus aureus (*MRSA) in Denmark: a nationwide study in a country with low prevalence of MRSA infection. J. Clin. Microbiol. **43**: 1836-1842.
17. **Figueiredo, T. A., R. G. Sobral, A. M. Ludovice, J. M. Almeida, N. K. Bui, W. Vollmer, H. de Lencastre, and A. Tomasz**. 2012. Identification of genetic determinants and enzymes involved with the amidation of glutamic acid residues in the peptidoglycan of *Staphylococcus aureus*. PLoS Pathog. **8**: e1002508.
18. **Gordon, R. J., and F. D. Lowy**. 2008 Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. Clin. Infect. Dis. **46**: S350-S359.
19. **Herbert, S., A. Bera, C. Nerz, D. Kraus, A. Peschel, C. Goerke, M. Meehl, A. Cheung and F. Gotz**. 2007. Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. PLoS Pathog. **3**: e102.
20. **Herold, B. C., L. C. Immergluck, M. C. Maranan, D. S. Lauderdale, R. E. Gaskin, S. Boyle-Vavra, C. D. Leitch, and R. S. Daum**. 1998. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. J.A.M.A. **279**: 593-598.
21. **Horner, C., P. Parnell, D. Hall, A. Kearns, J. Heritage, and M. Wilcox**. 2013. Methicillin-resistant *Staphylococcus aureus* in elderly residents of care homes: colonization rates and molecular epidemiology. J. Hosp. Infect. **83**: 212-218.
22. **Katayama, Y., D. A. Robinson, M. C. Enright, and H. F. Chambers**. 2005. Genetic background affects stability of *mecA* in *Staphylococcus aureus*. J. Clin. Microbiol. **43**: 2380-2383.
23. **Katayama, Y., H. Z. Zhang, and H. F. Chambers**. 2003. Effect of disruption of *Staphylococcus aureus pbp4* gene on resistance to beta-lactam antibiotics. Microb. Drug Resist. **9**: 329-336.
24. **Klevens, R. M., M. A. Morrison, S. K. Fridkin, A. Reingold, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, G. Fosheim, L. K. McDougal, F. C. Tenover and the Active Bacterial Core Surveillance of the Emerging Infections**

Program Network. 2006. Community-associated methicillin-resistant *Staphylococcus aureus* and healthcare risk factors. Emerg. Infect. Dis. **12** : 1991-1993.

25. **Kornblum, J., B. J. Hartman, R. P. Novick, and A. Tomasz**. 1986. Conversion of a homogeneously methicillin-resistant strain of *Staphylococcus aureus* to heterogeneous resistance by Tn*551*-mediated insertional inactivation. Eur. J. Clin. Microbiol. **5**: 714-718.
26. **Kos, V. N., C. A. Desjardins, A. Griggs, G. Cerqueira, A. Van Tonder, M. T. Holden, P. Godfrey, K. L. Palmer, K. Bodi, E. F. Mongodin, J. Wortman, M. Feldgarden, T. Lawley, S. R. Gill, B. J. Haas, B. Birren, and M. S. Gilmore**. 2012. Comparative genomics of vancomycin-resistant *Staphylococcus aureus* strains and their positions within the clade most commonly associated with Methicillin-resistant *S. aureus* hospital-acquired infection in the United States. M. Bio. **3**: e00112.
27. **Lowy, F. D**. 1998. *Staphylococcus aureus* infections. N. Engl. J. Med. **20**: 520-532.
28. **Ludovice, A. M., S. W. Wu, and H. de Lencastre**. 1998. Molecular cloning and DNA sequencing of the *Staphylococcus aureus* UDP-*N*-acetylmuramyl tripeptide synthetase (*murE*) gene, essential for the optimal expression of methicillin resistance. Microb. Drug Resist. **4**: 85-90.
29. **Marchese, A., L. Gualco, E. Maioli, and E. E. Debbia**. 2009. Molecular analysis and susceptibility patterns of meticillin-resistant *Staphylococcus aureus* (MRSA) strains circulating in the community in the Ligurian area, a northern region of Italy: emergence of USA300 and EMRSA-15 clones. Int. J. Antimicrob. Agents. **34**: 424-428.
30. **Maugat, S., A. de Rougemont, H. Aubry-Damon H, M. E. Reverdy, S. Georges, F. Vandenesch, J. Etienne, B. Coignard.** 2009. Methicillin-resistant *Staphylococcus aureus* among a network of French private-sector community-based-medical laboratories. Med. Mal. Infect. **39**: 311-318.
31. **McDougal, L. K., C. D. Steward, G. E. Killgore, J. M. Chaitram, S. K. McAllister, and F.C. Tenover**. 2003. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J. Clin. Microbiol. **41**: 5113-5120.
32. **Memmi, G., S. R. Filipe, M. G. Pinho, Z. Fu, and A. Cheung**. 2008. *Staphylococcus aureus* PBP4 is essential for beta-lactam resistance in community-acquired methicillin-resistant strains. Antimicrob. Agents Chemother. **52**: 3955-3966.
33. **Moran, G. J., A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, L. K. McDougal, R. B. Carey, D. A. Talan, Emergency ID Net Study Group**. 2006. Methicillin-resistant *S. aureus* infections among patients in the emergency department. N. Engl. J. Med. **355**: 666-674.
34. **Munch, D., T. Roemer, S. H. Lee, M. Engeser, S. H. Sahl, and T. Schneider**. 2012. Identification and *in vitro* analysis of the GatD/MurT enzyme-complex catalyzing lipid II amidation in *Staphylococcus aureus*. PLoS Pathog. **8**: e1002509.
35. **Murakami, K., and A. Tomasz**. 1989. Involvement of multiple genetic determinants in high-level methicillin resistance in *Staphylococcus aureus*. J. Bacteriol. **171**: 874-879.

36. **Nair, D., G. Memmi, D. Hernandez, J. Bard, M. Beaume, S. Gill, P. Francois and A. L. Cheung**. 2011. Whole-genome sequencing of *Staphylococcus aureus* strain RN4220, a key laboratory strain used in virulence research, identifies mutations that affect not only virulence factors but also the fitness of the strain. J. Bacteriol. **193**: 2332-2335.
37. **O'Brien, F. G., J. W. Pearman, M. Gracey, T. V. Riley, and W. B. Grubb.** 1999. Community strain of methicillin-resistant *Staphylococcus aureus* involved in a hospital outbreak. J. Clin. Microbiol. **37**: 2858-2862.
38. **Oliveira, D. C., A. Tomasz, and H. de Lencastre**. 2002. Secrets of success of a human pathogen: molecular evolution of pandemic clones of meticillin-resistant *Staphylococcus aureus*. Lancet Infect. Dis. **2**: 180-189.
39. **Oliveira, D. C., Crisóstomo, I., Santos-Sanches, I., Major, P., Alves, C. R., Aires-de-Sousa, M., Thege, M. K., and de Lencastre, H**. 2001. Comparison of DNA sequencing of the protein A gene polymorphic region with other molecular typing techniques for typing two epidemiologically diverse collections of methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **39**: 574-580.
40. **Oliveira, D. C., Tomasz, A., and de Lencastre, H**. 2001. The evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*: identification of two ancestral genetic backgrounds and the associated *mec* elements. Microb. Drug Resist. **7**: 349-361.
41. **Oshida, T., and A. Tomasz**. 1992. Isolation and characterization of a Tn*551*-autolysis mutant of *Staphylococcus aureus*. J Bacteriol. **174**: 4952-4959.
42. **Pereira, S. F., A. O. Henriques, M. G. Pinho, H. de Lencastre, and A. Tomasz**. 2007. Role of PBP1 in cell division of *Staphylococcus aureus*. J. Bacteriol. **189**: 3525-3531.
43. **Pinho, M. G., H. de Lencastre, and A. Tomasz.** 2000. Cloning, characterization, and inactivation of the gene *pbpC*, encoding penicillin-binding protein 3 of *Staphylococcus aureus*. J. Bacteriol. **182**: 1074-1079.
44. **Pinho, M. G., S. R. Filipe, H. de Lencastre, and A. Tomasz**. 2001. Complementation of the essential peptidoglycan transpeptidase function of penicillin-binding protein 2 (PBP2) by the drug resistance protein PBP2A in *Staphylococcus aureus*. J. Bacteriol. **183**: 6525-6531.
45. **Robinson, D. A., and M. C. Enright**. 2003. Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **47**: 3926-3934.
46. **Rolo, J., M. Miragaia, A. Turlej-Rogacka, J. Empel, O. Bouchami, N. A. Faria, A. Tavares, W. Hryniewicz, A. C. Fluit, H. de Lencastre, and CONCORD Working Group**. 2012. High genetic diversity among community-associated *Staphylococcus aureus* in Europe: results from a multicenter study. PLoS One. **7**: e34768.
47. **Sá-Leão, R., I. Santos Sanches, D. Dias, I. Peres, R. M. Barros, and H. de Lencastre.** 1999. Detection of an archaic clone of *Staphylococcus aureus* with low-level resistance to methicillin in a pediatric hospital in Portugal and in international samples: relics of a formerly widely disseminated strain? J. Clin. Microbiol. **37**: 1913-1920.
48. **Sobral, R. G., A. M. Ludovice, S. Gardete, K. Tabei, H. De Lencastre, and A. Tomasz**. 2003. Normally functioning *murF* is essential for the

optimal expression of methicillin resistance in *Staphylococcus aureus*. Microb. Drug Resist. **9**: 231-241.

