MARY HELLEN FABRES KLEIN

MASTITE BOVINA: AVALIAÇÃO DE ANTÍGENOS DE *Staphylococcus aureus* PARA DIAGNÓSTICO E O PAPEL DO REGULADOR LysR NA INTERAÇÃO PATÓGENO-HOSPEDEIRO

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Agrícola, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 19 de fevereiro de 2014

_____	_____
Luciano Gomes Fietto (Coorientador)	Juliana Lopes Rangel Fietto
_____	_____
Denise Mara Soares Bazzolli	Maria Aparecida V. de Paiva Brito

_____

Andrea de Oliveira Barros Ribon
(Orientadora)

A Deus

Aos meus pais, Sandra e Arildo (*in memoriam*)

Aos meus irmãos Bianca e José Arildo

Ao meu esposo, Raphael

Dedico

UMA VEZ FUI VIAJAR E NÃO VOLTEI

Não por rebeldia ou por ter decidido ficar; simplesmente mudei.
Cruzei fronteiras que eu nunca imaginaria cruzar.
Nem no mapa, nem na vida. Fui tão longe que olhar para trás não era confortante, era motivador.
Conheci o que posso chamar de professores e acessei conhecimentos que nenhum livro poderia me ensinar.
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BIOGRAFIA

Mary Hellen Fabres Klein, filha de José Arildo Fabres e Sandra Araújo Fabres, nasceu na cidade de Vitória, Espírito Santo, em 17 de julho de 1981.

Em dezembro de 1998 completou o ensino médio no Centro Educacional Neo em Vitória.

Em março de 2003, ingressou no bacharelado em Bioquímica da Universidade Federal de Viçosa, MG, graduando-se em janeiro de 2008.

Em março de 2008, ingressou no Programa de Pós Graduação em Bioquímica Agrícola, em nível de mestrado, na Universidade Federal de Viçosa, MG, defendendo a dissertação em fevereiro de 2010.

Ingressou no Programa de Pós Graduação em Bioquímica Agrícola em março de 2010, em nível de doutorado, na Universidade Federal de Viçosa, submetendo-se à defesa de tese em 19 de fevereiro de 2014.

SUMÁRIO

RESUMO

KLEIN, Mary Hellen Fabres, D.Sc., Universidade Federal de Viçosa, fevereiro de 2014. **Mastite bovina: Avaliação de antígenos de *Staphylococcus aureus* para diagnóstico e o papel do regulador LysR na interação patógeno-hospedeiro.** Orientadora: Andréa de Oliveira Barros Ribon. Coorientadores:Luciano Gomes Fietto e Sérgio Oliveira de Paula.

Infecções intramamárias causadas por *Staphylococcus aureus* são em sua maioria de natureza subclínica e evoluem frequentemente para persistente. O diagnóstico preciso dos animais infectados é fundamental para evitar a dissiminação do patógeno e prevenir o surgimento de novas infecções. A cultura bacteriológica é utilizada como padrão-ouro na identificação de patógenos causadores de mastite, mas a demora na obtenção de resultados ainda é um grande entrave. Os imunoensaios são uma abordagem alternativa para o diagnóstico que podem ser rápidos, específicos e, em alguns casos, realizados em campo. Na busca por marcadores para o diagnóstico da mastite bovina causada por *S. aureus*, avaliou-se a capacidade de três proteínas na identificação da bactéria em amostras de leite mastítico. O potencial de IsaA e Nuc para o immunodiagnóstico de *S. aureus* de origem bovina foi mostrado, embora seja necessária a padronização do método para que a sensibilidade aumente. Sugere-se que apenas uma região da proteína seja usada no teste. Neste trabalho, o papel de um regulador transcricional putativo da família LysR (SAB2209) também foi avaliado para expandir nosso conhecimento sobre os mecanismos envolvidos na patogênese de *S. aureus*. A superexpressão de LysR afetou a formação de biofilme e a susceptibilidade a estresse oxidativo, no entanto sem afetar o crescimento bacteriano e hemólise. LysR também reduziu a colonização da bactéria em ensaios *ex vivo* e *in vivo*. Análise do transcriptoma mostrou que 34,8% dos genes de *S. aureus* RF122 foram alterados em resposta à superexpressão de SAB2209. O gene sortase A (*srt*A), que codifica uma transpeptidase que ancora adesinas na parede da célula, e os genes *tag*X e *tag*B, envolvidos na produção de ácido teicóico foram reduzidos 3,0; 7,3 e 11,2 vezes, respectivamente. Em contraste, a transcrição dos genes responsáveis pela biossíntese de purinas e adenosina sintase (*ads*A) foi estimulada. Os nossos resultados sugerem que SAB2209 influencia passos iniciais para a colonização de *S. aureus* e evasão do sistema imune do hospedeiro, através da alteração da expressão de genes associados com a função da parede celular e estratégias evasivas.

ABSTRACT

KLEIN, Mary Hellen Fabres, D.Sc., Universidade Federal de Viçosa, February of 2014. **Bovine mastitis: Evaluation of antigens for diagnosis of *Staphylococcus aureus* and role of a LysR regulator in host-pathogen interaction.** Advisor: Andréa de Oliveira Barros Ribon. Co- Advisors:Luciano Gomes Fietto and Sérgio Oliveira de Paula

Intramammary infections caused by *Staphylococcus aureus* are mostly subclinical and frequently evolves to a persistent form. The accurate detection of infected animals is vital to avoid pathogen spread and to prevent the occurrence of new infections. Bacteriological culture remains the gold standard for the identification of mastitis pathogens, but it is also time-demanding. Immunoassays are an alternative approach of diagnosis that can be fast, specific, and in some cases, suitable for field analysis. In a continuous pursuit for biomarkers that could diagnose bovine mastitis caused by *S. aureus*, we evaluated the ability of three proteins to identify the pathogen in milk samples collected from infected animals. IsaA and Nuc showed a potential for immunodiagnosis of *S. aureus*, but specificity still needs improvement perhaps by targeting a smaller region of the protein in the assay. In this work the role of a putative LysR type transcriptional regulator (SAB2209) was also evaluated to expand our knowledge of the mechanisms involved in *S. aureus* pathogenesis. LysR overexpression affected biofilm formation and oxidative stress susceptibility but no changes on bacterial growth and hemolysis were seen. LysR also reduced colonization in *ex vivo* and *in vivo* assays. Transcriptome analysis showed that 34.8% of the staphylococcal genes were altered in response to SAB2209 overexpression. The gene sortase A (*srtA*), that codes a transpeptidase that anchors adhesins to the cell wall, and the genes *tagX* and *tagB,* involved in teichoic acid production were reduced 3.0, 7.3, and 11.2-fold, respectively. In contrast, genes responsible for purine biosynthesis and adenosine synthase (*adsA*) were up-regulated. Our results suggest that SAB2209 influences initial steps to *S. aureus* colonization and immune evasion, by altering the genes associated with cell wall function and evasive strategies.

1. INTRODUÇÃO GERAL

1.1. Mastite bovina

A América do Sul é responsável por 10% da produção mundial de leite, o que correspondeu a cerca de 65 milhões de toneladas em 2011 (FAO, 2013). Juntos, Brasil, Argentina, Equador e Colômbia são responsáveis por 85% da produção de leite da América do Sul (FAO, 2013). O Brasil contribui com 49% deste montante, o que o torna o maior produtor sul-americano e o quarto maior produtor mundial, ultrapassando a marca de 32 bilhões de litros em 2012 (IBGE, 2012). A região Sudeste concentra 35,9% da produção leiteira do país, sendo o estado de Minas Gerais o principal produtor com 8,9 bilhões de litros (IBGE, 2012). Minas Gerais também ocupa a posição de estado com o maior rebanho de vacas em lactação, aproximadamente 5,6 milhões (IBGE, 2012).

O Brasil possui boas oportunidades de se tornar um grande exportador de lácteos, pois existem disponibilidade de terra, água, tecnologia, além de custo de produção competitivo. Porém, contrariando as expectativas, a produtividade brasileira está bem aquém da média mundial (Embrapa, 2009). Um dos fatores que contribuem para essa baixa produtividade é a mastite bovina, doença caracterizada pela inflamação da glândula mamária causada pela invasão do úbere que danifica o tecido glandular e promove alterações físicas e químicas do leite produzido e redução da produção leiteira (Zhao e Lacasse, 2008).

A mastite bovina causa um prejuízo de, aproximadamente, US$ 35 bilhões em todo mundo (Ruegg, 2005). Nos Estados Unidos, as perdas anuais chegam a US$ 1,8 bilhões (Schroeder, 2010), com um custo em torno de US$ 200/animal. No Brasil, as estimativas mostram que o prejuízo causado pela mastite varia de R$ 700 a 2700 por vaca em lactação (Lopes et al., 2012). No custo total da mastite são computados, além das perdas devidas à queda na produção, gastos com medicamentos e assistência veterinária, descarte de leite contaminado após tratamento e descarte precoce de animais doentes (Petrovski et al., 2006).

A mastite bovina também apresenta impacto negativo na saúde pública pela possibilidade de veiculação de micro-organismos, toxinas e resíduos de antimicrobianos no leite (Peton e Le Loir, 2013). Os riscos à saúde do consumidor são representados principalmente pelo desencadeamento de fenômenos alérgicos em indivíduos sensíveis, pelos efeitos tóxicos, por alterações no equilíbrio da

microbiota intestinal e pela seleção de bactérias resistentes no trato digestivo dos consumidores (Oliveira et al., 1999).

A mastite pode ser causada por uma grande variedade de agentes, incluindo bactérias, leveduras, fungos e algas. Entretanto, a origem bacteriana é a mais freqüente (Hillerton e Berry, 2005). *Staphylococcus aureus* é um dos principais agentes infecciosos causadores de mastite bovina (Barkema et al., 2006), sendo considerado o patógeno de maior incidência em diversos países (Bramley et al., 1996) e também o mais comumente isolado do leite cru (Zecconi e Hahn, 2000). No Brasil, relatos de isolamento de *S. aureus* de mastite subclínica, manifestação onde sintomas típicos da doença não são observados, são conhecidos desde o início da década de 50 (Lacerda Jr. et al., 1954). Desde então, trabalhos realizados nas regiões Nordeste, Sudeste, Sul e Centro-Oeste do País mostraram a sua predominância sobre os demais agentes da doença (Brito e Brito, 1996; Rabello et al., 2005; Fagundes et al., 2010).

A prevalência de *S. aureus* pode variar de 27% a 69% de todos os casos de infecções intramamárias (IMI) em diversos países (Osteras et al., 2006; Reksen et al., 2006; Bradley et al., 2007; Ferguson et al., 2007; Ericsson-Unnerstad et al., 2009). A prevalência de *S. aureus* na mastite clínica é de 3,3% a 40% (Bradley et al., 2007; Ferguson et al., 2007; Olde Riekerink et al., 2008; Ericsson-Unnerstad et al., 2009), e na mastite subclínica, pode chegar a 62% (Tenhagen et al., 2006; Bradley et al., 2007).

Para iniciar uma infecção intramamária, *S. aureus* penetra o canal do teto mamário, principalmente durante a ordenha. Após a penetração, inicia-se a expressão de moléculas associadas à superfície bacteriana que reconhecem e se ligam às proteínas da matriz extracelular para a colonização do tecido glandular, considerada etapa fundamental da infecção (Hauck e Ohlsen, 2006). Dessa forma a bactéria pode multiplicar sem correr o risco de ser lavada pela saída do leite durante a ordenha. Segue-se a liberação de fatores de virulência (toxinas e exoproteínas) que rompem o tecido da glândula para a captação de nutrientes e a disseminação para os tecidos adjacentes (Hauck e Ohlsen, 2006). Nesta fase, o sistema imune do animal tenta combater a infecção em curso e os sintomas começam a aparecer, caracterizando um caso de mastite clínica. No entanto a mastite estafilocócica é um exemplo de infecção persistente, difícil de ser tratada e que, freqüentemente, evolui para uma forma crônica (Barkema et al., 2006). Este

aspecto muito se deve à expressão de genes de virulência, por exemplo proteína A, que neutralizam o sistema imune, à localização intracelular de *S. aureus* dentro da glândula mamária e a formação de biofilme, fatores que prolongam a sobrevivência da bactéria e a protege do sistema imune e da ação de antibióticos (Oviedo-Boyso et al., 2008; Sinha e Fraunholz, 2010; Tuchscherr et al., 2011).

1.2. Diagnóstico da mastite bovina

A redução na prevalência da mastite é conseguida pela implementação de um Programa Básico de Controle de Mastite onde são adotadas medidas preventivas e de higiene, que vão desde a limpeza dos equipamentos de ordenha até o descarte de animais com manifestação crônica (National Mastitis Council, 2001; Keefe, 2012). O sucesso deste programa requer a rápida identificação dos animais infectados com *S. aureus* para impedir a disseminação do patógeno no rebanho. Já foi demonstrado que a detecção precoce da mastite pode aumentar em 60% a taxa de cura e reduzir o tempo necessário para recuperar a produção de leite normal quando combinada com terapêutica antimicrobiana adequada (Milner et al., 1997).

A forma clínica da mastite bovina é facilmente diagnosticada pelos sinais evidentes de inflamação, como edema, aumento de temperatura, endurecimento e dor na glândula mamária, e/ou aparecimento de grumos, pus ou qualquer alteração das características visíveis do leite (Bradley, 2002). Na forma subclínica, mudanças visíveis no aspecto do leite ou do úbere não são percebidas (Persson-Waller et al., 2003). O diagnóstico da infecção subclínica é mais difícil uma vez que o leite tem aparência normal, e por isso é frequentemente adicionado ao tanque de resfriamento localizado na propriedade (LeJeune e Rajala-Schultz, 2009). A manifestação da doença em sua forma subclínica é a que mais preocupa não somente pela prevalência, mas também pela disseminação do patógeno de animais aparentemente sadios para os demais do rebanho (Peton e Le Loir, 2013).

Na mastite subclínica registra-se elevada contagem de células somáticas (CCS), um indicador de infecção intramamária que vem sendo utilizado desde a década de 60 (Pyörälä, 2003). O diagnóstico pode ser feito pela medição direta da CCS, ou indiretamente, pelo *California Mastitis Test* (CMT). O CMT é atualmente o teste mais usado por ser rápido, barato e acessível aos produtores, além de poder ser realizado em campo. Porém, não se recomenda que os

resultados do teste CMT sejam usados na definição do tratamento, para isso necessita-se a identificação do agente etiológico, o que auxilia na definição da estratégia de manejo a ser adotada para reduzir novos casos e influencia as decisões de terapia e escolha de antibióticos (Sears e McCarthy, 2003). Testes rotineiros usados em laboratórios de microbiologia constituem o procedimento mais correto para a detecção e a identificação do patógeno, embora não sejam rotina dada à demora na obtenção dos resultados. Assim, dificilmente o tratamento veterinário é subsidiado pelo resultado de uma cultura microbiológica.