49. **Sobral, R. G., A. M. Ludovice, H. de Lencastre and A. Tomasz**. 2006. Role of *murF* in cell wall biosynthesis: isolation and characterization of a *murF* conditional mutant of *Staphylococcus aureus*. J. Bacteriol. **188**: 2543-2553.
50. **Tavares, A., M. Miragaia, J. Rolo, C. Coelho, H. de Lencastre, and CA-MRSA/MSSA working group**. 2013. High prevalence of hospital-associated methicillin-resistant *Staphylococcus aureus* in the community in Portugal: evidence for the blurring of community-hospital boundaries. Eur. J. Clin. Microbiol. Infect. Dis. **32**: 1269-1283.
51. **Tonin, E. and A. Tomasz**. 1986. Beta-lactam-specific resistant mutants of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **30**: 577-583.
52. **Zapun, A., J. Philippe, K. A. Abrahams, L. Signor, D. I. Roper, E. Breukink and T. Vernet**. 2013. *In vitro* reconstitution of peptidoglycan assembly from the Gram-positive pathogen *Streptococcus pneumoniae*. ACS Chem. Biol. **8**: 2688-2696.

Supplementary Information

Supplementary Tables

Table S1. Primers used in this study.

Primer	Sequence 5'-3' [a]	Source or reference
PmurF'-R	CCCGGGCATTATCAGTAGCAACACCA	This study
PmurF'-F	CCCGGGCTGAGGTTGT**TATT**ATGATT	This study
PmurFdn	TGCTTTTTCGACATGTTGC	(3)
Pcad-R	GTTCAGACATTGACCTTCAC	(1)
PmurT-D1	CTTCGGTGAAATTGATATTATGG	(2)
PmurT-R1	GTTCTCTATCACTTCCACCACC	(2)

[a)] The restriction sequences included in the primers are underlined and the putative ribosome-binding site is indicated in boldface type.

Supplementary Figures

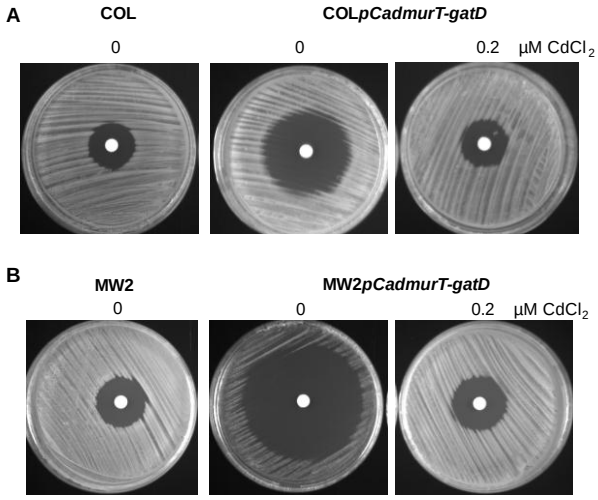

Figure S1. Effect of *murT-gatD* conditional expression on β-lactam resistance phenotype in COL and MW2 strains. Oxacillin inhibition halos (1-mg disks) were determined for COL, COL*pCadmurT-gatD,* MW2 and MW2*pCadmurT-gatD* grown under $CdCl_2$ concentrations of 0 and 0.2 µM.

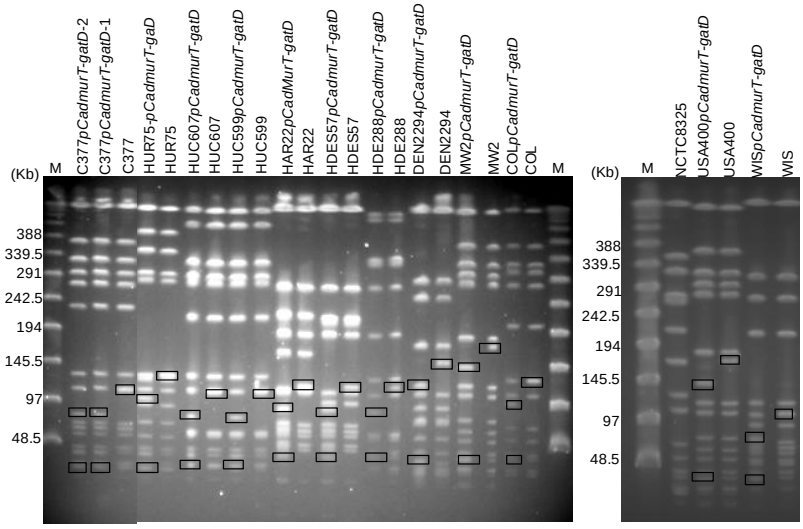

Figure S2. PFGE profiles of strains, listed in table 1, and of their respective *murT-gatD* conditional mutants. The boxes highlight the fragments where *murT-gatD* operon is located in the chromosome. The identification of these fragments was performed by the analysis of SmaI PFGE profile of the parental strain and the respective *murT-gatD* conditional mutant and through Southern blotting. Numbers on the left side indicate the size (Kb) of the molecular marker (M).

Supplementary References

1. **Charpentier, E., A. I. Anton, P. Barry, B. Alfonso, Y. Fang and R. P. Novick**. 2004. Novel cassette-based shuttle vector system for gram-positive bacteria. Appl. Environ. Microbiol. **70**:6076-6085.
2. **Figueiredo, T. A., R. G. Sobral, A. M. Ludovice, J. M. Almeida, N. K. Bui, W. Vollmer, H. de Lencastre, and A. Tomasz.** 2012. Identification of genetic determinants and enzymes involved with the amidation of glutamic acid residues in the peptidoglycan of *Staphylococcus aureus*. PLoS Pathog. **8**:e1002508.
3. **Sobral, R. G., A. M. Ludovice, H. de Lencastre and A. Tomasz**. 2006. Role of *murF* in cell wall biosynthesis: isolation and characterization of a *murF* conditional mutant of *Staphylococcus aureus*. J. Bacteriol. **188**:2543-2553.

Chapter V

Discussion and Future Perspectives

Discussion

The peptidoglycan of *Staphylococcus aureus* is a highly dynamic molecule that undergoes several secondary modifications to its primary structure. One of these modifications is the amidation of the γ-carboxyl group of the second residue of the stem peptide, D-glutamate, resulting in the formation of D-iso-glutamine. My doctoral Thesis describes identification of two genes - *murT* and *gatD* - in the genome of *S. aureus* strain COL, the new protein products of which are responsible for the amidation of the peptidoglycan. These determinants were unknown at the beginning of my work. My Thesis work also provided evidence for the existence of a physical interaction between the MurT and GatD proteins, resulting in the formation of a glutamine amidotransferase bi-enzymatic complex.

The findings reported in my Thesis allows one to conclude that amidation of *S. aureus* peptidoglycan requires the cooperative action of the MurT and GatD proteins, and to draw a functional model in which MurT is associated with the ATP-dependent synthetase activity, responsible for the recognition of the substrate and ATP, while GatD provides the glutaminase domain, involved in glutamine hydrolysis and in the subsequent amide nitrogen transfer from free glutamine to the peptidoglycan precursor.

Importantly, the availability of a *murT-gatD* conditional mutant allowed to demonstrate that amidation of peptidoglycan is important for bacterial growth, and is associated with the mechanism of resistance to β -lactams and lysozyme in *S. aureus*. Finally, the evaluation of the impact of peptidoglycan amidation on β-lactam and lysozyme resistance in different lineages of methicillin resistant *S. aureus* (MRSA) led to the suggestion that MRSA strains are able to develop different molecular mechanisms to overcome antibiotic pressure and to evade the host immune system during the infection process.

The amidation of *S. aureus* peptidoglycan is dependent on the activity of MurT-GatD enzymatic complex. In Chapter II of this Thesis, it is described a conditional mutant constructed for *murT-gatD* operon in the background of strain COL, COL*pCadmurT-gatD*, which showed abnormal peptidoglycan composition with decreased amidation of the D-glutamate residues, when grown in the absence of the promoter inducer. This provided direct evidence for the involvement of both genes in peptidoglycan amidation in *S. aureus* (8). Observations, described in Chapter II of this Thesis, raised the hypothesis that the protein products of *murT* and *gatD* genes interact functionally in order to amidate the peptidoglycan: i) *murT* and *gatD* genes are co-transcribed from the same promoter, and are organized as a small operon; ii) *murT* and *gatD* genes are present in most Gram-positive bacteria, as a syntenic block; and iii) the *murT-gatD* depletion phenotype is fully complemented, only when both *murT* and *gatD* are expressed extra-chromosomally (8).

The co-purification of MurT and GatD recombinant proteins from a vector expressing both *murT* and *gatD* genes, described in the chapter III, clearly indicating that the two proteins interact physically. Furthermore, the *in vitro* activity assays, with purified MurT-GatD and GatD proteins, showed that peptidoglycan amidation is dependent on both MurT and GatD activities, which is in agreement with the findings of Munch and colleagues (24).

The *in vivo* complementation assays, described in the Chapter II of this Thesis, constituted the first experimental evidence for the requirement of both MurT and GatD proteins in the peptidoglycan amidation reaction. These results indicated that MurT enzyme is essential and highly specific for the peptidoglycan amidation reaction, since the in trans complementation of the *murT-gatD* conditional mutant with several copies of *gatD* gene revealed no re-establishment of the normal peptidoglycan

composition (8). The specificity of MurT for *S. aureus* peptidoglycan amidation reported in this Thesis is in agreement with the findings of Munch and colleagues. Despite the fact that MurT shared high similarity with the sequence of *S. aureus* Mur ligases, MurT activity could not be replaced by purified MurC, D, E, and F proteins, in the lipid II amidation reaction *in vitro* (24). Regarding GatD specificity, *S. aureus* may contain other glutamine amidotransferases, besides GatD, able to catalyse the glutamine hydrolysis and the amino group transfer to peptidoglycan, as the in trans complementation with several copies of the *murT* gene was enough to obtain a partial amidated peptidoglycan. Nevertheless, the complementation assays showed that full expression of *murT*, together with a sub-optimal expression of *gatD,* are the minimal conditions to produce peptidoglycan fully amidated. Taking together, these results were not unexpected as the glutaminase domains of glutamine amidotransferases (GATases) have been described as highly conserved all through the entire GAT-family, while the synthetase domains tend to be more heterogeneous, since they are specific for the different nitrogen acceptor substrates in different biological reactions (22). In conclusion, the complete re-establishment of the *murT-gatD*-depletion phenotype requires the expression of *murT*, which is essential and highly specific for the recognition of peptidoglycan precursor, and at least a basal level of *gatD*, suggesting that *S. aureus* contains other glutamine amidotransferases that are able to partially replace GatD activity.

MurT amino acid sequence shares approximately 15% identity and 53% similarity with the sequence of the Mur ligases of *S. aureus,* which are responsible for the sequential addition of the five aminoacids to the cell wall precursor UDP-Mur*N*Ac, resulting in the formation of UDP-Mur*N*Ac–pentapeptide (30). The Mur ligases share the same type of enzymatic mechanism, consisting in the activation of the carboxyl group of the UDP

precursor by ATP, generating an acyl phosphate intermediate and ADP (1). Whereas several GATases do not need ATP for the amidation reaction, for some subtypes the ATP-hydrolysis is essential for the activation of the acceptor substrate (22).

The *in vitro* amidation assays, described in Chapter III, revealed that the MurT-GatD catalyzed reaction requires the presence of ATP, which allowed to infer mechanistically, that this reaction includes the activation of the substrate acceptor, resulting in the formation of an acyl phosphate intermediate. The phosphate group is then displaced by the incoming nitrogen group. Through similar *in vitro* amidation assays, Munch and colleagues also concluded that amidation of peptidoglycan is an ATP-dependent reaction (24). Interestingly, these authors reported that MurT could replace, *in vitro*, the MurE activity, suggesting that MurT is able to recognized UDP-Mur*N*Ac-dipeptide, bind L-lysine and activate the D-Glu carboxylate by phosphorylation. However, it is improbable that, *in vivo*, MurT can substitute MurE activity, as both genes are considered essential (16, 34).

The 243 amino acid GatD protein shows sequence similarities with the glutaminase domain of GATases. Also, *S. aureus* GatD harbored the conserved cysteine and histidine residues, commonly found in this family of proteins, but the third catalytic triad residue, glutamine, is missing (8). It was suggested by Munch and colleagues that the highly conserved glycine present in GatD aminoacid sequence, could be the third residue of its catalytic triad (24). The cysteine residue from the active site of the GATases is essential for glutaminase activity (22, 32, 37), since its nucleophilic sulfhydryl side chain initiates the amide transfer by the formation of a thioester with the substrate glutamine. Therefore, Munch and colleagues explored the importance of the cysteine residue in the catalytic

activity of GatD by site-directed mutagenesis. The inability of the GatD mutant, wherein the canonical cysteine of the proposed active site was replaced by glycine, to use glutamine and consequently to amidate the peptidoglycan precursor allowed to confirm the active site of the enzyme and provided clear evidence for the function of GatD as a glutaminase (24).

The analysis of MurT-GatD amino acid sequence and the experimental data allowed to propose that both proteins together contain the domains required for the amidation of *S. aureus* peptidoglycan to occur. Therefore, considering the modular organization of the GATases, the peptidoglycan amidation should require the synchronization of MurT and GatD activities: MurT acting as the synthetase domain, responsible for the recognition of the acceptor substrate and ATP, while GatD acting as the glutaminase domain, responsible for the hydrolysis of glutamine and the transfer of the resultant amino group to the peptidoglycan precursor (Figure 1).

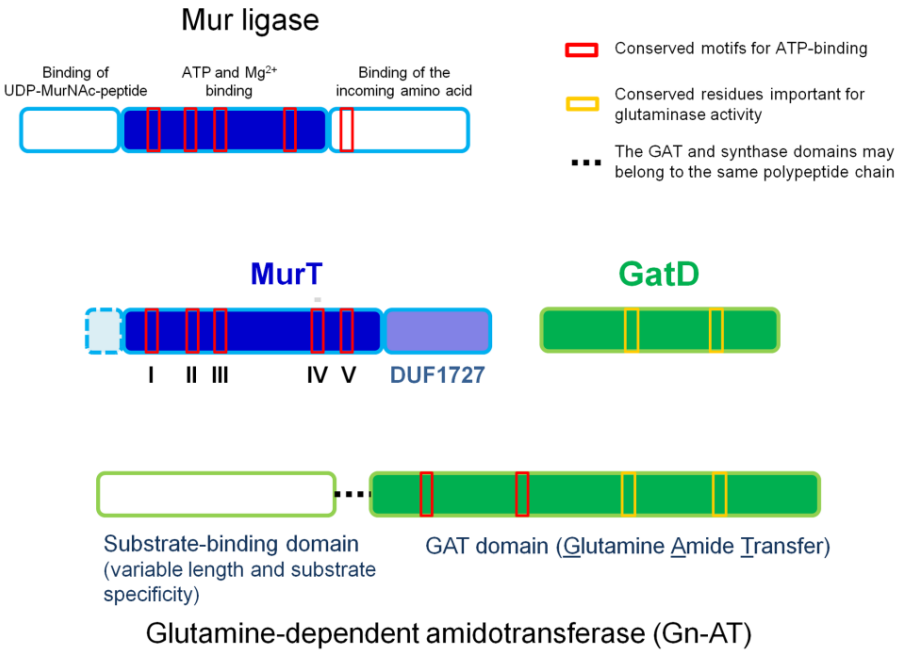

Figure 1. Protein regions necessary for the cooperative function of MurT and GatD proteins. The top panel illustrates the topology of Mur ligase family proteins. The MurT protein shows similarities only with the central domain, including the conserved motifs for ATP binding, which are indicated by red boxes.The lower panel illustrates the modular structure of glutamine-dependent amidotransferases, which includes a synthetase domain and a glutamine amide transfer domain, containing the glutaminase activity motifs (yellow boxes) and ATP-binding motifs (red boxes). GatD only shows similarities with the glutaminase domain, namely with the conserved residues important for glutaminase activity. (Reproduced from reference 8).

The peptidoglycan lipid-linked intermediates are acceptor substrates of the peptidoglycan amidation reaction. It was previously described by Siewert and Strominger that, upon addition of ATP and NH_4Cl or glutamine, amidated lipid I or lipid II could be detected in membranes of *S. aureus*. The composition of the peptidoglycan precursor pool of the *murT-gatD* conditional mutant and the respective parental strain, described in Chapter II of this Thesis, showed that *S. aureus* cytoplasmic precursors are not amidated (8). Hence, probably the D-glutamic acid amidation occurs at the membrane stage, confirming the previous findings described above (29). The *in vitro* amidation of lipid II in the presence of MurT-GatD, glutamine

and ATP, described in Chapter III, supports the proposal that this step occurs at the membrane stage of peptidoglycan biosynthesis and shows that lipid II is an acceptor substrate of the reaction catalyzed by the MurT-GatD enzyme complex. In order to identify the primary acceptor substrate, Munch and colleagues also included purified lipid I and lipid II-Gly_5 in the *in vitro* amidation assay, and concluded that the both lipid intermediates can serve as acceptor substrates for the MurT-GatD enzymatic complex (24). On the other hand, the presence of a 10 fold excess of Mur*N*Ac-pentapeptide did not interfere with the amidation of lipid II, indicating that the cytoplasmic precursors are not substrates for MurT-GatD dependent amidation. Furthermore, these authors also clarified that this reaction does not occur during MurC-F catalyzed stem peptide formation, highlighting once again that the soluble cell wall precursor UDP-Mur*N*ac-pentapeptide does not serve as a substrate for the amidation reaction (24).