Um esquema simplificado para identificação de *S. aureus* de origem bovina inicia-se com o cultivo de uma amostra do leite em meio ágar-sangue. Colônias brancas que apresentam halos de hemólise completos são submetidas a coloração de Gram e visualizadas em microscópio. Cocos Gram-positivos são diferenciados pelos testes de catalase e esculina. Bactérias catalase positiva e esculina negativa são classificadas como *Staphylococcus*. A separação de *S. aureus* de outros Staphylococci é feita pela produção de coagulase e acetoína. Os testes de produção de β-galactosidase e utilização anaeróbica do manitol são também úteis para diferenciar *S. intermedius* e *S. aureus* quando necessário (Breed et al., 1957; Brito et al., 2002).

Como alternativa, o diagnóstico da mastite pode ser feito pela avaliação de sinais que indicam os níveis de inflamação. A medição da temperatura corporal do animal pode ser realizada utilizando uma câmera térmica capaz de detectar mudanças de temperatura de 1 a 1,5°C (Hovinen et al., 2006; Colak et al., 2008). No entanto, nem todos os casos de mastite levam ao aumento na temperatura corporal. Além disso, essa abordagem não-invasiva também pode ser afetada pela temperatura ambiente (Viguier et al., 2009). Testes que avaliam o pH e a condutividade elétrica do leite podem ser utilizados para diagnosticar uma inflamação no úbere, mas não são sensíveis o suficiente para o diagnóstico conclusivo (Viguier et al., 2009). Existem ainda ensaios colorimétricos e fluorimétricos que medem a concentração de enzimas que se encontram em níveis elevados durante a mastite, como as enzimas N-acetil-β-D-glicosaminidase (NAGase) e a lactato desidrogenase (LDH) (Pemberton et al., 2001; Day, 2005; Hiss et al., 2007). Esses ensaios, além de não identificarem o patógeno, necessitam de pessoas treinadas e de laboratórios especializados (Viguier et al., 2009).

Os avanços tecnológicos, juntamente com o aumento da informação proteômica e genômica, resultaram em melhorias na sensibilidade de testes utilizados para a detecção da mastite (Viguier et al., 2009). Imunoensaios, como ELISA (*Enzyme-Linked Immunosorbent Assay*), foram desenvolvidos para a identificação de *S. aureus* e outras bactérias como *Escherichia coli* e *Listeria monocytogenes (*Arora et al., 2006). Mas os autores concluíram que a cultura microbiológica dos resultados positivos ainda se faz necessária para a confirmação do patógeno. Testes para identificação do micro-organismo baseados na reação em cadeia da polimerase (PCR), como PCR quantitativo e PCR *multiplex*, também foram desenvolvidos para diagnóstico e embora sejam sensíveis e específicos, o alto custo associado e a necessidade de pessoal capacitado inviabiliza o uso rotineiro (Phuektes et al., 2003; Cai et al., 2005; Cremonesi et al., 2005; Gillespie e Oliver, 2005; Viguier et al., 2009).

Novas tecnologias de diagnóstico utilizando dispositivos com microfluidos, os chamados *biochips* (também chamados de "*lab-on-a-chip*") vêm sendo testadas na detecção da mastite (Garcia-Cordero e Ricco, 2008). Choi e colaboradores (2006) desenvolveram um *chip* para monitorar simultaneamente patógenos, células somáticas e o pH em amostras de leite cru. Anticorpos contra o patógeno e contra células somáticas foram imobilizados no *chip* e os complexos antígeno-anticorpo formados foram detectados por microscopia de fluorescência. Um *biochip* capaz de detectar sete espécies comuns na mastite incluindo *Corynebacterium bovis*, *Mycoplasma bovis*, *S. aureus*, *Streptococcus agalactiae*, *Streptococcus bovis*, *Streptococcus dysgalactiae*, e *Streptococcus uberis* foi desenvolvido por Lee e colaboradores (2008). Testes preliminares foram realizados utilizando 82 amostras de leite e comparados com métodos microbiológicos convencionais, das quais apenas um não ratificou a análise bacteriológica. Na teoria estes testes poderiam ser realizados em campo, mas ainda é necessário reduzir os custos para disponibilizá-los comercialmente (Viguier et al., 2009). No momento, a maioria dessas tecnologias está em fase de validação e continua inacessível aos produtores.

Um outro grande desafio é encontrar marcadores que identifiquem as diferentes cepas de *S. aureus* encontradas nos rebanhos. A dificuldade se deve principalmente pela alta diversidade presente em populações de *S. aureus*, pela variedade de mecanismos envolvidos na patogenicidade e pela variação dos

fatores de virulência expressos por cada isolado (Le Maréchal et al., 2011a; Hecker et al., 2012; Klein et al., 2012). A busca por proteínas biomarcadoras de *S. aureus* utilizando técnicas como análise sorológica do subproteoma, eletroforese bidimensional e *screening* de biblioteca genômica tem proporcionado a descoberta de inúmeras proteínas candidatas, algumas são específica para *S. aureus* (Vytvytska et al., 2002; Weichhart et al., 2003; Le Maréchal et al., 2011a; Fabres-Klein et al., 2013).

1.3. *Staphylococcus aureus*: interação patógeno-hospedeiro

A caracterização de genes expressos durante a infecção intramamária é um passo fundamental para maior esclarecimento dos mecanismos envolvidos na patogênese de *S. aureus* (Koprivnjak et al., 2010; Malachowa et al., 2011). Ela permite não somente identificar novos fatores de virulência relevantes ao processo infeccioso, mas também entender as bases moleculares que levam a diferentes isolados da mesma espécie a causarem diferentes manifestações de uma mesma doença. Outro aspecto a se considerar é o potencial biotecnológico dessa estratégia que revela novos alvos que podem ser usados no diagnóstico ou prevenção de infecções causadas por *S. aureus*.

Genes que codificam proteínas hipotéticas representam uma fração importante dos genomas já sequenciados, podendo alcançar cerca de 50% das sequências anotadas (Minion et al*.,* 2004; Powers et al., 2008). Essa fração ainda não conhecida dos genomas guarda muita informação que pode ser explorada para o melhor entendimento da biologia de diferentes organismos.

O sequenciamento do genoma de *S. aureus* RF122 (ET-3), um dos clones mais comumente isolado de mastite bovina no mundo, revelou um total de 2589 *open reading frames* (ORFs), 44% dos quais codificavam proteínas hipotéticas (Herron-Olson et al., 2007). Estudos de tipagem molecular, como MLST (Multilocus Sequence Typing) e Spa Typing, têm revelado a alta diversidade das populações de *S. aureus* e também mostram que alguns complexos clonais (CC), definido como um grupo de tipos de sequência (ST) estreitamente relacionadas, são predominantes e relacionados com hospedeiros específicos. RF122 faz parte do complexo clonal CC705 (ST151) o qual possui apenas isolados bovinos (Peton e Le Loir, 2013). Genes associados especificamente a isolados de *S. aureus* de origem bovina foram identificados a partir da comparação com genomas de

isolados humanos, sugerindo uma especialização molecular do patógeno com o hospedeiro bovino (Kozytska et al., 2010). Estes genes pertencem a várias categorias incluindo proteínas hipotéticas, ilhas de patogenicidade e reguladores de transcrição (SAB2083c).

Uma busca por potenciais fatores de virulência relacionados a doenças cardiovasculares revelou uma ORF conservada no genoma de treze cepas de *S. aureus* de origem humana que possuía 96-100% de identidade entre todas as sequências analisadas (Malachowa *et al*., 2011). A sequência protéica predita dessa ORF revelou 59% de identidade com antígeno miosina *cross* reativo de *Streptococcus pyogenes*. A proteína foi chamada de SOK (*Surface factor promoting resistance to oxidative killing*) por se localizar na superfície celular e aumentar a resistência à morte oxidativa. Experimentos *in vitro* mostraram o envolvimento de SOK na resistência bacteriana ao estresse oxidativo e também na morte induzida por neutrófilos. Os ensaios *in vivo* revelaram o importante papel de SOK nas infecções cardiovasculares estafilocócicas.

Estudo comparativo entre os genomas de duas linhagens de *S. aureus* foi realizado para investigar as bases genéticas e moleculares da patogênese na mastite em ruminantes (Le Maréchal et al., 2011b). Duas cepas, geneticamente semelhantes mas que causam sintomas diferentes de mastite, mostraram pequenas divergências gênicas e notáveis alterações no perfil de expressão gênica. Estes resultados sugerem que fatores como elementos genéticos móveis, metabolismo de ferro, reguladores de transcrição e produção de exoproteínas contribuem para a virulência de *S. aureus* e podem influenciar na severidade da mastite (Le Maréchal et al., 2011b).

O estudo do perfil transcricional de *S. aureus* isolado de leite de vacas infectadas experimentalmente identificou genes que podem estar relacionados com a virulência da bactéria, alguns dos quais expressos em mais de uma vaca infectada e em mais de um tempo de infecção (Allard et al., 2013). Dentre os genes, foi identificado um regulador transcricional SACOL2325 que também foi induzido pelo crescimento da bactéria em leite. O gene SACOL2325 faz parte da família de reguladores transcricionais LysR (LTTR), a qual representa um dos tipos mais abundantes de reguladores transcricionais em procariotos (Momany e Neidle, 2012).

Os membros da família LTTR têm uma estrutura conservada com um motivo N-terminal hélice-volta-hélice e um domínio C-terminal de ligação ao DNA. Reguladores LysR se ligam a regiões promotoras mas também podem se ligar a *downstream* do promotor (Maddocks e Oyston, 2008). Reguladores LysR podem ser induzido por substratos, metabólitos intermediários ou produtos finais das vias nas quais os genes alvo estão envolvidos (Momany e Neidle, 2012). As características estruturais principais dos LTTRs incluem: um domínio de conservado ligação ao DNA, chamado de motivo HTH (Hélice-Volta-Hélice) e um motivo menos conservado, denominado motivo LysR (Schell, 1993). Os reguladores transcricionais da família LysR regulam um conjunto diversificado de genes, que podem estar envolvidos na virulência, divisão celular, metabolismo, quorum sensing, respostas a estresse oxidativo, adesão e motilidade da bactéria (Maddocks e Oyston, 2008).

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CAPÍTULO 1

Moving towards the immunodiagnosis of staphylococcal intramammary infections

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Moving towards the immunodiagnosis of staphylococcal intramammary infections

Fabres-Klein, M.H., Aguilar, A.P., Silva, M.P., Silva, D.M., Ribon, A.O.B.

Laboratorio de Biotecnologia Molecular, Departmento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, 36570-000, Viçosa, MG, Brazil.

*Corresponding author: Tel: 55 31 3899 2837; Fax: 55 31 3899 2373

E-mail addresses: abribon@ufv.br (A.O.B. Ribon)

Abstract

Bovine mastitis is the primary disease of dairy cattle and is associated with the loss of U.S. $ 141.50/year. It is estimated that worldwide economic losses due to mastitis range between U.S. $ 82-131 per cow/year. A fast and efficient diagnosis of the disease remains a major bottleneck that directly influences the speed with which treatment decisions and management are undertaken. Microbiological culture remains the gold standard in the identification of bacteria that cause mastitis, but the method has inherent limitations, such as a delay in obtaining results and cost, and requires special care during the collection and processing of the sample. For this reason, multiple groups have devoted efforts to develop alternative methods that preferably can be easily accomplished in the field. The specificity of the antigen-antibody reaction has enabled the emergence of major diagnostic methods used in clinical practice, such as immunoassays, which have major advantages in speed, sensitivity, specificity, and portability. Commercially, immunodiagnostics have been used in the detection of various diseases in cattle. However, in several cases, only a presumptive diagnosis can be made, which requires confirmation using culture-based methods. This review discusses the immunological-based assays developed since the 1990s for the detection of *Staphylococcus aureus*, which is considered the primary pathogen of contagious bovine mastitis. Although no ideal antigens ensure the accurate performance of tests and the costs need to be reduced to allow for good market competitiveness, immunoassays, particularly lateral flow immunoassay and immunoagglutination, have emerged as promising tests to be used in the field.

Keywords: *Staphylococcus aureus*, Bovine mastitis, Microbiological Diagnosis, Immunodiagnosis

The Staphylococci group

Staphyloccocci are a group of Gram-positive bacteria, and this group contains numerous species of clinical significance. These species are microbiologically characterized as facultative anaerobes that have no motility and no capacity to form spores but may aggregate in grapelike clusters. Forty-nine species have been described and are divided in two groups based on the ability to produce coagulase [1, 2]. Coagulase-negative staphylococci (CNS) are common commensal bacteria found in human skin and mucous membranes and are normally described as non-pathogenic; however, reports regarding their involvement in different infections have been increasing lately [3, 4, 5]. *Staphylococcus epidermidis* is by far the most important member of the group because of its association with hospital-acquired infections [6, 7, 8]. Coagulase-positive staphylococci (CPS) comprise a broad group that includes many pathogenic species, such as *S. aureus*, *S. intermedius*, and *S. hyicus*. *S. aureus* is a pathogen of major importance in human and veterinary medicine due to its ability to cause chronic infections and its great potential to develop resistance to antimicrobials [9].

Staphylococcus aureus

S. aureus is considered a major causative agent of bovine mastitis, although this bacterium also causes mastitis in other hosts such as humans, goats, sheep, and pigs [10, 11]. Molecular typing, particularly MLST (Multilocus Sequence Typing), has revealed that *S. aureus* has great population diversity. The MLST database (http://saureus.mlst.net) contains more than 2200 types of sequences (ST) determined using different strains isolated from a variety of hosts,

although the human source is the most prevalent. The data show that the clonal complex (CC), defined as a group of closely related STs, is predominant and clearly associated with certain hosts. For example, the *S. aureus* strains that cause mastitis in ruminants are mostly associated with the clonal complexes CC97, CC133, CC130, CC126, and CC705 [10, 12]. CC705 includes ST151, which has strains exclusively of bovine origin [10]. *S. aureus* strains of human origin are rarely found in CC97 and CC126 (www.mlst.net) [13]; however, several authors suggest that these complexes are composed exclusively of strains that cause intramammary infections in ruminants [14].

Staphylococcal intramammary infection and immune response

Intramammary infections begin when *S. aureus* penetrates the teat canal primarily at or after milking when the teat sphincter is still open. Bacteria that are not eliminated by the immune system disseminate and colonize the secretory tissue through the expression of molecules associated with the bacterial surface that bind to extracellular matrix components of the host [15]. After damage to the cistern and larger ducts tissues, the bacteria reach internal areas of the mammary gland [16]. At this stage, the release of toxins and exoproteins is essential to damage the glandular tissue to enable nutrient uptake and to spread to adjacent tissues [17]. The expression of virulence genes that neutralize the immune system, the ability to survive in the mammary gland and the capability to form biofilms are additional factors that ensure the persistence of *S. aureus* and promote chronic infection [18, 19, 20].

The immune system is characterized by its ability to recognize and discriminate between foreign invaders and molecules produced by the body. The

mammary gland is protected by innate immunity and acquired immunity [21]. Innate immunity is the first line of defense during the early stages of infection and starts when specific pattern recognition receptors (PRR) located on the surface or inside the mammary gland cells recognize and bind to microbial molecules called pathogen-associated molecular patterns (PAMPs) [22, 23]. Lipoteichoic acid (LTA) is a PAMP present on the surface of *S. aureus* that is recognized by the receptors and weakly induces proinflammatory cytokine expression compared to Gram-negative PAMPs [24]. Bovine mammary epithelial cells (bMECs) infected with heat-killed *S. aureus* or exposed to LTA do not sustain cytokine expression [25, 26]. The reduced levels of cytokine have been suggested to be insufficient to eliminate the pathogen from the mammary gland, which may help explain the chronic infections typically caused by *S. aureus*.

In addition to PRR, the innate immunity of the organism also relies on physical and biochemical barriers such as the teat sphincter muscle, teat canal keratins, lysozyme, and lactoferrin [22, 27]. Once these barriers are overcome, the innate immune system initiates an active defense that consists of cellular components (macrophages, neutrophils, dendritic cells, natural killer cells, and soluble factors) and acute-phase proteins (cytokines and eicosanoids) [24, 27]. The acquired response is activated by the production of immunoglobulins (Igs) and memory cells that recognize specific antigenic determinants if the innate immune response fails [22, 24]. The concentration of Igs is the highest in the mammary secretions during inflammation and also during colostrogenesis [22]. Four classes of Igs (IgG1, IgG2, IgM, and IgA) act in defense of the mammary gland, and the former three classes enhance the opsonization of *S. aureus*. The IgG1 isotype is found in the primary mammary secretions of healthy animals;

however, IgG2 increases substantially during the inflammation process. IgA promotes the clumping of bacteria and neutralizes several toxins [22]. In addition, a two-week delay occurs in the increase of antibody levels upon intramammary instillation of *S. aureus* [28]. The levels remained elevated in cows with postpartum infections but were below the threshold in cows where intramammary infections were cured during the non-lactating period.

Microbiological diagnosis – the gold standard

The early diagnosis of bovine mastitis is imperative for animal management and therapeutics, which are two aspects that reduce economic losses. Several methods are used to detect mastitis, but only a few methods identify the etiologic agent [29].

Culture-based methods are the gold standard for the identification of pathogens in milk and are frequently used as a reference when other methods need to be validated [30]. The isolation of bacteria directly from milk samples has >75% sensitivity and achieves 100% specificity [31, 32]. However, major drawbacks associated with culture-based methods are the time needed for a conclusive diagnosis and the final cost of the analysis [29, 33].

Another shortcoming of this method is that the number of bacteria present in the milk may reach levels lower than the detectable limit for microbiological cultures (10 CFU/0.01 mL), which may be due to the intermittent pattern and low shedding of *S. aureus* in the milk of infected animals [31]. The shedding pattern varies significantly between animals and mammary quarters and is influenced by the bacterial strain, type of infection (natural or experimental), and other factors

[34, 35]. However, the shedding pattern does not affect the bacteria detection in milk samples for microbiological cultures conducted with 0.1 mL of inoculum.

For a reliable microbiological diagnosis, several parameters should be taken into account, such as asepsis during milk collection, the temperature that the samples are maintained during transport to the lab, and the time of storage and processing of the milk [36]. According to the European Directive CD92/46in 1992 [37] the milk has to be maintained at 8 °C immediately after milking or at least 6 °C if the milking was performed the previous day. The National Mastitis Council (NMC) [38] also recommends teat disinfection and the disposal of several streams of milk before the final milking.

Several protocols suggest the use of various culture media to identify mastitis pathogens [39, 40]. In the case of mastitis caused by *S. aureus*, differential and/or selective media such as Blood Agar (BA), Mannitol Salt Agar (MSA), Baird-Parker (BP), and Vogel Johnson (VJ) (or variations of these) are used during the initial steps of identification [40]. In addition, several serological and biochemical tests such as the coagulase test and/or acetoin production are needed to identify bacteria at the species level [41].

Routinely, BA is initially used for bacterial culture because it provides an overview of the bacteria present in the sample [40, 42]. BA has an important advantage, especially when employed in the diagnosis of infections caused by *Staphylococcus* because BA allows the evaluation of the hemolysis patterns of the isolates [43], a criterion to identify the species [44]. MSA, VJ, and DNAse Test Agar are also used for the isolation and differentiation of staphylococci [33, 43, 45]; however, the identification of *Staphylococcus aureus* is not conclusive.

Other approaches describe the use of selective media with chromogenic substrates such as CHROMagar Staph. Aureus for the diagnosis of *S. aureus*, and a specificity and sensitivity greater than 95% and 98%, respectively are reported [42, 46, 47]. A comparative study between specific media for the identification of *S. aureus* in mastitic milk samples showed 100% specificity and sensitivity when blood agar (BA) followed by CHROM medium was used [40]. The diagnosis was considered definitive only if the bacteria were Gram positive and catalase positive and had double incomplete hemolytic activity.

Petrifilm plates are "ready for use" culture media that enable the rapid isolation of bacteria present in milk. The Petrifilm ™ Staph Express Count Plate contains a selective chromogenic medium for the differential identification of *Staphylococcus* sp. The confirmation of *S. aureus* needs a disc embedded with deoxyribonuclease and a dye that produces a pink zone around the colonies. According to manufacturers, this method is simple and takes 24 h for a rapid diagnosis. The Petrifilm concept was tested as a diagnostic method for mastitis caused by *S. aureus* [48], and high sensitivity (87.5%) and specificity (98.5%) were reported; however, the interpretation of the test varied between persons. Recent approaches have reported that the Petrifilm™ method does not provide information at the species level and may be simply used to identify Gram-positive and Gram-negative bacteria and the absence of growth in milk samples. Therefore, the method of microbial culture is required for the accurate identification of the infectious pathogen [49].

Immunodiagnosis and veterinary pathogens

The development of rapid, sensitive, and specific assays for the detection

of disease-causing organisms has become a priority among the initiatives for animal health. In recent years, many researchers have demonstrated the potential of immunoassays in the rapid diagnosis of infectious diseases, particularly diseases caused by bacteria [50, 51, 52, 53]. Immunoassays are based on the specific recognition between antigen and antibody, and the performance of these tests is primarily evaluated by the following two parameters: sensitivity and specificity. Analytical specificity is the ability of the assay to distinguish the antibody from non-target analytes, including matrix components [54]. The limit of detection is a measure of the analytical sensitivity of the assay. Immunology-based tests often appear as a method to improve the diagnosis of veterinary pathogens, allowing the detection of very low antibody concentrations. Agglutination reactions have sensitivities of 0.4 and 0.8 µg antibody/mL for direct and passive reactions, respectively. ELISA and radioimmunoassays detect even lower antibody concentrations, ranging between 0.0008 and 0.008 µg antibody/mL [55].

The following several steps must be considered in the development of an immunoassay for reliable diagnosis: target selection (preference should be given to very immunogenic and highly specific targets), the nature of the matrix (blood, urine, milk, and others), and the method of detection (depends on the choice for point-of-use or instrument-compatible assays). When using an antigen capture design, the next step will be to produce an antibody, and a decision regarding monoclonal or polyclonal antibodies has to be made [56].

Mycobacterium tuberculosis and *Brucella abortus* cause, respectively, bovine tuberculosis (TB) and brucellosis, two important zoonoses distributed worldwide. The economical importance attributed to these diseases is the direct

losses resulting from the death of animals, decreasing weight gain and milk production, early disposal and the condemnation of carcasses at slaughter [57]. Several immunoassays are recommended by the World Organization for Animal Health for the diagnosis of these pathogens [58, 59].

In cattle, clinical evidence of tuberculosis is usually lacking until very extensive lesions have developed [58]. The control of the disease in a number of countries is based on the implementation of a "test-and-slaughter" control program using the tuberculin skin test, which measures delayed hypersensitivity responses to a purified protein derivative (PPD) prepared from *Mycobacterium bovis* [60]. Different cocktails of proteins have now been tested to develop a more specific skin test for bovine TB, and sensitivities as high as 93% have been achieved [61, 62, 63].

Several animals do not respond to the skin test; for these cases, serological assays have emerged as a method to diagnose *M. bovis* infections. ELISA appears to be the most suitable of the antibody-detection tests and can be used as a complement, rather than an alternative, to tests based on cellular immunity [58]. Recently, the performance of a commercial kit based on ELISA and developed by IDEXX was tested on milk samples [64]. The study showed that milk samples and serum samples can be used for the screening of individual cows for *M. bovis* infection, although milk pools from 10 to 20 animals may result in a reduction in sensitivities by approximately 50%. Other rapid tests for the diagnosis of bovine TB that relies on the antibody-antigen interaction is the Anigen Rapid Bovine TB Ab Test Kit (Bionote Inc., Hwaseong-si, Gyeonggi-do, Korea), which is a rapid strip test with 83.6% sensitivity and 83% specificity [61].

Suitable immunoassays for screening herds and individual animals for the presence of *Brucella abortus* are recommended, such as the Rose Bengal test, the buffered plate agglutination test, the complement fixation test, ELISA or the fluorescence polarization assay [59]. Abernethy et al [65] conducted a field study to evaluate six serological tests for the diagnosis of bovine brucellosis and observed that no single test identified all infected cattle, and bacteriological cultures were still needed to define several cases.

Several kits are available that offer high specificity and sensitivity for detecting brucellosis in blood samples. The SVANOVIR® Brucella-Ab I-ELISA detects *Brucella*-specific antibodies in bovine serum/plasma or milk samples with high specificities (> 99%), according to the manufacturers. However, several companies suggest that other methods such as ELISA should be applied to guarantee higher accuracy, and the same recommendation is found in the OIE Manual [59, 59].

Despite significant advances in the immunodiagnosis of bovine TB and brucellosis, the confirmatory diagnosis still relies on culture-based methods; although these methods are not very rapid and efficient, they remain the gold standard for the routine confirmation of infection.

Approaches for the immunodiagnosis of staphylococcal infections

The major challenges involved in the immunodiagnosis of *S. aureus* remain the identification of the best antigens and the reduction in the costs to make the tests affordable to producers. Studies have identified potential serological markers for the diagnosis of staphylococcal mastitis [66, 67, 68, 69] although they still need to be validated. In recent years, several immunoassays have been patented

(Table 1). Although these assays showed good in-house performance, only field trials will determine their market potential.

Protein-A stands out as the most widely used biomarker in patents targeting *S. aureus* immunodiagnosis (Table 1). This protein, encoded by the *spa* gene, is anchored on the bacterial surface and has a fundamental role in the evasion of the immune system [70]. Studies have shown variations in the prevalence and expression of the *spa* gene in different bovine strains, the challenges to test sensitivity and justifications to search for new targets [71, 72, 73, 74].

The thermonuclease (Nuc) is another important virulence factor of *S. aureus* that plays an important role in the degradation of nucleic acids and microbial defense [75]. Thermonuclease is present in all strains, which is the reason this factor is used in the molecular diagnosis of the pathogen. According to Sasaki et al. [76] the *nuc* gene discriminates *S. aureus* from other coagulase-positive staphylococci with total specificity.

Table 1. Relevant immunoassays developed and patented for the diagnosis of *Staphylococcus aureus*.

Immunoassay	Biomarker	Performance[1]	Sample	Ref.
ELISA	Exoproteins (18-26 kDa)	n.d	Milk	**81
Immuno magnetic ELISA	Whole cell	10^4-10^5 CFU/mL	Bacterial culture and milk	84
Immuno magnetic -ELISA	Thermonuclease	89%/70%	Milk	80
Enzymatic (catalase) immunosensor	Protein A	>10^4 CFU/mL	Bacterial culture, beef and milk*	97
Enzymatic (alkaline phosphatase) immunosensor	Protein A	> 10^4 CFU/mL	Bacterial culture, beef, cheese and milk*	98
Enzymatic (alkaline phosphatase) immunosensor	Protein A	> 10^3 CFU/ mL	Bacterial culture, beef, cheese and milk*	99
Lateral flow immuno-assay	Several	n.d	Milk*	**94
Immuno agglutination	Fibrinogen binding proteins and CP type 5/8.	100%	Bacterial culture and biological fluid	**87
Biosensor	Whole cell	10^2 CFU/mL	Bacterial culture and biological fluid	**103
Lateral flow strip	Protein A	100/93-100%,	Food homogenate*	95
Immunosensor (tyrosinase–modified)	Protein A	2.3×10^3 CFU/mL	Bacterial culture and milk*	100
ELISA and immuno-chromatography	IsaA	n.d	n.d	**104
Immunological test strips	Protein A	n.d	n.d	**105
Lateral flow strip	Leucocidin	79%/100%	Sample from human infections	96
Lateral flow immune assay	Whole cell	n.d	n.d	**106
immunofluorescent probe	Protein A	10^3 CFU/mL	Bacterial culture	**86
Lateral flow immune assay	PBP2a	n.d	n.d	**107
Sandwich ELISA	Whole cell	10^5 CFU/mL	Milk*	**85

Immunocapture PCR	Thermonuclease	10^3 CFU/mL	Milk, yogurt and juice	**108
Superparamagnetic enrichment and immuno-chromatographic test strip	Whole cell	10^4 CFU/mL	Bacterial culture	**109
Immunomagnetic separation	Protein A	10^2 CFU/mL	Bacterial culture	**110
Magnetoimmunosensor	Protein A	1 CFU/mL	Milk*	111
Immuno-chromatography test paper	Protein A	6 x 10^5 ufc/mL	Frozen food*	**112
Microarray immunoassay	n.d	n.d	Bacterial culture	**113

* Artificially contaminated.