Glutamine is the nitrogen donor substrate of the peptidoglycan amidation reaction. The abnormal peptidoglycan composition of *murT-gatD* depletion mutant had already been observed for *glnRA* transposition mutant RUSA208 (27). In RUSA208, the transposon insertion occurred in the glutamine synthetase repressor gene (*glnR*), resulting in a polar effect on the transcription of the glutamine synthetase gene (*glnA*). The inhibition of the *glnRA* operon produced a decrease in the glutamine synthetase activity and most probably, a reduction in the availability of glutamine in the cells, which explains the decrease of the amount of amidated D-glutamate in the mutant strain RUSA208 peptidoglycan. In the present study, the fully non amidated peptidoglycan of the construct RUSA208*pCadmurT-gatD*, in which both expression of *murT-gatD* and *glnRA* are inhibited, allowed us to conclude that their protein products are together essential for the amidation of the glutamic acid residue of *S. aureus* peptidoglycan, indicating that MurT-GatD enzymatic complex uses glutamine as the nitrogen source (8).

The analysis of the peptidoglycan composition of RUSA208 strain revealed that amidation of the muropeptides still occurred partially (27), suggesting that other sources of nitrogen, besides glutamine, may be used, although less efficiently. In fact, although most GATases seem to be highly specific for glutamine utilization as an amide nitrogen donor, they can also accept exogenous ammonia as nitrogen source (36). Munch and colleagues demonstrated that only at optimal conditions for the ammonia-dependent activity at pH 8.5, is the MurT-GatD complex able to use free ammonia, as nitrogen donor substrate (24). Taking into account the neutral pH within the cytoplasm and the fact that the peptidoglycan composition of a *glnRA* transposition mutant, unable to synthesize glutamine, is identical to that of the *murT-gatD* depletion mutant (8), it is most likely that MurT-GatD enzymatic complex uses preferentially glutamine as nitrogen donor substrate. Additionally, the complete absence of amidated muropeptides in the peptidoglycan of *glnRA* and *murT-gatD* depletion mutant highlight that D-glutamate amidation is dependent of the presence of glutamine, as the source of the amino group, and no other nitrogen donor is accepted by MurT-GatD enzymatic complex.

Peptidoglycan amidation is required for bacterial growth, and for optimal expression of β-lactam and lysozyme resistance in *S. aureus*. The growth rate of *murT-gatD* depleted cells was greatly reduced in the conditional mutant, indicating that amidation of peptidoglycan is required, but not essential, for normal growth (8), which contrasts with the findings of Munch and colleagues who found the *murT-gatD* genes essential for *S. aureus* growth (24). The residual growth and the partial amidation of the peptidoglycan of the conditional mutant, grown with no $CdCl_2$, can be explained by the leaky expression of *murT-gatD* operon through the *pCad* promoter in the absence of $CdCl_2$. However, the viability of the *glnRA* and *murT-gatD* depleted mutant, the peptidoglycan of which is totally

deamidated, suggests that D-glutamic acid amidation is not essential for bacterial survival (8). The different genetic strategies used in the two studies can account for the divergent results regarding the essentiality of *murT-gatD* in *S. aureus.* In the study of Munch and collaborators, *murT* and *gatD* were recognized as essential genes for *S. aureus*, through the transformation of the MRSA COL strain with a plasmid containing a xylose inducible antisense interference fragment, specifically targeting *murT* and *gatD* genes (9, 21). Upon addition of xylose, the antisence expression targets the complementary mRNA which totally blocks the transcription and translation of both proteins. In contrast, the *murT-gatD* conditional mutant used in our work, wherein the *murT-gatD* operon was placed under the control of an inducible promoter, showed a residual level of transcription in the absence of inducer, which probably is the cause for the observed residual growth.

The impact of peptidoglycan amidation on growth rate is consistent with previous results, which suggested that non-amidated lipid II is an inefficient substrate for one or more transpeptidases, as crosslinking of two adjacent stem peptides requires that at least one of them is amidated (26, 31). On the other hand, considering that the cell viability depends on the close coordination between the peptidoglycan precursor translocation, transglycosylation and transpeptidation reactions, MurT-GatD dependent amidation may function as a check-point signal, and could regulate, spatially and temporally, these biochemical events. In *Streptococcus pneumoniae,* it was recently demonstrated that the efficient peptidoglycan crosslinking, catalyzed by penicillin binding proteins 2a, 2b, and 2x, is dependent on the presence of D-iso-glutamine in lipid II structure (38). Curiously, the PBP2x and PBP2b are the two essentials PBPs of *S. pneumoniae* (18), which are also the main targets of β-lactams, and PBP2a seems to be the main transglycosylase, since the mutant affected in *pbp2a*

gene showed a higher sensitivity to moenomycin, a inhibitor of the transglycosylase activity in *Escherichia coli* PBP1b (14, 28). Therefore, it appears that amidation of peptidoglycan is involved in the growth of *S. pneumoniae*, by interfering with the activity of essentials proteins in cell wall biosynthesis.

It is possible that lack of the amide group may create an unbalance between the biosynthetic and the hydrolytic machineries of the cell. Interestingly, the structure of the amidase catalytic domain of the autolysin Atl of *Staphylococcus epidermidis,* referred to as AmiE, revealed the existence of extended contacts between the D-iso-glutamine residue of the cell wall muropeptide and conserved residues in the putative ligand-binding groove of AmiE, which suggested an essential role for the D-iso-glutamine in substrate recognition (39). This data raised the hypothesis that cell wall degradation during cell division, required for splitting the equatorial septum between two dividing daughter cells (13, 35), can be regulated by the presence of D-iso-glutamine in the stem peptide. However, the morphology of the *murT-gatD* depleted cells did not show significant abnormalities, which suggests that the cell division process is not affected by the presence of D-glutamate in the stem peptide of peptidoglycan. Interestingly, these findings can lead to new insights into *S. aureus* cell division mechanism, as Atl can be replaced by the activity of Sle1, the other *S. aureus* hydrolase responsible for daughter cells separation during cell division.

The impact of amidation on oxacillin resistance level observed in *murT-gatD* depleted cells, is consistent with previously results described for *glnRA* mutant (RUSA208) (12). It has been shown that several genes (the so called auxiliary genes), many of them cell wall related, are linked to the optimal expression of methicillin resistance (7). However, the mechanism

by which these genes contribute to the expression of methicillin resistance is not well understood. One of the existing theories is that the inactivation of genes involved in cell wall biosynthesis leads to the production of structurally abnormal muropeptides, which are poorer substrates for PBP2A. Consequently, due to the lack of effective muropeptide competitors, methicillin molecules would bind more frequently to the active site of PBP2A, resulting in a decrease of methicillin resistance levels (6).

In *S. aureus*, several modifications of cell wall structure, namely the *O*-acetylation of Mur*N*ac and the D-alanylation of wall teichoic acids (WTA), are involved in lysozyme resistance (3). In this Thesis, glutamate amidation was also found to contribute to *S. aureus* lysozyme resistance, since purified non-amidated peptidoglycan was more susceptible to the muramidase activity of this enzyme (8). However, it appears that the lack of amidation in the stem peptide of peptidoglycan does not affect the cationic antimicrobial peptide activity of lysozyme, which was an unexpected result, as the presence of D-glutamate leads to a more negatively charged cell wall. These observations suggest that the negative charge induced by the presence of glutamic acid may be neutralized by same other factor, such as the level of D-alanylation of WTAs.

The impact of peptidoglycan amidation on lysozyme resistance of the cell wall may play a role in bacterial pathogenesis, as it may encumber bacterial clearance by the host's immune system. The impact of peptidoglycan structure on pathogenesis has been studied extensively. The products of peptidoglycan degradation, the muropeptides, are recognized by receptors from the human innate immune system, namely by NOD1, that senses the minimal structure D-Glu-meso-diaminopimelic acid (DAP) dipeptide, commonly found in Gram-negative bacteria (5, 10), and NOD2, which detects the muramyl dipeptide and the lysine-containing muramyl tripeptide

(11, 15). It was reported that the presence of D-isoglutamine hampered recognition of peptidoglycan via NOD1, while the binding activity of NOD2 was not impaired (33). On the other hand, it appears that this peptidoglycan modification is unrelated with pro-inflamatory activity, as amidated *S. aureus* peptidoglycan did not affect the production of cytokines (20).