** Patented test

1 – Performance, reported as detection limit, sensitivity/specificity

n.d- not described.

Nevertheless, ELISA assays using Nuc showed low specificity (70%), which is most likely due to the use of the entire protein in the immunoassay and its cross-reaction with other pathogens [77]. A PCR immunocapture assay using the *nuc* gene has the advantage of exploring only the gene region that is specific to *S. aureus*, making the test highly specific [76].

A. Enzyme-linked Immunosorbent Assay (ELISA)

ELISA is used to diagnose numerous human and animal diseases; therefore, this assay has been shown special interest by the market. ELISA has a low cost ($ 4-6/sample), and the results are obtained on the same day; however, the tests have to be performed in diagnostic laboratories. Different types of ELISAs were developed for the detection of *S. aureus* or inflammation markers in milk samples with suspected mastitis [78, 79, 80].

One of the earliest patented assays detected specific antibodies for *S. aureus* proteins with molecular weights ranging between 18 and 26 kDa [81]. This test was launched in the market in the 1990s (Prostaph 1©) as an alternative to the microbiological culture-based method but had a variable performance when compared to the standard microbiological methods [78, 82, 83]. According to Watts and colleagues [82], the test was shown to be more effective in detecting healthy than infected animals.

Potential sources of the disagreement between ELISA and culture-based tests may be due to variations in antibody production in response to infection and intermittent shedding of *S. aureus* in milk. Fox and Adams [28] evaluated the dynamics of antibody production in animals experimentally infected with *S. aureus* and showed that animals with incipient infections were misclassified as

false negative by ELISA. Additionally, healthy animals or other *Staphylococcus* types were diagnosed as false positives. These misclassifications may have precluded the use of this technique as a method of diagnosis although other successful cases have been described.

Interestingly, 100% sensitivity was achieved using monoclonal antibodies produced against *S. aureus* extracts in ELISA using monodisperse magnetic particles as the solid phase (MPP) [84]. When anti-thermonuclease antibodies (Nuc) produced against the entire protein were used to coat the magnetic particles, the test performance was significantly reduced, showing the importance of the choice of antigen in the development of these assays [80]. A sandwich ELISA was recently patented [85] to detect *S. aureus* and may be useful for the diagnosis of staphylococcal mastitis (Table 1) because this test uses two antibodies with different specificities, making the test more sensitive but also more costly.

B. Immunofluorescent probe

In an attempt to reduce the detection limit of bacteria in food samples, immunofluorescent probes that specifically bind to protein A have been developed [86]. This technology uses an antibody connected to a fluorescent particle to capture free *S. aureus* in the sample. The *S. aureus*-probe complex is collected and analyzed by fluorescence intensity. The probes detected up to 10^3 CFU/mL; however, the test is not appropriate for a diagnosis in the field due to the need for very specific equipment, trained personnel and the high cost of the test.

C. Immunoagglutination

Immunoagglutination tests employ latex or polystyrene particles coated with fibrinogen and/or antibodies. When these molecules contact the bacteria present in the sample, they form complexes that precipitate in the test tube. Several agglutination tests developed for the detection of *S. aureus* have been validated for human diagnosis [87, 88] and are now being tested for the detection of *S. aureus* in milk samples. The performance of six commercially available tests were evaluated: Staphylase® (Test-Oxoid), Masta-Staph® (Mast Diagnostics), Staphyloslide Latex Test® (Becton Dickinson), Staphytect-Plus® (Oxoid), Dry Spot Staphytect Plus® (Oxoid) and Slidex Staph Plus® (Merieux) [88]. The best result was achieved using Masta-Staph®. For example, 86% sensitivity and 90% specificity were achieved using this test. However, these values are still lower than reported for human strains [88, 89], most likely due to variations in the expression of the virulence factors that differ among bovine and human strains [74, 90, 91, 92]. The use of antibodies against specific antigens found in bovine isolates may improve test performance. The immunoagglutination tests are rapid (10-20 min), cheap (around $1/sample) and user-friendly; therefore, they represent a good alternative for staphylococcal mastitis diagnosis on-site.

D. Lateral flow immunoassay (LFA)

LFA, also known as ILFST (*immunochromatographic lateral flow strip test*), is a well-established technique that is easy to interpret, rapid, does not require refrigeration, and can be accomplished in the field for the diagnosis of bovine mastitis. This method is based on the principle of immunochromatography, in which a sample spreads by capillary action on a strip and reacts with antibodies or specific antigens. The test was initially developed as a pregnancy test [93] and

has become a popular platform for the development of diagnostic tests. Several commercially available tests based on LFA cost between $ 0.8-2/sample, and the result is ready within 20 min.

LFA-based tests for *S. aureus* detection in milk samples have been patented since 2002 [94]. The patents that use this strategy do not report the sensitivity and specificity of the tests, which is key information for assessing performance; however, the potential of this methodology cannot be dismissed. LFA is the basis of Singlepath and Duopath (Merck Millipore), Anthrax BioThreat Alert (BTA) test strips, Bot-Tox-BTA (Tetracore), and Lepto lateral flow kits (KIT), which are marketed for the diagnosis of *Escherichia coli*, *Salmonella*, *Campylobacter*, *Clostridium botulinum*, *Bacillus anthracis*, and *Leptospira.*

A lateral flow strip to detect *S. aureus* in samples of raw and processed foods was developed using purified polyclonal anti-protein A [95]. LFA was initially tested on 28 *S. aureus* and 23 non-*S. aureus* strains grown in culture medium. The sensitivity and specificity were determined as 100% and 93-100%, respectively. *S. aureus* leukocidin producers have also been identified by this technique [96]. Monoclonal anti-lukS-PV immobilized on a nitrocellulose membrane was used to test 185 body fluid samples. Although leukocidin non-producers have not been identified, the test was less sensitive (79.1%) to PVL-positive samples.

E. PCR immunoassay

Two PCR immunoassays have been developed for *S. aureus* detection in food samples (Table 1). In the PCR immunocapture, bacteria present in the samples are captured by polyclonal antibodies prior to the amplification of a region of the *nuc* gene by polymerase chain reaction (PCR). The microarray immunoassay identified the following six different mastitis-related pathogens: *S. aureus*, *Corynebacterium bovis*, *Mycobacterium bovis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*. The genes of each species are amplified by tagged-primers, and the amplicons are recognized by antibodies immobilized on a nitrocellulose membrane. These strategies are highly specific, and the results can be obtained in 3 h. However, similar to ELISA and the microbiological methods, the samples need to be processed in specialized laboratories.

F. Biosensor

Several biosensors have been developed for the immune diagnosis of *S. aureus* in food samples including milk. This technology uses antibodies labeled with molecules that produce a signal that is measured by a detector [97, 98, 99, 100].

Mirhabibollahi and coworkers [97] were the first to describe an enzyme-linked immunosensor to detect and quantify *S. aureus* by a sandwich ELISA using anti-protein A antibodies labeled with catalase. The detection and quantification was performed by measuring the O_2 levels. Although sensitive, electrical detection for each sample is slow and technically difficult. In addition, the assay utilizes antibody-coated membranes in glass containers hampering the automation of the process. These types of assays decrease the time for results and favor the

automation process [98, 99, 100], but the associated cost and the need for sophisticated equipment remain the primary bottlenecks of this strategy.

Conclusions and Perspectives

Staphylococcus aureus is a contagious pathogen that cannot be completely eliminated from dairy herds, but its incidence can be reduced to an acceptable level. The success in controlling staphylococcal mastitis depends on an accurate diagnosis, proper treatment and the implementation of preventive and hygiene practices, ranging from cleaning the milking equipment to discarding animals with chronic manifestation [101].

The rapid and accurate diagnosis is useful to make decisions regarding which treatment and proper management should be adopted. The early detection of mastitis may increase the cure rate by 60% and reduce the time required to recover normal milk production when combined with appropriate antimicrobial therapy [102].

One of the most widely utilized methods for subclinical mastitis diagnosis is the California Mastitis Test; however, this method only discriminates sick from healthy animals and is unable to identify the causative agent of infection. Therefore, microbiological culture is still considered the gold standard for diagnosing mastitis pathogens, allowing for a targeted control and treatment decision in addition to presenting high sensitivity and specificity. Another advantage of microbial culture-based methods is the possibility of performing the antibiotic susceptibility of bacteria; however, the major limitations of this method include delays in obtaining results and the final cost of the analysis.

The speed, sensitivity, ease of handling and low cost are desired characteristics for an ideal diagnostic test, which are requirements met by methodologies based on serology. The market contains several commercialized immunoassays for the diagnosis of diseases of veterinary importance. Kits for serodiagnosis of bovine brucellosis and tuberculosis are recommended by the World Organization for Animal Health for treatment choices. Thus, immunodiagnostics create new perspectives for the diagnosis of bovine mastitis as an alternative to microbiological culture. Several immunoassays have been described for the identification of *S. aureus* and may be suitable for the detection of staphylococcal mastitis. Several assay require a laboratory infrastructure and trained personnel; however, other assays may be suitable for the field by using trained staff at the farm, reducing costs and allowing a greater number of analyses. These tests still require validation and several adaptations for the diagnosis of mastitis on the farm; however, their potential cannot be ruled out.

Conflict of interest

The authors declare that they have no conflict of interest.

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CAPÍTULO 2

Avaliação de candidatos antigênicos para diagnóstico da mastite estafilocócica

RESUMO

Staphylococcus aureus é um dos principais patógenos bacterianos envolvidos com infecções intramamárias em bovinos. A cultura bacteriológica é considerada o padrão-ouro para o diagnóstico da mastite estafilocócica, porém limitações inerentes ao método podem retardar as decisões de manejo e comprometer a saúde e bem-estar do rebanho, acarrentando perdas econômicas substanciais. O imunoensaio se destaca como um estratégia viável para o diagnóstico de doenças bacterianas em animais, cuja eficácia está intimamente ligada à escolha do antígeno usado no ensaio. Vários imunoensaios vêm sendo patenteados para a identificação de *S. aureus* de diferentes tipos de amostras porém a grande maioria necessita de validação, comprometendo o seu potencial de mercado. Nesse trabalho, a presença de 16 genes foi avaliada em 195 isolados bovinos de *S. aureus*. A maioria foi detectada em todas a cepas estudadas e apenas *eap*, *isdA* e SACOL1912 apresentaram baixa prevalência. As proteínas Aux, IsaA e Nuc foram expressas em sistema heterólogo e a sua reatividade com anticorpos policlonais presentes no soro de leite mastítico foi avaliada. IsaA e Nuc apresentaram sensibilidade de 83% e especificidade de 62,5% e 75%, respectivamente. No entanto, essas proteínas exibiram atividade cruzada com *Staphylococcus* coagulase-negativos e com *Streptococcus agalactiae*. A proteína Aux, embora seja a mais específica, teve a pior sensibilidade (50%). Conclui-se que IsaA e Nuc se mostraram mais adequadas para o imunodiagnóstico de *S. aureus*, embora sugere-se que sejam expressas apenas partes das proteínas como forma de aumentar a especificidade da reação.

Palavras-chave: *Staphylococcus aureus*, diagnóstico, antígeno

1. INTRODUÇÃO

A mastite bovina é uma inflamação da glândula mamária que afeta produtores de leite em todo mundo. A doença causa uma considerável perda econômica devido a redução do leite produzido e pelos gastos com tratamento, assistência veterinária e descarte precoce de animais doentes (Petrovski et al., 2006; Keefe, 2012). A mastite também tem implicações na saúde pública pela possibilidade de veiculação de micro-organismos, antimicrobianos e toxinas termoestáveis que permanecem estáveis no leite (Peton e Le Loir, 2013)

Mais de 140 tipos diferentes de organismos podem causar a mastite bovina, entretanto, a origem bacteriana é a mais freqüente (Hillerton e Berry, 2005). *Staphylococcus aureus* é um dos principais agentes etiológicos da doença, responsável por quase 30% de todas as infecções intramamárias reportadas (IMI) (Barkema et al., 2006; Bradley et al., 2007; Olde Riekerink et al., 2008). A prevalência de *S. aureus* na mastite clínica é de 3,3% a 40% (Bradley et al., 2007; Olde Riekerink et al., 2008), podendo chegar a 62% na manifestação subclínica (Giannechini et al., 2002).

O diagnóstico precoce da mastite é de extrema importância para o manejo e controle da doença nos rebanhos leiteiros. Atualmente, os testes mais usados para a detecção da mastite são a contagem de células somáticas e o *California Mastitis Test* (CMT). Entretanto, nestes testes o agente etiológico não é identificado o que dificulta a escolha do antimicrobiano a ser aplicado, além do monitoramento e do controle da taxa de infecção no rebanho (Sears e McCarthy, 2003). O diagnóstico microbiológico é o mais utilizado para identificação do patógeno, mesmo com a demora na obtenção dos resultados. Outras formas de diagnóstico podem ser empregadas, embora necessitem de pessoal capacitado o que inviabiliza sua rotina na propriedade rural (Viguier et al., 2009).

Na busca por novas metodologias de diagnóstico se destacam os ensaios rápidos, sensíveis, de fácil execução e interpretação. Essas características podem ser encontradas nos imunoensaios, que dependendo do teste pode ainda possuir boa estabilidade a temperatura ambiente. Porém, um dos maiores desafios no campo do imunodiagnóstico é a busca por antígenos altamente sensíveis e específicos.

Vários grupos têm voltado esforços para a identificação de proteínas que possam ser usadas não somente para o diagnóstico de *S. aureus* de origem bovina, mas também para o desenvolvimento de vacinas (Le Maréchal et al., 2011a; Le Maréchal et al., 2011b; Fabres-Klein et al., 2013). Inúmeras proteínas imunogênicas ou especificamente expressas por *S. aureus* durante a mastite bovina foram indicadas como candidatas para desenvolvimento de vacinas e de testes de diagnóstico, mas trabalhos direcionados para a validação do uso dessas proteínas ainda são poucos (Holtfreter et al., 2010; Allard et al., 2013).

A análise sorológica do proteoma expresso por *S. aureus* durante infecção (Serological proteome analysis - SERPA) tem identificado inúmeras proteínas imunoreativas candidatas (Vytvytska et al., 2002; Tedeschi et al., 2009; Le Maréchal et al., 2011a). A comparação do SERPA (Serological proteome analysis) de *S. aureus* com antisoro de 12 animais com mastite, severa ou subclínica, revelou 74 proteínas reativas para ambos os grupos e 15 diferencialmente reativas entre os grupos. Os fatores de virulência α-hemolisina (Hla) e termonuclease (Nuc) foram identificados tanto em antisoro de animais com mastite subclínica quanto clínica, enquanto o antígeno imunodominante A (IsaA) foi reativo apenas em antisoro de animais sem sinais clínicos. A imunoreatividade de Hla, Nuc e IsaA também já foi descrita em diversos outros trabalhos (Etz et al., 2002; Vytvytska et al., 2002; Clarke et al., 2006; Holtfreter et al., 2009).

Trabalho prévio realizado por nosso grupo, identificou 65 proteínas imunoreativas entre elas proteínas relacionadas ao metabolismo, adesão, parede celular, toxinas, proteínas regulatórias, adesinas, proteínas de parede celular e proteínas hipotéticas (Fabres-Klein et al., 2013). Porém, uma avaliação *in silico* revelou que entre as proteínas identificadas, apenas o fator de agregação A (ClfA), a proteína de ligação ao fibrinogênio (Efb) e uma proteína hipotética (SAB0166) foram encontradas exclusivamente em *S. aureus*.

Dessa forma este estudo teve por objetivo analisar a viabilidade de proteínas previamente identificadas como alvos para o diagnóstico da mastite bovina causada por *S. aureus*. Inicialmente, a presença de 16 genes foi avaliada em 195 isolados de *S. aureus* envolvidos com mastite bovina. Três delas foram selecionadas para expressão em sistema heterólogo e a reatividade à leite mastítico foi testada.

2. OBJETIVOS

Avaliar a reatividade de antígenos alvo para o diagnóstico da mastite bovina.

OBJETIVOS ESPECÍFICOS

- Clonar os genes de interesse em vetor de expressão pDEST17;
- Expressar os antígenos selecionados em sistema heterólogo;
- Purificar as proteínas de interesse em condições desnaturantes;
- Avaliar a reatividade das proteínas purificadas por meio de *western blotting* utilizando soro de leite.

3. MATERIAL E MÉTODOS

3.1. Micro-organismos e condições de cultivo

Um total de 195 isolados clínicos de *Staphylococcus aureus* envolvidos com mastite bovina pertencentes a biblioteca do Canadian Bovine Mastitis Research and Milk Quality Network (CBMRN) foram utilizados para determinação da prevalência dos genes de interesse. O isolado *S. aureus* 3993, proveniente de animal com manifestação de mastite subclínica, foi selecionado para amplificação dos genes alvos. Para osprocedimentos de clonagem e expressão gênica foram utilizadas as cepas hospedeiras *Escherichia coli* DH5α, BL21 (DE3) pRARE2, BL21 (DE3) pLyss e C41 (DE3). As culturas foram crescidas em meio infusão cérebro-coração (BHI, Himedia, Mumbai, India) ou Luria Bertani (LB) por 16 h a 35 °C, com agitação de 225 rpm. As culturas estoques foram mantidas em BHI ou LB acrescidas de 20 % de glicerol a -80 °C.

3.2. Extração de DNA genômico

O DNA genômico total dos isolados foi extraído a partir de cultivo celular crescido em BHI por 16 h sob agitação de 225 rpm a 35 °C. As células foram coletadas por centrifugação e ressuspendidas em TE (10 mM Tris-HCl, 1mM EDTA, pH 8,0) contendo 45 mg/mL de lisozima (SIGMA- L6876, Oakville, ON, Canada) e 200 µg/mL de lisostafina (SIGMA-L7386). Após 1 h de incubação a 37 °C, o DNA foi extraído utilizando GenElute™ Bacterial Genomic DNA Kit (Sigma-NA2110) seguindo as recomendações do fabricante. A qualidade e a concentração do DNA extraído foram averiguadas por espectrofotometria em leitora de microplacas Epoch (BioTek, Winooski, VT, USA). As amostras foram armazenadas a -20 ºC para uso posterior.

3.3. PCR multiplex

A prevalência dos genes foi determinada por PCR *multiplex* para cada um dos grupos descritos na Tabela 1. A reação de amplificação continha 200 ng de DNA genômico, 2,0 µL de tampão de amplificação 10X, 3,0 mM de $MgCl_2$, 0,2 mM de cada desoxirribonucleotídeo (dNTP), 1,25 U de *Taq* DNA Polimerase Thermopol (New England Biolabs-M0267, Whitby, ON, Canadá) e uma mistura

dos pares de *primers* de cada grupo (Tabela 1), totalizando um volume final de 20 µL.

Tabela 1. Lista de *primers* utilizados neste trabalho.

Gene	**Grupo**	**TM (°C)**	**Concentração na reação (µM)**	**Amplicon (pb)**	***Primers* (5'-3')**
aux	G1	57	1,0	634	F-CTTTTAGTTCTGTGTGATGGTATGG R-CAATAGCCGCGAGTATGAAAGT
SACOL2365	G1	57	1,0	569	F-TTTAGCTGCATGTGGTCAAGA R-CAGTAACCATTGCTTGTCCAGT
SACOL2295	G1	57	1,0	445	F-AATCGGCACAGCATTAGTAGGT R-TTACCTGCTTCACTTGCAGAAA
eap	G1	57	1,0	330	F-CGCTACTGTGGCAGGTAATGA R-TCAAACAAGTTTGCTGTGTAGC
SAB0166	G1	57	1,0	214	F-CAATTCAGACCGAAAAGAAAGC R-GATGATATTGGAACCCCACCT
hla	G2	57	1,0	831	F-TCCAGTGCAATTGGTAGTCATC R-CAACAACACTATTGCTAGGTTCCA
LP309	G2	57	1,0	690	F-AAAGTACGATTGTCGCACCACT R-TAGGGATAGTTTGTGGTACAGTTGG
SACOL1912	G2	57	1,0	484	F-GATACAGATCAAGCGCCAGTT R-AAGCAAAGCGTGACGTAAAGT
fib	G2	57	1,0	383	F-CAGATGCGAGCGAAGGATAC R-TCGCTCTTGTAAGACCATTTTC
SACOL*2325*	G3	57	1,0	741	F-GCCAGCCATCTTTAACTGCT R-GTTCGGAGTGTTTGCGTTTT
*isa*A	G3	57	1,0	619	F-GTTGGTCAACAGTGTTTGTTGG R-TCATTAGCAGTGGCATTAGGTG
nuc	G3	57	1,0	430	F-AAATCGCTTGCTATGATTGTGG R-TCTCTCTAGCAAGTCCCTTTTCC
SACOL2385	G3	57	1,0	168	F-TCAAGCTACTAGAAGCGCAAAA R-CTTGGTTTGATTTTAGGCAAGG
SACOL2171	G4	58	1,0	830	F-CTGGAGAACACGCCTTTGAT R-CGCCTAAATGGTTTTGAACG
*isd*A	G4	58	1,0	350	F-CGTAGCTGGAGTAGCTTCCTTC R-ACCAAACTCTCGTCCAATTGAT
lip	G4	58	1,0	280	F-CGTTGCTTCACCAGTGTATGTT R-GCAGCTGAGAAGCAAGTGAATA
aux (clonagem)	-	50	1,0	783	F-AAAAGCAGGCTTCACAATGCTAGAGGCACAATTT R-AGAAAGCTGGGTGCTTTCATACTTTATCTCCTTC
fib (clonagem)	-	55	1,0	498	F-AAAAAGCAGGCTTCATGAAAAATAAATTGATAGC R-AGAAAGCTGGGTCTTATTTTACTAATCCTTGTT
hla (clonagem)	-	50	1,0	999	F-AAAAAGCAGGCTTCACAATGAAAACACGTATAGTC R-AGAAAGCTGGGTGCTTTTAATTTGTCATTTCTTC
*isa*A (clonagem)	-	50	1,0	741	F-AAAAGCAGGCTTCACAATGAAAAAGACAATTATG R-AGAAAGCTGGGTGCTTTTAGAATCCCCAAGCGCT
nuc (clonagem)	-	55	1,0	687	F-AAAAAGCAGGCTTCATGAAGTCAAATAAATCGCT R-AGAAAGCTGGGTCTTATTTACTCCAATATTTAA
attB 1	-	52	1,0	-	5'GGG GAC AAG TTT GTA CAA AAA AGC AG 3'
attB 2	-	52	1,0	-	5'GGG GAC CAC TTT GTA CAA GAA AGC TG 3'

As condições de amplificação consistiram de uma etapa de desnaturação a 94 °C por 5 min, seguidos por 30 ciclos de 30 seg a 94 °C, 40 seg a 57 °C (ou 58 °C para o grupo 4) e 90 seg a 68 °C, com uma extensão final a 68 °C por 10 min. Os amplicons foram analisados por eletroforese em gel de agarose 1,5% contendo 1µL de Gel Red (Biotium-41003, Hayward, CA, USA). As imagens foram registradas em sistema de fotodocumentação Gel Doc™ XR+ (BIO-RAD, Hercules, CA, USA).

3.4. Preparo de *Escherichia coli* competentes

Para o preparo de *Escherichia coli* DH5α competente, uma colônia foi inoculada em 10 mL de meio LB e incubada *overnight* a 37 ºC. Foram transferidos 2 mL do pré-inóculo para 250 mL de meio SOB fresco seguido por incubação a 18 ºC com agitação de 180 rpm. Quando a cultura atingiu densidade ótica ($D.O._{600nm}$) entre 0,5-0,7, o material foi transferido para tubos Falcon e resfriado em gelo durante 10 min. A cultura foi centrifugada por 15 min a 3300 x g a 4 ºC. O sobrenadante foi descartado e as células foram delicadamente ressuspendidas em 80 mL de solução TB (10mM de Pipes, 55mM de $MnCl_2.4H_2O$, 15mM de $CaCl_2.2H_2O$ e 250mM de KCl, pH 7,0) estéril e gelada e incubadas em gelo por 10 min. As células foram centrifugadas por 15 min a 3300 x g a 4 ºC. O pellet foi ressupendido em 8mL de solução TB gelada acrescida de 560 µL de DMSO e novamente incubado em gelo por 10 min. Alíquotas de 40 µL foram congeladas com nitrogênio líquido e armazenadas a -80 ºC para uso posterior.

Para o preparo de células de *E. coli* competentes utilizadas para expressão, um pré-inóculo foi feito em meio LB e incubado overnight a 37 °C, sob agitação de 180 rpm. A cultura foi, então, diluída 1:10 e incubada novamente a 37 °C, 180 rpm por 3 h. Centrifugou-se 1,2 mL desse inóculo por 2 min, 3500 x g, a 4°C e o pellet foi cuidadosamente ressuspendido em 1 mL de cloreto de cálcio 0,1 M gelado. Após outra centrifugação, o sobrenadante foi novamente descartado e o pellet foi ressuspendido em 150 µL de $CaCl_2$ 0,1 M gelado.

3.5. Clonagem dos genes de interesse em vetor de expressão

Os genes selecionados para clonagem (Tabela 1) foram amplificados pela reação em cadeia da polimerase (PCR) a partir do DNA total de *S. aureus* 3993

com *primers* para clonagem sítio-específica em sistema Gateway (Invitrogen, Carlsbad, CA, USA). A reação consistiu de 300 ng/µL do DNA molde, 2,5 µL de tampão de amplificação 10X, 1,5 mM de $MgCl_2$, 0,2 mM de cada dNTP, 1U de *AccuTaq* DNA Polimerase (Sigma-D4184) e o 1,0 µM de cada *primer*, totalizando um volume final de 25 µL. Os fragmentos de DNA amplificados foram analisados em gel de agarose 1% (p/v) em TAE (40 mM de Tris-acetato e 1 mM de EDTA). Uma segunda reação de amplificação foi realizada para completar os sítios de recombinação ATTB1 e ATTB2. Essa reação consistiu de 3,0 µL da primeira PCR, 5,0 µL de tampão de amplificação 10X, 1,5 mM de $MgCl_2$, 0,2 mM de cada dNTP, 2,5 U de *AccuTaq* DNA Polimerase (Sigma) e 1,0 µM de cada *primer*, com volume final de 50 µL. Os amplicons foram purificados com 10% de polietilenoglicol (PEG), visualizados em gel de agarose 1% em TAE e quantificados por $OD_{260\,nm}$ em espectrofômetro SP 220 (Biospectro, Curitiba, PR, Brasil). A inserção do gene no vetor de entrada pDONR201 (Invitrogen) foi feita em reação de recombinação contendo 150 ng do fragmento de DNA purificado, 150 ng de pDONR201, 2 µL e BP clonase II (Invitrogen) e o volume completado para 10 µL com TE (10 mM de Tris-HCl, 1mM de EDTA e pH 8,0) por 16 h a 25ºC. Essa reação foi utilizada para transformação de *Escherichia coli* DH5α ultracompetentes, e os transformantes selecionados em meio LB contendo canamicina (50 µg/mL), a 37 ºC. Posteriormente, o DNA plasmidial dos transformantes foi utilizado para transferir o gene alvo do vetor de entrada para o vetor de expressão pDEST17 (Invitrogen, Carlsbad, CA, USA) em uma segunda reação de recombinação utilizando a LR clonase II, conforme descrito anteriormente. *E. coli* DH5α foi transformada com a segunda reação de recombinação e cultivada em LB contendo ampicilina (100 µg/mL). Os mapas físicos dos vetores usados nesse trabalho podem ser encontrados na Figura 1A (apêndice).

3.6. Sequenciamento do DNA plasmidial

O DNA plasmidial dos diferentes clones foi sequenciado usando o método de di-desoxi de terminação de cadeia em sequenciador ABI3730XL (Applied Biosystems) utilizando *primers* T7 *foward* e *reverse.* As sequências obtidas foram analisadas utilizando os programas de alinhamento BLAST (*Basic Local*

Alignment Search Tool) disponíveis na página do National Center for Biotechnology Information (NCBI-www blast.ncbi.nlm.nih.gov/Blast.cgi).