Despite the clear evidences, suggesting a direct involvement of amidation of peptidoglycan glutamic acid residue in bacterial pathogenesis, other physiological roles must also be attributed to this cell wall modification, as it is commonly found in many no pathogenic bacterial species.

The impact of peptidoglycan amidation on β-lactam and lysozyme resistance is determined by the genetic background of the strain. As mentioned above, despite *mecA* gene being the main determinant of methicillin resistance in MRSA strains, other genes are required for the optimal expression of methicillin resistance (4, 7, 19, 25).

Chapter IV of this Thesis describes that lack of amidation can generate different levels of decrease in oxacillin resistance in the most important MRSA clones, including community acquired MRSA (CA-MRSA) and hospital acquired MRSA (HA-MRSA). Overall, the impairment of *murT-gatD* caused a higher decrease of oxacillin resistance in CA-MRSA or related strains, than in HA-MRSA. Actually, this differential impact in oxacillin resistance can be correlated with the genetic background of each strain. Therefore, according to the ability of each genetic background to receive and maintain *mecA* (17), these results seem to suggest that the backgrounds less fit to receiving *mecA* gene need to recruit preferentially specific housekeeping genes, such as *murT-gatD*, for their β-lactam resistance mechanism. Thus, the absence of *murT-gatD* expression would, in these strains, produce a higher decrease of oxacillin resistance. Interestingly, the impairment of *murT-gatD* also caused a decrease of β-

lactam resistance in a *mecA*-independent resistant strain, suggesting that peptidoglycan amidation is important for expression of β-lactam resistance, even in the absence of *mecA* gene.

Furthermore, the impairment of *murT-gatD* and *murF* caused different levels of oxacillin resistance in some CA-MRSA clones. In these strains, it appears that *murT-gatD* is more important for the optimal expression of methicillin resistance, than the *murF* gene, suggesting that various auxiliary genes may be involved in methicillin resistance through different mechanisms.

Overall, these results allowed us to speculate that MRSA strains are able to develop different strategies, in which different auxiliary genes are recruited, in order to overcome the antibiotic selective pressure.

In this line of thought, and considering that PBP4 is a major player for methicillin resistance specifically in CA-MRSA (23), a strategy for β-lactam resistance that relies on peptidoglycan secondary modifications, such as secondary cross-linking (*pbp4*) and amidation (*murT-gatD*), may also exist.

Despite the lack of evidence indicating a dependence of *S. aureus* PBPs catalytic “efficiency” on the amidation status of the substrate molecule, the results reported in this Thesis allows one to speculate that PBP4 and/or PBP2 may also require amidated muropeptides to perform optimal transpeptidation, both proteins being involved in methicillin resistance although through different mechanisms.

The impairment of *murT-gatD* operon produced a strong impact on lysozyme resistance in CA-MRSA backgrounds. For the HA backgrounds, the impact of this mutation was more variable, depending on the genetic background. However, the lysozyme resistance levels of purified

peptidoglycan were similar for strains from both CA and HA settings. We propose that additional factor(s) conferring lysozyme resistance that are triggered/controlled by peptidoglycan amidation, may exist in the CA-MRSA strains analyzed.

Future perspectives

The studies performed in this Thesis led to the conclusion that amidation of the γ-carboxyl group of the D-glutamate of the stem peptide of the *S. aureus* peptidoglycan is important for the rate of bacterial growth and also for several other physiologically important properties. However, the exact mechanism through which peptidoglycan amidation influences growth remains unknown. To explore if deaminated peptidoglycan is a poor substrate for *S. aureus* autolysins, we plan to perform zymogram, using cell walls from the *murT-gatD* depleted mutant. This approach should clarify if peptidoglycan amidation is required for the proper cell wall degradation by the action of autolysins during cell division. In addition, this strategy may also provide insights regarding the role of the major staphylococcal autolysins in cell division, as this event is not affected by peptidoglycan amidation.

It is clear that lipid-linked intermediates of peptidoglycan synthesis are the acceptor substrates for the reaction catalyzed by the MurT-GatD enzymatic complex. However, it is not yet understood if amidation of D-glutamate takes place in the cytoplasm, or in the outer side of the cytoplasmic membrane, where the polymerization of peptidoglycan occurs. The determination of the intra- or extra-cellular presence of MurT-GatD complex by chemical cross-linking followed by immunoprecipitation should allow one to determine the precise location of amidation reaction. The immunoprecipitation assays would also provide evidence for the existence of other partners able to interact with MurT and GatD proteins.

Considering the frequent presence of MurT-GatD among gram-positive bacterial pathogens, the importance of these proteins in *S. aureus,* and their involvement in the resistance to β-lactams and lysozyme, the amidation step offers a new target for the development of inhibitors as monotherapeutics and/or as combination agents for existing β-lactam antibiotics, thereby boosting and/or restoring their activity against MRSA. The identification of the specific region responsible for the interaction between MurT and GatD is essential for the design of the proper inhibitor of peptidoglycan amidation. The C-terminal domain of unknown function (DUF1727) present in the MurT structure constitutes a likely candidate for the region responsible of MurT-GatD interaction. The construction of a MurT-GatD-His_6 co-expression system, in which expression of DUF1727 is blocked by the insertion of a stop codon in the 3' end of *murT*, would clarify the role of the N-terminal domain of MurT in the assembly of MurT-GatD enzymatic complex. This strategy would test if DUF1727 is required for the co-purification of GatD-His_6 and MurT, by affinity chromatography. Furthermore, the construction of a His_6-DUF-GatD co-expression system, wherein the *duf-gatD* sequence is fused with the N-terminal His_6-tag, would also provide some clues regarding the role of DUF1727 in the interaction between MurT and GatD proteins. The co-purification of His_6-DUF1727 with GatD, through affinity chromatography, would indicate that the N-terminal domain of MurT (DUF1727) is able to interact with GatD and therefore is important for the interaction between MurT and GatD. Additionally, the performance of "Bacterial two-hybrid system", using GatD and different versions of MurT, should clarify and confirm the results obtained by affinity chromatography, described above. On the other hand, in a more detailed approach, the performance of Isothermal Titration Calorimetry assays would define not only the requirement of DUF1727 for MurT-GatD interaction, but also would identify the forces that stabilize the interaction.

Since peptidoglycan amidation is directly involved in the intrinsic resistance of *S. aureus* to lysozyme, it is expected that this structural modification provides protection against macrophage killing and may limit host cytokine responses, promoting bacterial survival in the infected host, similarly to OatA acetyltransferase in *Listeria monocytogenes* (2). The evaluation of the impact *S. aureus* MurT-GatD inhibition on the stimulation of an inflammatory response by macrophages, (using murine infection models and animal cells lines), should begin to explore the role of glutamate amidation of peptidoglycan in host evasion. The effect of reduced peptidoglycan amidation on lysozyme resistance was clearly distinct between CA and HA MRSA backgrounds, with a more pronounced effect on MRSA strains predominant in the community. For this reason exploring the impact of the *murT-gatD* mutation should be done in *S. aureus* genetic backgrounds, representing both hospital derived as well as community strains of MRSA.