3.7. Expressão e purificação das proteínas recombinantes em condições desnaturantes

A expressão das proteínas foi induzida pela adição de 0,4 mM de IPTG (isopropil-β-D-1-tiogalactopiranosídeo) à cultura bacteriana de DO_{600nm} 0,6-1,0 em meio LB. Após 12 h de indução a 20 °C e 180 rpm, as células foram coletadas por meio de centrifugação 4000 x g por 10 min. O pellet foi ressuspendido em tampão de lise desnaturante (100 mM de NaH_2PO_4, 10 mM de Tris e 10 M de Uréia), contendo 1 mg/mL de lisozima, 0,8 mM de PMSF (Sigma, Oakville, Canada) e 0,1% v/v de Triton X-100 e incubado em gelo por 30 min. As células foram rompidas por meio de sonicação até que a amostra ficasse homogênea e em seguida centrifugadas por 30 min a 14000 x g a 4 °C. O sobrenadante foi coletado em um novo tubo e a ele foram adicionados a 500 µL de resina Ni-NTA agarose (Quiagen, Hilden, Germany) previamente equilibrada em tampão de lise. A interação da proteína recombinante com a resina ocorreu por 12 h a 4 °C sob leve agitação, quando então a resina foi coletada por cetrifugação por 2 min a 1600 x g. A resina foi lavada 5 vezes com o tampão de lavagem desnaturante (100 mM de NaH_2PO_4, 10 mM de Tris, 10 M de Uréia e 20 mM de Imidazol). Após as lavagens, adicionaram-se à resina 500 µL de tampão de eluição (100 mM de NaH_2PO_4, 10 mM de Tris, 10 M de Uréia e 250 mM de Imidazol) e a mistura foi incubada sob agitação por 16 h a 4 °C e cetrifugada por 2 min a 1600 x g. Este passo foi repetido mais duas vezes porém com um tempo de eluição de apenas 1 h. A análise da purificação foi realizada por eletroforese em gel de poliacrilamida-dodecil sulfato de sódio (SDS-PAGE) e por *Western Blotting* utilizando anticorpo específico para a cauda de histidina (GE-27-4710-10-Buckinghamshire, Inglaterra).

3.8. Avaliação da reatividade das proteínas alvo com leite

As proteínas recombinantes purificadas na etapa anterior foram separadas por eletroforese em gel de poliacrilamida 12 % contendo dodecil sulfato de sódio (SDS) e eletrotransferidas para membrana de nitrocelulose utilizando o aparelho *mini Trans Blot Cell* (BIO-RAD, Hercules, CA, USA). Após a transferência a

membrana foi bloqueada em solução de bloqueio (TBS-T contendo 3% de soro albumina bovina) por 1 h sob leve agitação. A membrana foi lavada três vezes com TBS-T 1x e incubada por 2 h com anticorpos policlonais presentes no soro de leite, o qual foi obtido a partir da centrifugação do leite mastítico por 30 min a 13000 x g e 4°C. A membrana foi novamente lavada com TBS-T e incubada com anticorpo secundário anti-IgG bovino conjugado com peroxidase (Sigma-SAB3700020 diluição 1:5000) por 1 h. Após a lavagem da membrana, a revelação foi feita durante 15 min utilizando 10 mL de 50 mM de Tris-HCl, pH 7,6, 1 mg/mL de DAB e 3% de H_2O_2. As 20 amostras de leite provenientes do Programa de desenvolvimento da pecuária leiteira da região de Viçosa (PDPL - UFV) usadas nesse experimento foram coletadas de animais com mastite subclínica e foram selecionadas de acordo com o patógeno identificado por cultivo microbiológico e testes bioquímicos rotineiros.

3.9. Cálculo da sensibilidade e especifícidade

A sensibilidade e a especificidade de cada antígeno foram calculadas considerando o o teste microbiológico das amostras de leite como teste padrão. As fórmulas utilizadas estão descritas abaixo:

$$\text{Sensibilidade} = a / (a + c)$$

$$\text{Especificidade} = b / (b + d)$$

onde *a* é o número de amostras verdadeiramente positivas, *b* é o número de amostras verdadeiramente negativas, *c* é o número de amostras falsos negativas e *d* é o número de amostras falsos positivas.

4. RESULTADOS

O primeiro passo para a seleção de um antígeno ideal para diagnóstico mastite estafilocóccica é a determinação da prevalência do gene entre isolados de *S. aureus* bovinos. Dezesseis genes que codificam proteínas de *S. aureus* descritas como imunogênicas, específicas ou que foram expressas durante uma infecção intramamária foram selecionados para estudo da prevalência por meio de PCR multiplex (Etz et al., 2002; Vytvytska et al., 2002; Clarke et al., 2006; Holtfreter et al., 2009; Holtfreter et al., 2010; Le Maréchal et al., 2011; Allard et al., 2013; Fabres-Klein et al., 2013).

Os resultados de prevalência obtidos estão descritos na Tabela 2. Dos 16 genes estudados, onze genes estavam presentes nos 195 isolados analisados. Os genes *hla* e SACOL2325, apesar de não estarem presentes em 100% dos isolados, também apresentaram alta prevalência (Figuras 2A-21A). As menores prevalências foram observadas para os genes *eap*, *isd*A e SACOL1912 com 5,1%, 48,7% e 47,7% respectivamente.

Tabela 2. Prevalência de genes encontrados em isolados clínicos de *Staphylococcus aureus* de origem bovina.

Gene	kDa*	Localização**	Produto gênico	Prevalência
aux	28,0	SC	Proteína relacionada com resistência à meticilina	100%
eap	15,8	PC	Proteína de aderência extracelular	5,1%
fib	18,8	EC	Proteína de ligação à fibrinogênio	100%
hla	35,9	EC	Alfa-hemolisina	94,4%
*Isa*A	24,2	EC	Antígeno imunodominante A	100%
*isd*A	38,7	SC	Proteína de parede regulada por ferro	48,7%
lip	76,5	EC	Lipase	100%
LP309	34,7	SC	Transportador ABC regulado por ferro	100%
nuc	20,3	EC	Termonuclease	100%
SAB0166	12,3	D	Proteína hipotética	100%
SACOL2325	35,0	C	Regulador transcricional LysR	99,5%
SACOL2295	17,4	EC	Proteína de biossíntese de estafiloxantina putativa	100%
SACOL2365	23,3	D	Proteína hipotética	100%
SACOL2385	16,3	PC	Proteína da família Hsp20	100%
SACOL1912	22,3	C	Proteína hipotética	47,7%
SACOL2171	76,0	C	Proteína de biossíntese de aerobactina	100%

* UniProtKB/Swiss-Prot

** Localização subcelular do antígeno prevista pelo programa PSORTb v3.0.2. SC- superfície celular; PC- parede celular; EC- extracelular; C-citoplasmática; D-desconhecida.

Para auxiliar na seleção dos antígenos, a localização subcelular das proteínas selecionadas também foi predita (Tabela 2). A maioria das proteínas a predição indicou ser extracelular ou fazer parte da parede ou membrana celular de *S. aureus.* Com base na prevalência gênica e na localização subcelular, foram selecionados os genes *aux*, *isa*A, *fib*, *hla*, *lip* e SAB0166 para clonagem em vetor de expressão pDEST17. A confirmação da clonagem foi feita por sequenciamento dos plasmídios de expressão contendo o gene de interesse.

Os vetores de expressão contendo os genes de interesse foi utilizado para transformar as células hospedeiras BL21 (DE3) pRARE2, BL21 (DE3) pLyss e C41 (DE3) e os testes de expressão foram conduzidos conforme descrito na Tabela 3.

Tabela 3. Condições usadas nos testes de expressão das proteínas recombinantes.

Cepa	Temperatura de Indução	Coleta de amostras
BL21 (DE3) pRARE2	37°C	0, 2, 4 e 6 horas após a indução
BL21 (DE3) pLyss	28°C	0, 2, 4 e 6 horas após a indução
C41 (DE3)	20°C	0, 12, 24 e 36 horas após a indução

A expressão de Aux (28 kDa) e Hla (35,9 kDa) foram testadas em todas as condições (Figuras 1 e 2), porém apenas a cepa de *E. coli* C41 foi eficiente na expressão das proteínas recombinantes (Figuras 1C e 2C). A confirmação da expressão foi feita por *Western blotting* utilizando o anticorpo primário anti-his (Figuras 1D e 2D). Os extratos celulares das células expressando Aux e Hla foram utilizados para a purificação das proteínas recombinantes por meio de cromatografia de afinidade em resina contendo níquel. Porém, apenas Aux foi purificada (Figuras 2E e 2F).

A expressão de Fib também foi testada em todas as condições descritas na Tabela 3, mas nas temperaturas de 37 ° e 28 °C a indução da expressão foi tóxica para as células hospedeiras. Dessa forma apenas *E. coli* C41 a 20 °C foi capaz de expressar a proteína recombinante de 18,8 kDa (Figura 3A), sendo confirmadas por *Western blotting* (Figura 3B). Duas tentativas de purificação das proteínas Hla e Fib foram realizadas sem sucesso, o que mostra que ajustes

deverão ser realizados no protocolo utilizado para uma purificação eficiente da proteína recombinante.

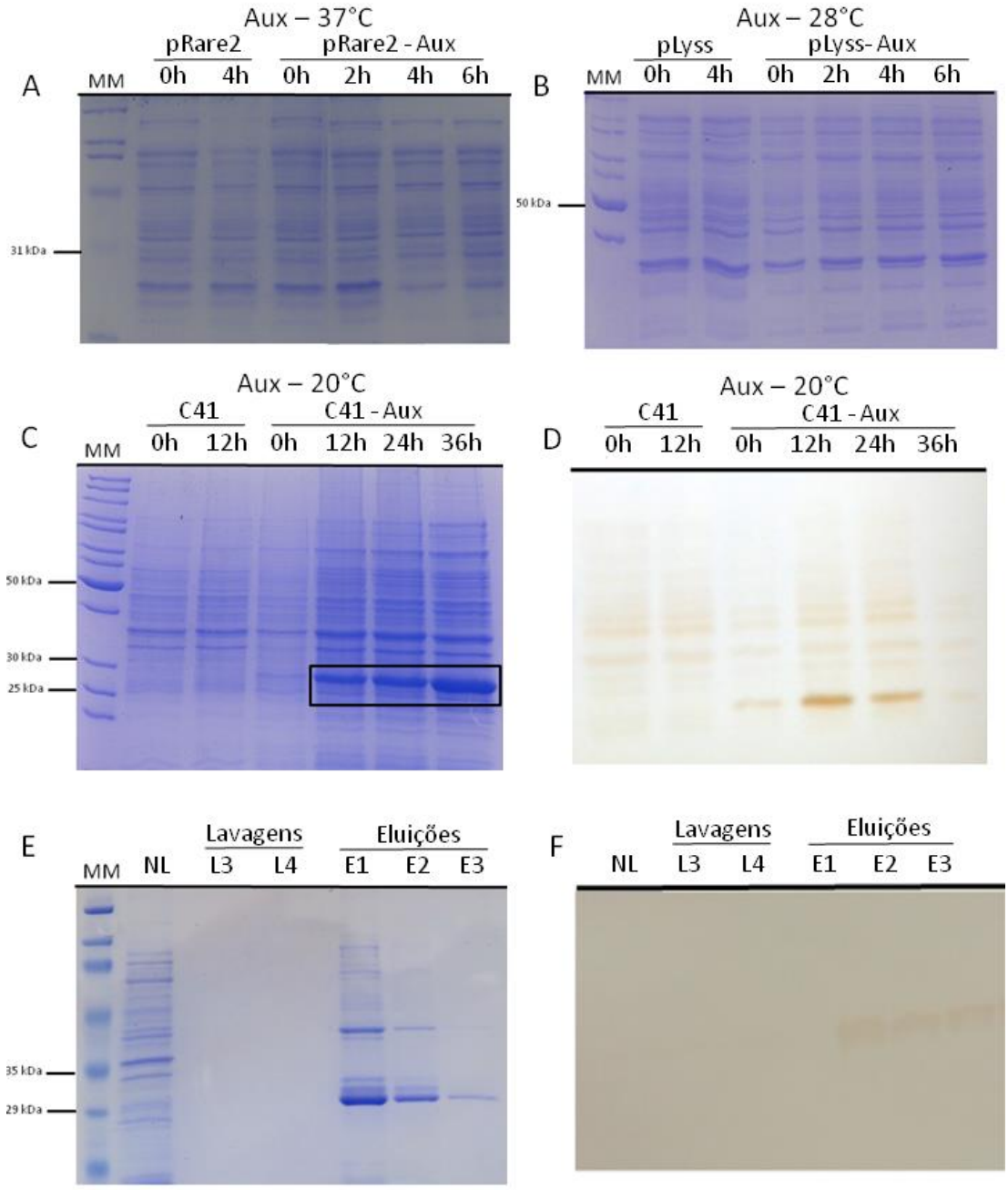

Figura 1. Expressão da proteína recombinante Aux. A expressão da proteína recombinante foi avaliada por eletroforese em gel SDS-PAGE 12% (A, B, C, E) e *Western Blotting* (D e F). Expressão de Aux nas células hospedeiras *Escherichia coli* BL21 pRARE2 a 37 °C (A), *E. coli* BL21 pLyss a 28 °C (B) e *E coli* C41 (C). As bandas induzidas estão indicadas pelo retângulo. A purificação da proteina recombinante foi verificada por SDS-PAGE (E) e confirmada por *Western Blotting* (F). Fração não ligada (NL), terceira (2) e quarta (3) lavagens, primeira (4), segunda (5) e terceira eluições (6). Marcador molecular broad range BIO-RAD ou Fermentas.

Tendo em vista que a célula hospedeira *E. coli* C41 se mostrou mais eficiente para a expressão das proteínas recombinantes descritas acima, ela foi utilizada para os testes posteriores de expressão das proteínas IsaA, Lip e SAB0166. No ensaio de expressão, o antígeno imunodominante A (IsaA) de 24,3 kDa foi expresso eficientemente em *E. coli* C41 induzidas por IPTG (Figuras 4A e B). A purificação por cromatografia de afinidade também foi eficiente (Figuras 4B e C). Porém, foi observado que tanto na purificação de Aux quanto na de IsaA, várias outras proteínas foram co-purificadas no processo, provavelmente devido à presença de muitas proteínas ricas em histidina naturalmente expressas por *E. coli*.

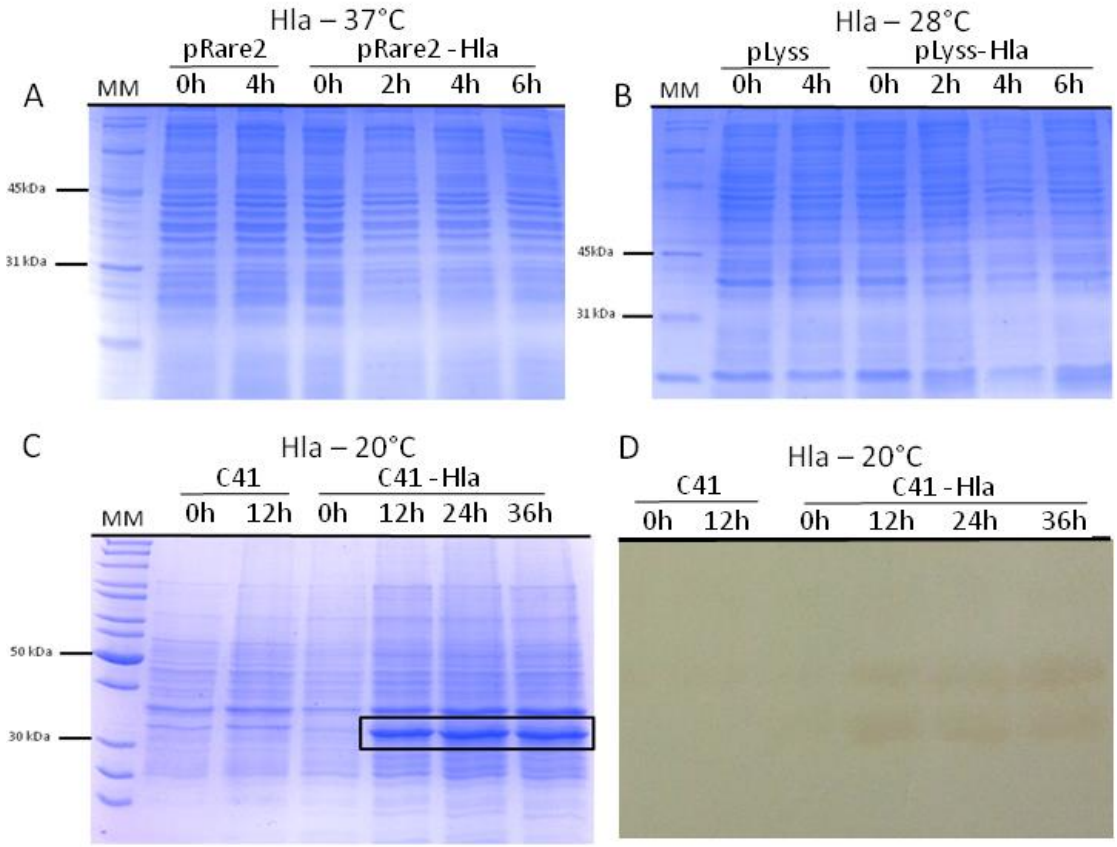

Figura2. Expressão da proteína recombinante Hla. A expressão da proteína recombinante foi avaliada por eletroforese em gel SDS-PAGE 12% (A, B, C) e *Western Blotting* (D). Expressão de Hla foi testada nas células hospedeiras *Escherichia coli* BL21 pRARE2 a 37 °C(A), *E. coli* BL21 pLyss a 28 °C (B) e *E. coli* C41 (C). As bandas induzidas estão indicadas no retângulo. Marcador molecular broad range BIO-RAD ou Fermentas.

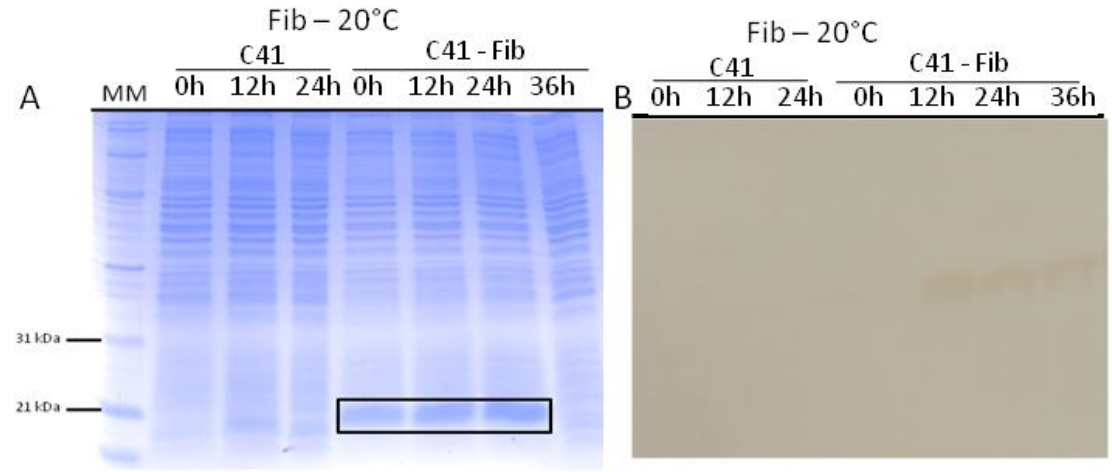

Figura 3. Expressão da proteína recombinante Fib. A expressão da proteína recombinante em *E. coli* C41 a 20 °C foi avaliada por eletroforese em gel SDS-PAGE 12% (A) e *Western Blotting* (B). As bandas induzidas estão indicadas no retângulo. A expressão foi confirmada por *Western Blotting* (B).

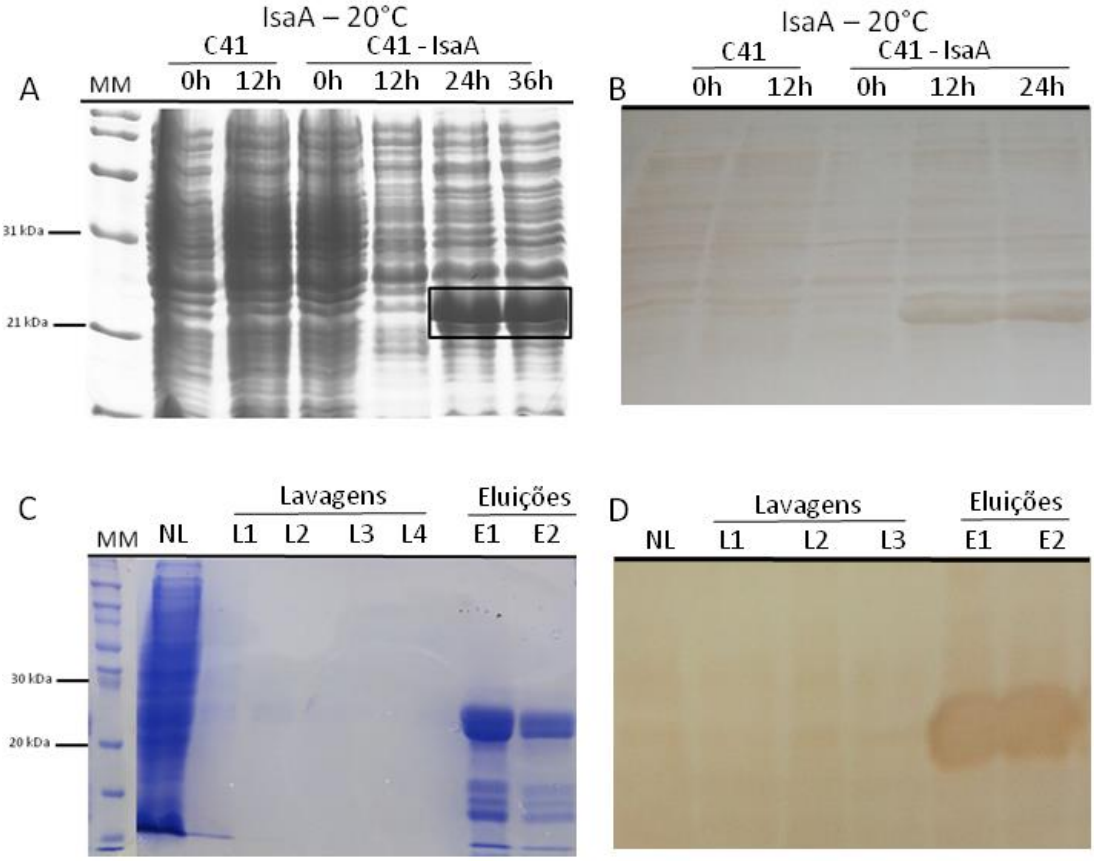

Figura 4. Expressão e purificação da proteína recombinante IsaA. A expressão da proteína recombinante em *E. coli* C41 foi avaliada por eletroforese em gel SDS-PAGE 12% (A e C) e *Western Blotting* (B e D). As bandas induzidas estão indicadas no retângulo. Marcador molecular broad range BIO-RAD ou Fermentas.

As várias tentativas de expressão das proteínas recombinantes SAB0166 e Lip na linhagem hospedeira *E. coli* C41 (Figura 5A e 5B) não foram bem sucedidas. Novas tentativas devem ser feitas variando-se a linhagem da célula, temperatura, concentração celular de indução e o tempo de cultivo em busca da melhor condição para a expressão das proteínas recombinantes.

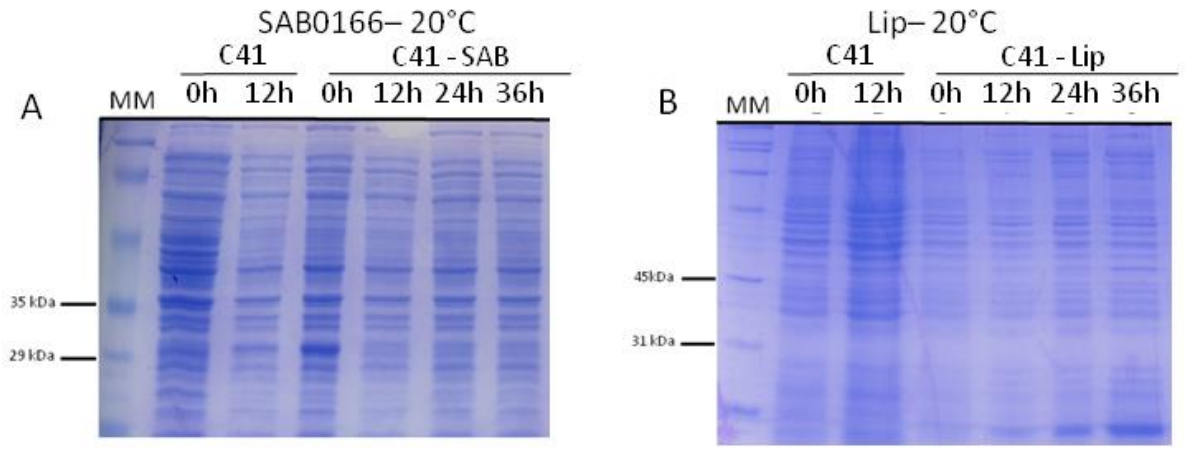

Figura 5 Teste de expressão das proteínas recombinantes SAB0166 e Lip. A expressão das proteínas recombinantes SAB0166 (A) e Lip (B) em *E. coli* C41 a 20 °C foi avalida por eletroforese em gel SDS-PAGE 12%.

Para avaliar o potencial para o imunodiagnóstico foram utilizadas as proteínas parcialmente purificadas Aux e IsaA. A proteína recombinante Nuc previamente purificada em nosso laboratório (Silva, 2013) também foi adicionada a esse estudo. A reatividade entre proteínas e anticorpos presentes em soro de leite coletados de animais com mastite subclínica foi avaliada por *Western blotting* e está exemplificada na Figura 6.

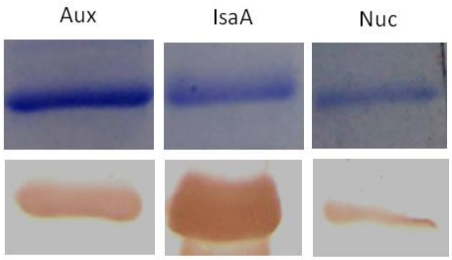

Figura 6. Avaliação da reatividade das proteínas Aux, IsaA e Nuc por *Western Blotting*.

IsaA e Nuc foram reativas para 5 das 6 amostras de soro provenientes de animais infectados com *S. aureus*, enquanto Aux reagiu apenas em 3 amostras. Foi observada reatividade cruzada de IsaA com soro de animais infectados por *Streptococcus agalactiae* em 75% das amostras. Já Nuc foi falso positivos em duas amostras, uma de *S. agalactiae* e uma de *Staphylococcus* coagulase-negativo. Aux não apresentou nenhum falso positivo.

Para cada antígeno foi calculada a sensibilidade e especificidade utilizando os resultados da cultura microbiológica como padrão (Tabela 4). Seis amostras de leite que não tiveram o patógeno identificado de acordo com os meios de cultivo usados para identificação de *Streptococcus* sp. e *Staphylococcus* sp. foram descartadas das análises. Dentre os antígenos escolhidos, a termonuclease (Nuc) apresentou os melhores valores de sensibilidade (83%) e especificidade (75%). O antígeno imunodominante A (IsaA) também apresentou sensibilidade de 83%, mas foi menos específico que Nuc. Aux obteve a sensibilidade de apenas 50%, mas foi 100% específico na identificação de *S. aureus*.

Tabela 4. Sensibilidade e especificidade dos antígenos Aux, IsaA e Nuc.

Antígeno	Sensibilidade	Especificidade
Aux	50%	100%
IsaA	83%	62,5%
Nuc	83%	75%

Já foi reportado que outras espécies de *Staphylococcus* possuem termonucleases e por isso podem gerar reatividade cruzada com Nuc de *S. aureus*. Para verificar se a reatividade cruzada de Nuc e IsaA era devido à presença de proteínas semelhantes em outros patógenos da mastite foi realizada uma busca de homologia em banco de dados não redundante disponível no site do NCBI (http://www.ncbi.nlm.nih.gov/blast/). IsaA apresentou 72% de identidade com uma transglicosilase de *S. agalactiae* e 59% com transglicosilases de *Staphylococcus* sp. Já Nuc apresentou uma faixa de identidade com nucleases de outras espécies de *Staphylococcus* que variou entre 41-66%

Para identificar as regiões específicas de IsaA e Nuc foi feita uma análise de antigenicidade por meio da predição de epítopos lineares reconhecidos por

células B (Bepipred Linear Epitope Prediction) presentes na sequência protéica (Figuras 7 e 8) utilizando programa disponível em http://tools.immuneepitope.org/tools/bcell/iedb_input.