References

1. **Anderson, M. S., S. S. Eveland, H. R. Onishi and D. L. Pompliano**. 1996. Kinetic mechanism of the *Escherichia coli* UDPMur*N*Ac-tripeptide D-alanyl-D-alanine-adding enzyme: use of a glutathione S-transferase fusion. Biochem. **35**: 16264-16269.
2. **Aubry, C., C. Goulard, M. A. Nahori, N. Cayet, J. Decalf, M. Sachse, I. G. Boneca, P. Cossart and O. Dussurget**. 2011. OatA, a peptidoglycan O-acetyltransferase involved in *Listeria monocytogenes* immune escape, is critical for virulence. J. Infect. Dis. **204**: 731-740.
3. **Bera, A., R. Biswas, S. Herbert, E. Kulauzovic, C. Weidenmaier, A. Peschel, and F. Gotz**. 2007. Influence of wall teichoic acid on lysozyme resistance in *Staphylococcus aureus*. J. Bacteriol. **189**: 280-283.
4. **Berger-Bächi, B.** 1983. Insertional inactivation of staphylococcal methicillin resistance by *Tn*551. J. Bacteriol. **154**: 479-487.
5. **Chamaillard, M., M. Hashimoto, Y. Horie, J. Masumoto, S. Qiu, L. Saab, Y. Ogura, A. Kawasaki, K. Fukase, S. Kusumoto, M. A. Valvano, S. J. Foster, T. W. Mak, G. Nunez and N. Inohara**. 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat. Immunol. **4**:702-707.
6. **de Lencastre, H., and A. Tomasz**. 1994. Reassessment of the number of auxiliary genes essential for expression of high-level methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents. Chemother. **38**: 2590-2598.
7. **de Lencastre, H., S. W. Wu, M. G. Pinho, A. M. Ludovice, S. Filipe, S. Gardete, R. Sobral, S. Gill, M. Chung, and A. Tomasz**. 1999. Antibiotic resistance as a stress response: complete sequencing of a large number of chromosomal loci in *Staphylococcus aureus* strain COL that impact on the expression of resistance to methicillin. Microb. Drug Resist. **5**: 163-175.
8. **Figueiredo, T. A., R. G. Sobral, A. M. Ludovice, J. M. Almeida, N. K. Bui, W. Vollmer, H. de Lencastre, and A. Tomasz.** 2012. Identification of genetic determinants and enzymes involved with the amidation of glutamic acid residues in the peptidoglycan of *Staphylococcus aureus*. PLoS Pathog. **8**: e1002508.
9. **Forsyth, R. A., R. J. Haselbeck, K. L. Ohlsen, R. T. Yamamoto, H. Xu, J. D. Trawick, D. Wall, L. Wang, V. Brown-Driver, J. M. Froelich, P. King, M. McCarthy, C. Malone, B. Misiner, D. Robbins, Z. Tan, Z. Y. Zhu Zy, G. Carr, D. A. Mosca, C. Zamudio, J. G. Foulkes and J. W. Zyskind**. 2002. A genome-wide strategy for the identification of essential genes in *Staphylococcus aureus*. Mol. Microbiol. **43**: 1387-1400
10. **Girardin, S. E., I. G. Boneca, L. A. Carneiro, A. Antignac, M. Jehanno, J. Viala, K. Tedin, M. K. Taha, A. Labigne, U. Zahringer, A. J. Coyle, P. S. DiStefano, J. Bertin, P. J. Sansonetti and D. J. Philpott**. 2003. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science. **300**: 1584-1587.
11. **Girardin, S. E., L. H. Travassos, M. Herve, D. Blanot, I. G. Boneca, D. J. Philpott, P. J. Sansonetti and D. Mengin-Lecreulx**. 2003. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. J. Biol. Chem. **278**: 41702-41708.

12. **Gustafson, J., A. Strassle, H. Hachler, F. H. Kayser, and B. Berger-Bachi**. 1994. The *femC* locus of *Staphylococcus aureus* required for methicillin resistance includes the glutamine synthetase operon. J. Bacteriol. **176**: 1460-1467.
13. **Hobot, J. A., and H. J. Rogers**. 1991. Intracellular location of the autolytic N-acetylmuramyl-L-alanine amidase in *Bacillus subtilis* 168 and in an autolysis-deficient mutant by immunoelectron microscopy. J. Bacteriol. **173**: 961-967.
14. **Hoskins, J., P. Matsushima, D. L. Mullen, J. Tang, G. Zhao, T. I. Meier, T. I. Nicas and S. R. Jaskunas**. 1999. Gene disruption studies of penicillin-binding proteins 1a, 1b, and 2a in *Streptococcus pneumoniae*. J. Bacteriol. **181**: 6552-6555.
15. **Inohara, N., Y. Ogura, A. Fontalba, O. Gutierrez, F. Pons, J. Crespo, K. Fukase, S. Inamura, S. Kusumoto, M. Hashimoto, S. J. Foster, A. P. Moran, J. L. Fernandez-Luna and G. Nunez**. 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. J. Biol. Chem. **278**: 5509-5512.
16. **Jana, M., T. T. Luong, H. Komatsuzawa, M. Shigeta and C. Y. Lee**. 2000. A method for demonstrating gene essentiality in *Staphylococcus aureus*. Plasmid. **44**: 100-104.
17. **Katayama, Y., D. A. Robinson, M. C. Enright, and H. F. Chambers**. 2005. Genetic background affects stability of *mecA* in *Staphylococcus aureus*. J. Clin. Microbiol. **43**: 2380-2383.
18. **Kell, C. M., U. K. Sharma, C. G. Dowson, C. Town, T. S. Balganesh and B. G. Spratt**. 1993. Deletion analysis of the essentiality of penicillin-binding proteins 1A, 2B and 2X of *Streptococcus pneumoniae*. FEMS Microbiol. Lett. **106**: 171-175.
19. **Kornblum, J., B. J. Hartman, R. P. Novick, and A. Tomasz**. 1986. Conversion of a homogeneously methicillin-resistant strain of *Staphylococcus aureus* to heterogeneous resistance by Tn*551*-mediated insertional inactivation. Eur. J. Clin. Microbiol. **5**: 714-718.
20. **Kraus, D., H. Kalbacher, J. Buschmann, B. Berger-Bachi, F. Gotz and A. Peschel**. 2007. Muropeptide modification-amidation of peptidoglycan D-glutamate does not affect the proinflammatory activity of *Staphylococcus aureus*. Infect. Immun. **75**: 2084-2087.
21. **Lee, S. H., L. W. Jarantow, H. Wang, S. Sillaots, H. Cheng, T. C. Meredith, J. Thompson, and T. Roemer**. 2011. Antagonism of chemical genetic interaction networks resensitize MRSA to beta-lactam antibiotics. Chem. Biol. **18**: 1379-1389.
22. **Massiere, F., and M. A. Badet-Denisot**. 1998. The mechanism of glutamine-dependent amidotransferases. Cell Mol. Life Sci. **54**: 205-222.
23. **Memmi, G., S. R. Filipe, M. G. Pinho, Z. Fu, and A. Cheung**. 2008. *Staphylococcus aureus* PBP4 is essential for beta-lactam resistance in community-acquired methicillin-resistant strains. Antimicrob. Agents Chemother. **52**: 3955-3966.
24. **Munch, D., T. Roemer, S. H. Lee, M. Engeser, S. H. Sahl, and T. Schneider**. 2012. Identification and *in vitro* analysis of the GatD/MurT enzyme-complex catalyzing lipid II amidation in *Staphylococcus aureus*. PLoS Pathog. **8**: e1002509.

25. **Murakami, K., and A. Tomasz**. 1989. Involvement of multiple genetic determinants in high-level methicillin resistance in *Staphylococcus aureus*. J. Bacteriol. **171**:874-879.
26. **Nakel, M., J. M. Ghuysen, and O. Kandler**. 1971. Wall peptidoglycan in *Aerococcus viridans* strains 201 Evans and ATCC 11563 and in Gaffkya homari strain ATCC 10400. Biochem. **10**: 2170-2175.
27. **Ornelas-Soares, A., H. de Lencastre, B. de Jonge, D. Gage, Y. S. Chang, and A. Tomasz**. 1993. The peptidoglycan composition of a *Staphylococcus aureus* mutant selected for reduced methicillin resistance. J. Biol. Chem. **268**: 26268-26272.
28. **Paik, J., I. Kern, R. Lurz and R. Hakenbeck**. 1999. Mutational analysis of the *Streptococcus pneumoniae* bimodular class A penicillin-binding proteins. J. Bacteriol. **181**: 3852-3856.
29. **Siewert, G., and J. L. Strominger**. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XI. Formation of the isoglutamine amide group in the cell walls of *Staphylococcus aureus*. J. Biol. Chem. **243**: 783-790.
30. **Smith, C. A.** 2006. Structure, function and dynamics in the mur family of bacterial cell wall ligases. J. Mol. Biol. **362**: 640-655.
31. **Stranden, A. M., M. Roos and B. Berger-Bachi**. 1996. Glutamine synthetase and heteroresistance in methicillin-resistant *Staphylococcus aureus*. Microb. Drug Resist. **2**: 201-207.
32. **Tesmer, J. J., T. J. Klem, M. L. Deras, V. J. Davisson, and J. L. Smith**. 1996. The crystal structure of GMP synthetase reveals a novel catalytic triad and is a structural paradigm for two enzyme families. Nat. Struct. Biol. **3**: 74-86.
33. **Wolfert, M. A., A. Roychowdhury and G. J. Boons**. 2007. Modification of the structure of peptidoglycan is a strategy to avoid detection by nucleotide-binding oligomerization domain protein 1. Infect. Immun. **75**: 706-713.
34. **Xu, H. H., J. D. Trawick, R. J. Haselbeck, R. A. Forsyth, R. T. Yamamoto, R. Archer, J. Patterson, M. Allen, J. M. Froelich, I. Taylor, D. Nakaji, R. Maile, G. C. Kedar, M. Pilcher, V. Brown-Driver, M. McCarthy, A. Files, D. Robbins, P. King, S. Sillaots, C. Malone, C. S. Zamudio, T. Roemer, L. Wang, P. J. Youngman and D. Wall**. 2010. *Staphylococcus aureus* TargetArray: comprehensive differential essential gene expression as a mechanistic tool to profile antibacterials. Antimicrob. Agents Chemother. **54**: 3659-3670.
35. **Yamada, S., M. Sugai, H. Komatsuzawa, S. Nakashima, T. Oshida, A. Matsumoto, and H. Suginaka**. 1996. An autolysin ring associated with cell separation of *Staphylococcus aureus*. J. Bacteriol. **178**: 1565-1571.
36. **Zalkin, H.** 1993. The amidotransferases. Adv. Enzymol. Relat. Areas. Mol. Biol. **66**: 203-309.
37. **Zalkin, H., and J. L. Smith**. 1998. Enzymes utilizing glutamine as an amide donor. Adv. Enzymol. Relat. Areas Mol. Biol. **72**: 87-144.
38. **Zapun, A., J. Philippe, K. A. Abrahams, L. Signor, D. I. Roper, E. Breukink and T. Vernet**. 2013. *In vitro* reconstitution of peptidoglycan assembly from the Gram-positive pathogen *Streptococcus pneumoniae*. ACS Chem. Biol. **8**: 2688-2696.

39. **Zoll, S., B. Patzold, M. Schlag, F. Gotz, H. Kalbacher, and T. Stehle**. 2010. Structural basis of cell wall cleavage by a staphylococcal autolysin. PLoS Pathog. **6**:e1000807.

Appendix I

Purification, crystallization and preliminary X-ray diffraction analysis of GatD, a glutamine amidotransferase-like protein from *Staphylococcus aureus* peptidoglycan

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Abstract

Amidation of peptidoglycan is an essential feature in *Staphylococcus aureus* that is necessary for resistance to β-lactams and lysozyme. GatD, a 27 kDa type I glutamine amidotransferase-like protein, together with MurT ligase, catalyses the amidation reaction of the glutamic acid residues of the peptidoglycan of *S. aureus*. The native and the selenomethionine-derivative proteins were crystallized using the sitting-drop vapour-diffusion method with polyethylene glycol, sodium acetate and calcium acetate. The crystals obtained diffracted beyond 1.85 and 2.25 A°, respectively, and belonged to space group P212121. X-ray diffraction data sets were collected at Diamond Light Source (on beamlines I02 and I04) and were used to obtain initial phases.

Introduction

Peptidoglycan, the major component of the Gram-positive bacterial cell wall, is a polymer composed of glycan chains cross-linked by short peptides and is responsible for cell-shape maintenance and for counterbalancing turgor pressure (5). The importance of peptidoglycan for cell survival and its exclusivity to the bacterial kingdom renders most enzymatic steps involved in its biosynthesis excellent targets for antimicrobial therapy.

Staphylococcus aureus, an opportunistic bacterium responsible for a wide range of infections (2, 6) owes its success as a human pathogen mainly to its capacity to acquire antibiotic-resistance traits. Most genes involved in the peptidoglycan biosynthesis pathway are intimately related to the mechanism of β-lactam resistance, enhancing their potential as targets for antimicrobial therapy (3).

The main biosynthesis of peptidoglycan is well understood (9, 12). Additional steps are responsible for secondary modifications to the main structure (13). In *S. aureus*, one such modification is the amidation of the γ-carboxyl group of the second residue of the stem peptide, d-isoglutamate, resulting in the formation of d-isoglutamine (10). Recently, the *murT-gatD* operon was identified as the genetic determinant of peptidoglycan amidation in *S. aureus*, which is found to be widespread among bacteria as a syntenic block, almost exclusively in Gram-positives. The impaired expression of this operon impacts bacterial growth, β-lactam resistance and intrinsic lysozyme resistance (4). Moreover, the two proteins physically interact and form a glutamine amidotransferase bienzymatic complex (8). We have cloned and expressed the GatD protein in *Escherichia coli* and purified and crystallized it. X-ray diffraction data were collected both from

native as well as SeMet-containing protein and were used to obtain preliminary phases of the model.

Materials and methods

Cloning, overexpression and purification of GatD. The coding sequence of the *gatD* gene was amplified from *S. aureus* and cloned into the vector pOPINF using the In-Fusion method to generate the construct OPPF12143 (1). The protein was produced in *E. coli* using the auto-induction method (11). Macromolecule production information is given in Table 1.

Table 1. Macromolecule-production information.

Source organism	*S. aureus* COL
DNA source	*S. aureus* COL
Forward primer	AAGTTCTGTTTCAGGGCCCGCATGAATTGACTATTTATCATTTTATGTCAG
Reverse primer	ATGGTCTAGAAAGCTTTAACGAGATTTCTTCTGTCTATTTG-CTC
Cloning vector	pOPINF
Expression vector	pOPINF
Expression host	*E. coli* strain Lemo21(DE3)
UniProt accession code	Q5HEN2
Complete amino-acid sequence of the construct produced	GPHELTIYHFMSDKLNLYSDIGNIIALRQRAKKRNIKVNVVEINE TEGITFDECDIFFIGGGSDREQALATKELSKIKTPLKEAIEDGMP GLTICGGYQFLGKKYITPDGTELEGLGILDFYTESKTNRLTGDI VIESDTFGTIVGFENHGGRTYHDFGTLGHVTFGYGNNDEDKK EGIHYKNLLGTYLHGPILPKNYEITDYLLEKACERKGIPFEPKEI DNEAEIQAKQVLIDRANRQKKSR

After 20 h of incubation, the cells were harvested and resuspended in lysis buffer: 50 mM Tris–HCl pH 7.5, 500 mM NaCl, 20 mM imidazole and 0.2% Tween 20 supplemented with protease inhibitors (Sigma) and 400 U/ml DNAse type I. The cells were lysed using a Basic-Z cell disruptor at 207 MPa and clarified by centrifugation at 30 000g for 30 min at 4ºC. The supernatant was loaded onto a 5 ml HisTrap FF column (GE Healthcare)

equilibrated with wash buffer (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 20 mM imidazole), extensively washed with this buffer and eluted with elution buffer (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 500 mM imidazole). The sample was subsequently loaded onto a Superdex 200 HiLoad 16/60 column (GE Healthcare) equilibrated with gel-filtration buffer (20 mM Tris–HCl pH 7.5, 200 mM NaCl, 1 mM TCEP). Fractions containing GatD protein were pooled and the N-terminal hexahistidine tag was removed by cleavage with 3C protease. The mixture was then purified by reverse Ni-affinity chromatography. The protein was concentrated to 10, 20 and 45 mg/ml in gel-filtration buffer for crystallization. The expressed protein differs from the native in its N-terminus, where the original M is substituted by GP. A selenomethionine derivative was expressed using the Seleno-Methionine Expression Media kit (Molecular Dimensions) and the purification protocol was followed as described above.

Crystallization. Crystallization screens were performed at the Oxford Protein Production Facility (OPPF-UK) using a Cartesian instrument (Digilab MicroSys liquid-handling system). 100 nl GatD sample was mixed with 100 nl crystallization solution and equilibrated over 90 ml reservoir solution (see Table 2 for details). Crystals appeared in several conditions from the Emerald Wizard 1 and 2 crystallization screen (Rigaku Reagents). The best crystals (native and SeMet derivative) grew after 48 h (Fig. 1). The crystals were cryoprotected in 50%(v/v) PEG 400 and flash-cooled in liquid nitrogen prior to data collection.

Table 2. Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	Greiner Bio-One
Temperature (K)	294
Protein concentration (mg ml^{-1})	45
Buffer composition of protein solution	20 mM Tris–HCl pH 7.5, 200 mM NaCl, 1mM TCEP
Composition of reservoir solution	30%(w/v) PEG 400, 100 mM sodium acetate/ acetic acid pH 4.5, 200 mM calcium acetate
Volume and ratio of drop	200 nl; 1:1
Volume of reservoir (ml)	90

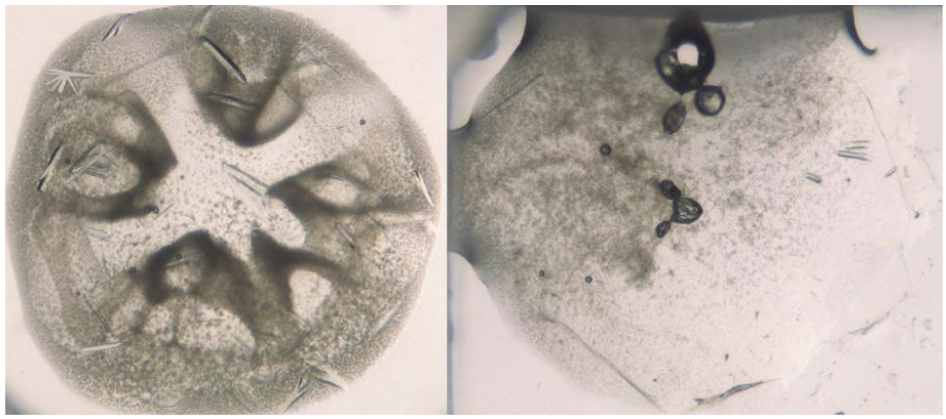

Figure 1. GatD crystals obtained using the crystallization robot (native, left; SeMet derivative, right). The dimensions of the best crystal were 250x30x30 mm.

Data collection and processing. Data were collected on beamlines I02 (on a Pilatus 6M detector) and I04 (on an ADSC Quantum Q315r detector) at the Diamond Light Source (DLS). Crystals of the native protein diffracted beyond 1.9 A° resolution and those of the SeMet-derivatized protein diffracted beyond 2.3 A° resolution (Fig. 2). The data were automatically processed using xia2 (14). Data-collection and processing statistics are given in Table 3.

Initial phases were obtained by single-wavelength anomalous diffraction (SAD) using data collected from SeMet derivative at the Se edge peak.

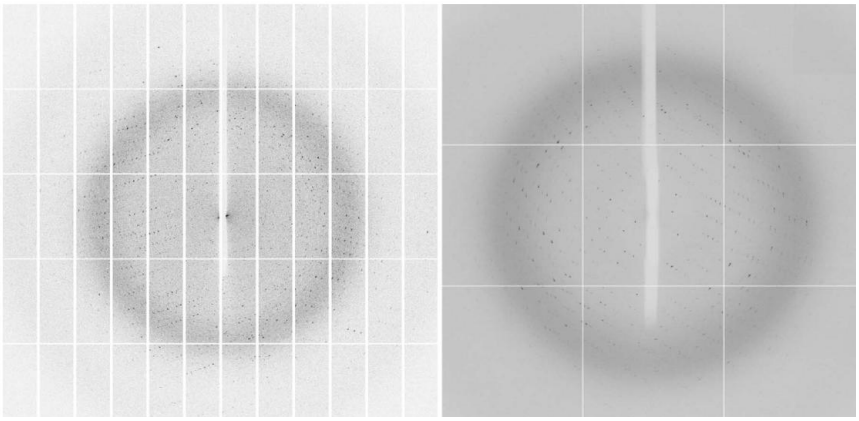

Figure 2. Representative diffraction patterns of the crystals (native, left; SeMet derivative, right). The resolution at the edge of the detector is 2.1 and 2.5 A°, respectively.

Table 3. Data collection and processing. Values in parentheses are for the outer shell.

	SAD	**Native**
Diffraction source	I04, DLS	I02, DLS
Wavelenght (Å)	0.9796	1.0000
Temperature (K)	100	100
Detector	ADSC Q315r	Pilatus 6M
Crystal-to-detector distance (mm)	375	407.4
Rotation range per image (º)	1.0	0.5
Total rotation range (º)	360	1200
Exposure time per image (s)	0.5	0.04
Space group	$P2_12_12_1$	$P2_12_12_1$
a, b, c (Å)	48.28, 93.00, 109.30	48.61, 93.92, 110.08
α, β, γ (Å)	90, 90, 90	90, 90, 90
Mosaicity (º)	0.387	0.141
Resolution range (Å)	47.12-2.25	36.43-1.85
Total No. of reflection	301512	839410
No. of unique reflection	24020	42987
Completeness (%)	98.7 (89.2)	97.9 (83.7)
Multiplicity	12.6 (6.7)	19.5 (10.1)
I/σ(I)	16.5 (2.2)	20.7 (2.2)
$R_{p.i.m.}$	0.078 (0.477)	0.028 (0.313)
Overall B factor from Wilson plot ($Å^2$)	9.429	15.237
Anomalous completeness (%)	98.7 (89.2)	
Anomalous multiplicity	6.7 (3.4)	

Results and discussion

The recently identified MurT–GatD enzymatic complex represents an unexplored step as a potential antimicrobial target. MurT shares considerable similarity with the sequence of the Mur ligases of *S. aureus*, which are cytoplasmic enzymes that are responsible for the sequential addition of amino-acid residues to the growing muropeptide stem. GatD shows similarity to the glutamine amidotransferases (GATases), with glutamine amide-transfer activity to a wide variety of substrates (7). Typically, GATases catalyze two distinct reactions: the glutaminase reaction, in which glutamine is converted into ammonia and glutamate, and the synthase reaction, in which ammonia is transferred to an acceptor substrate. These two reactions occur at distinct active sites, which may sit on the same polypeptide chain or on independent protein subunits. GatD protein corresponds to a glutaminase subunit, most probably being responsible for the production of ammonia from glutamine.

In order to determine the structure of *S. aureus* GatD protein, the encoding region of the *gatD* gene was cloned into pOPINF plasmid and expressed in *E. coli* Lemo21(DE3) as an N-terminal His-tag fusion. The purity of the recombinant protein was estimated by SDS–PAGE, which showed a single band corresponding to a molecular weight of 27 kDa.

The crystallization trials were performed at a high-throughput crystallization facility. Several crystallization hits were obtained using the Emerald Wizard 1 and 2 screens from Rigaku Reagents.

Diffraction data were collected on I02 and I04 at DLS to a resolution beyond 1.9 A°. Initial phases were obtained by single-wavelength anomalous diffraction (SAD) using data collected from SeMet derivatives at the Se edge peak.

The crystals belonged to space group P212121, with unit-cell parameters a = 48.29, b = 93.00, c = 109.31 A°. The structure of GatD, together with complete biochemical studies, will provide important insights into the molecular basis of the mechanism responsible for the amidation of the glutamic acid residues of the peptidoglycan of *S. aureus*.

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References

1. **Bird, L. E.** 2011. High throughput construction and small scale expression screening of multi-tag vectors in *Escherichia coli*. Methods. **55**: 29-37.
2. **Boucher, H. W., G. H. Talbot, J. S. Bradley, J. E. Edwards, D. Gilbert, L. B. Rice, M. Scheld, B. Spellberg and J. Bartlett**. 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin. Infect. Dis. **48**: 1-12.
3. **de Lencastre, H., S. W. Wu, M. G. Pinho, A. M. Ludovice, S. Filipe, S. Gardete, R. Sobral, S. Gill, M. Chung, and A. Tomasz**. 1999. Antibiotic resistance as a stress response: complete sequencing of a large number of chromosomal loci in *Staphylococcus aureus* strain COL that impact on the expression of resistance to methicillin. Microb. Drug Resist. **5**: 163-175.
4. **Figueiredo, T. A., R. G. Sobral, A. M. Ludovice, J. M. Almeida, N. K. Bui, W. Vollmer, H. de Lencastre, and A. Tomasz.** 2012. Identification of genetic determinants and enzymes involved with the amidation of glutamic acid residues in the peptidoglycan of *Staphylococcus aureus*. PLoS Pathog. **8**:e1002508.
5. **Holtje, J. V.** 1998. Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. Microbiol. Mol. Biol. Rev. **62**: 181-203.
6. **Klevens, R. M., M. A. Morrison, J. Nadle, S. Petit, K. Gershman, S. Ray, L. H. Harrison, R. Lynfield, G. Dumyati, J. M. Townes, A. S. Craig, E. R. Zell, G. E. Fosheim, L. K. McDougal, R. B. Carey, S. K. Fridkin**. 2007. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. J.A.M.A. **298**: 1763-1771.
7. **Massiere, F., and M. A. Badet-Denisot**. 1998. The mechanism of glutamine-dependent amidotransferases. Cell Mol. Life Sci. **54**: 205-222.
8. **Munch, D., T. Roemer, S. H. Lee, M. Engeser, S. H. Sahl, and T. Schneider**. 2012. Identification and *in vitro* analysis of the GatD/MurT enzyme-complex catalyzing lipid II amidation in *Staphylococcus aureus*. PLoS Pathog. **8**: e1002509.
9. **Scheffers, D. J., and M. G. Pinho**. 2005 Bacterial cell wall synthesis: new insights from localization studies. Microbiol. Mol. Biol. Rev. **69**: 585-607.
10. **Siewert, G., and J. L. Strominger**. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XI. Formation of the isoglutamine amide group in the cell walls of *Staphylococcus aureus*. J. Biol. Chem. **243**: 783-790.
11. **Studier, F. W.** 2005. Protein production by auto-induction in high density shaking cultures. Protein Expr. Purif. **41**: 207-234.
12. **Veiga, P., M. Erkelenz, E. Bernard, P. Courtin, S. Kulakauskas and M. P. Chapot-Chartier**. 2009. Identification of the asparagine synthase responsible for D-Asp amidation in the *Lactococcus lactis* peptidoglycan interpeptide crossbridge. J. Bacteriol. **191**: 3752-3757.
13. **Vollmer, W.** 2008. Structural variation in the glycan strands of bacterial peptidoglycan. FEMS Microbiol. Rev. **32**: 287-306.
14. **Winter, G., S. Dokel, A. K. Jones, P. Scheerer, N. Krauss, W. Hohne and B. Friedrich**. 2010. Crystallization and preliminary X-ray

crystallographic analysis of the [NiFe]-hydrogenase maturation factor HypF1 from Ralstonia eutropha H16. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. **66**: 452-455.