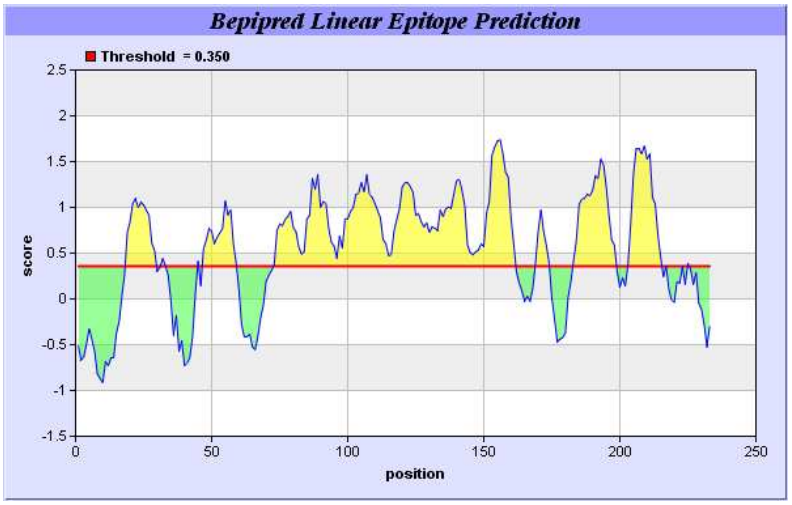

Figura 7. Gráfico de predição dos epítopos lineares de IsaA.

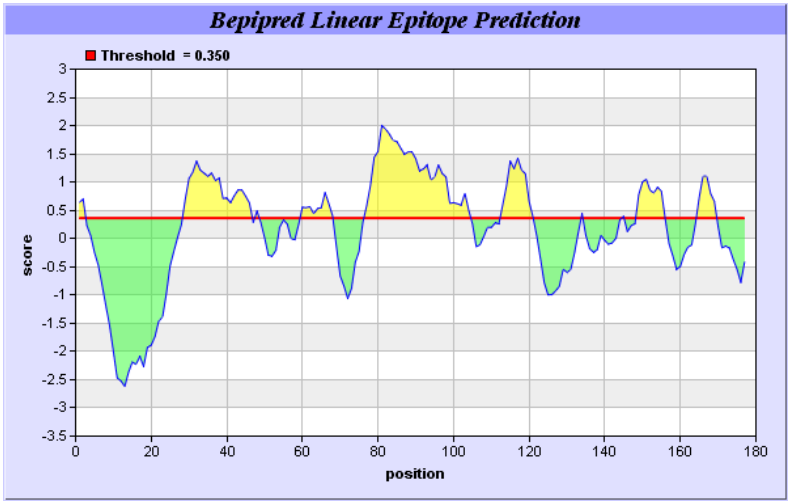

Figura 8. Gráfico de predição dos epítopos lineares de Nuc.

Foram preditas 11 epítopos para IsaA e 10 para Nuc. Posteriormente, apenas os epítopos com mais de 10 aminoácidos foram alinhados utilizando o programa BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) para identificação das sequências específicas de proteína. A sequência esepcífica identificada em IsaA compreende do aminoácido 102 ao 120 (YTTSYNQGSNVQSVSYNAQ) e de Nuc compreende do aminoácido 29 ao 46 (GPFKKGTNHETVQDLNGK).

5. DISCUSSÃO

Entre as doenças que afetam o rebanho leiteiro, a mastite é considerada a de maior impacto econômico em decorrência da diminuição da secreção de leite e dos gastos com o tratamento. Outro agravante é o número de agentes etiológicos associados à mastite o que dificulta o controle e diagnóstico baseado apenas na sintomatologia (Hillerton e Berry, 2005). O diagnóstico precoce da mastite bovina é de extrema importância na gestão dos animais infectados, controle da doença e principalmente para a redução das perdas econômicas (Sears e McCarthy, 2003). O diagnóstico da mastite subclínica é feito rotineiramente pelo CMT, um teste rápido e barato, baseado na contagem indireta de células somáticas no leite, que apenas discrimina animais sadios dos doentes. A identificação precisa do patógeno deve ser feita por métodos mais confiáveis, como a cultura microbiológica. No entanto, uma grande desvantagem da cultura bacteriológica se deve a demora na obtenção dos resultados o que pode atrasar o tratamento, permitindo a disseminação da bactéria no rebanho (Viguier et al., 2009; Kateete et al., 2010). Isso vem motivando a busca por métodos de diagnóstico mais precisos e sensíveis e que, de preferência possam ser utilizados em campo em conjunto com o CMT.

Um dos maiores desafios no imunodiagnóstico de *S. aureus* continua sendo a identificação do melhor antígeno a ser utilizado. Os antígenos mais visados para diagnóstico de infecções estafilocóccicas são fatores de virulência. Mas sabe-se que inúmeros isolados causadores de mastite podem coexistir num rebanho e que uma variedade de mecanismos estão envolvidos na patogenicidade de *S. aureus*, portanto os fatores de virulência expressos não são necessariamente os mesmos entre eles. Por isso, a necessidade inicial de avaliar a prevalência de um possível gene como forma de definir seu potencial biotecnológico.

Neste trabalho, os resultados disponibilizados por diferentes grupos (Le Maréchal et al., 2011a; Le Maréchal et al., 2011b; Fabres-Klein et al., 2013; Mansor et al., 2013) foram analisados e chegou-se a uma lista de 16 genes cuja presença foi avaliada em 195 isolados de *S. aureus* bovinos, partindo-se sempre da hipótese que um antígeno ideal para o diagnóstico deve estar presente em todos isolados de *S. aureus*. Dos genes selecionados apenas eap (5,1%), *hla* (94,4%), *isdA* (48,7%), SACOL1912 (47,7%) e SACOL2325 (99,5%) não estavam presentes em todos os isolados estudados. Dentre os genes mais prevalentes,

apenas *aux*, *isa*A, *fib*, *hla*, *lip* e SAB0166 foram clonados em vetor de expressão pDEST17. A expressão dos genes *aux*, *isa*A, *fib*, *hla*, *lip* e SAB0166 foi testada em diferentes linhagens de *Escherichia coli* e temperatura. A melhor condição de indução foi observada quando *E. coli* C41 transformada era crescida na temperatura de 20 °C.

Existem vários dados na literatura, incluindo patentes e artigos científicos, sobre o imunodiagnóstico de *S. aureus* a partir de amostras de soro humano ou alimento, mas poucos foram avaliados com leite mastítico. Dos voltados para a doença, faltam informações mais detalhadas de seu desempenho e quando existe, falta a validação em rebanhos leiteiros. As proteínas parcialmente purificadas nesse estudo, Aux, IsaA e Nuc, por serem componentes da superfície bacteriana ou proteína extracelular, estão mais acessíveis às células B e portanto induziriam mais eficientemente a produção de anticorpos (Holtfreter et al., 2010). Assim, elas tiveram a reatividade avaliada usando anticorpos presentes no soro de leite coletados de animais com mastite subclínica. Apenas uma amostra infectada com *S. aureus* não foi reativa com nenhum dos três antígenos testados, possivelmente devido a uma infecção inicial na qual os anticorpos ainda não atingiram título necessário para reagir no *immunoblotting*. Nuc e IsaA apresentaram os melhores valores de sensibilidade (83%), mas a termonuclease foi mais específica (75%) que o antígeno imunodominante A (62,5%). A especificidade de Nuc e IsaA foram afetadas por reações cruzadas que ocorreram com *Staphylococcus* coagulase-negativo e *Streptococcus agalactiae*, respectivamente.

Yazdankhah e colaboradores (1999) reportaram valores semelhantes de sensibilidade (89%) e especifidade (70%) em ensaios de ELISA utilizando Nuc, valores que são compatíveis aos encontrados neste trabalho. O gene *nuc* é altamente conservado em espécies de *Staphylococcus*, exceto em *S. sciuri* (Hirotaki et al, 2011) e já foi relatado tanto em *Staphylococcus* coagulase-positivos quanto em coagulase-negativos (Gudding, 1983). Regiões do gene *nuc* permitem a separação de *S. aureus* de outros staphylococci coagulase-positivos com absoluta especificidade (Sasaki et al., 2010). Desta forma, é possível que a expressão de apenas essa porção contribua para aumentar a especificidade do imunoensaio.

IsaA (Antígeno imunodominante A) é uma proteína altamente imunogênica associada a parede celular de *S. aureus* (Stapleton et al., 2006) que

foi originalmente identificada em triagem de antígenos que poderiam servir como candidatos vacinais ou alvos para imunoterapia (Lorenz et al., 2000). Em estudos comparando soro de pessoas saudáveis com aquelas com infecção estafilocócica mostrou-se que IsaA é muito eficiente em elicitar os mecanismos de defesa imunológica e a produção de anticorpos (Lorenz et al., 2000; Etz et al., 2002), características importantes também para o diagnóstico.

Vytvytska e colaboradores (2002) analisando soroproteoma de *S. aureus* identificado por anticorpos presentes no soro humano, indicaram IsaA e Aux como bons marcadores de infecção causada por *S. aureus* que poderiam ser utilizados em formulações vacinais ou para diagnóstico. Porém, dentre as proteínas estudadas, Aux foi a que apresentou o pior desempenho na sensibilidade (50%), apesar de ser 100% específica. Este resultado pode ser um indício de que Aux não é expressa eficientemente durante infecções intramamárias em bovinos, e por isso a presença de anticorpos contra esta proteína no leite é muito baixa.

A variação na produção de anticorpos no leite em respostas a infecções pode influenciar nos resultados de imunodiagnósticos para a mastite. Fox e Adams (2000) avaliaram a dinâmica da produção de anticorpos em animais experimentalmente infectados com *S. aureus* e mostraram que animais com infecções incipientes apresentam baixos níveis de anticorpos específicos e poderiam ser classificados como falso-negativos por ELISA. Da mesma forma, animais não infectados ou infectados com outra espécie de *Staphylococcus* apresentaram altos níveis de anticorpos nos 21 dias seguintes ao parto e seriam falso-positivos. Uma alternativa seria a detecção do antígeno na amostras de leite, testes utilizando esta estratégia tem reportado 79-100% de sensibilidade e 93-100% especificidade na detecção de *S. aureus* (Huang et al., 2007; Badiou et al., 2010).

6. CONCLUSÃO

Os genes *isaA, fib, aux, lip,* LP309, *nuc*, SAB0166, SACOL2365, SACOL2385 e SACOL2171 foram encontrados em 100% dos isolados bovinos estudados. A reatividade de IsaA e Nuc a anticorpos presentes em amostras de leite foi demonstrada, e portanto essas proteínas têm um potencial para imunodiagnóstico Entretanto, elas mostraram reatividade cruzada com outros patógenos da mastite bovina. Novos estudos devem ser conduzidos para expressar e testar regiões da proteína com maior especificidade para *S. aureus*.

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CAPÍTULO 3

Effect of a LysR-type regulator on colonization of *Staphylococcus aureus*

Effect of a LysR-type regulator on colonization of *Staphylococcus aureus*

Mary Hellen Fabres-Klein[1], Celine Ster[2], Eric Brouillette[2],**Andréa de Oliveira Barros Ribon[1], **François Malouin[2]

[1]Laboratório de Biotecnologia Molecular, Departmento of Bioquímica e Biologia Molecular, 36570-000, Viçosa, MG, Brazil.

[2]Centre d'Étude et de Valorisation de la Diversité Microbienne (CEVDM), Département de biologie, Faculté des sciences, Université de Sherbrooke, Sherbrooke, QC, J1K 2R1, Canada.

*Co-Corresponding authors:

Francois.Malouin@usherbrooke.ca (F.Maloiun) Tel.: +1 819 821 8000x61202; fax: +1 819 821 8049.

abribon@ufv.br (A.O.B.Ribon), Tel: 55 31 3899 2837; Fax: 55 31 3899 2373

ABSTRACT

Staphylococcus aureus is a well-armed pathogen that has been extensively studied to elucidate the mechanisms involved in pathogenicity that could aid in the definition of new strategies to prevent infections. Recently, a transcriptional profile of *S. aureus* during intramammary infection demonstrated that SACOL2325, a putative LysR-type transcriptional regulator, was highly induced *in vivo*. In this work, we investigated the role of SACOL2325, here identified as LysR, in virulence of *Staphylococcus aureus* RF122 under different biochemical approaches. LysR overexpression affected biofilm formation and oxidative stress susceptibility, with no changes on bacterial growth and hemolysis. Assays using MAC-T cells and a mastitis murine model indicated that LysR reduced colonization. Transcriptome analysis showed that 34.8% of staphylococcal genes were altered in response to SACOL2325 overexpression. The sortase A (*srt*A) gene and some genes involved in teichoic acid production (*tagX* and *tagB*) were down-regulated 3.0, 7.3, and 11.2-fold, respectively. In contrast, genes responsible for purine biosynthesis and adenosine synthase (*ads*A) were up-regulated. Our results suggest that SACOL2325 influences initial steps in *S. aureus* colonization and immune evasion, by altering the expression of genes associated with cell wall function and evasive strategies.

Keywords: *Staphylococcus aureus*, bovine mastitis, LysR, colonization, adhesion, transcriptome

1. INTRODUCTION

Staphylococcus aureus is a well-armed pathogen that has been extensively studied for a better understanding of the molecular mechanisms that contribute to its pathogenicity. Besides being considered an important human pathogen, *Staphylococcus aureus* is also considered one of the most frequent pathogens that causes mastitis in dairy cattle. Several reports describe its prevalence in herds from different countries (Bramley et al., 1996; Tenhagen et al., 2006; Bradley et al., 2007). Infections caused by *S. aureus* are mainly subclinical and frequently develop into a chronic form that is difficult to eradicate.

The molecular mechanisms by which *S. aureus* causes bovine mastitis remain poorly understood. Some genes are associated uniquely with bovine strains but the relevance in the infection has not been proved (Kozytska et al., 2010). In recent years a effort has been attempted to elucidate the global response of bovine isolates to environmental conditions found in the udder (Le Maréchal et al., 2011a; Le Maréchal et al., 2011b; Allard et al., 2006). Several authors have compared the transcriptome and proteome of *S. aureus* strains to build a complete scenario that allows the bacteria to cause different forms of mastits (Allard et al., 2013; Le Maréchal et al., 2011a, Tedeschi et al., 2009).

Allard et al (2013) experimentally infected cows with four different strains of *S. aureus*. A complete transcriptional profile of the bacteria recovered from milk at three time points during infection was determined by microarray. Thirty-six genes showed a significant difference in their expression in at least two strains of the *S. aureus* tested. Only 5.4 to 16.7% of the genes detected by microarray had significant variation in the expression level. Four genes that showed a strong expression and with putative functions in pathogenesis had their differential expression validated by qPCR. This analysis revealed the gene SACOL2325 previously found to be induced by milk *in vitro* (Allard et al., 2013).

Gene annotation defined SACOL2325 as a LysR-type transcriptional regulator (LTTR) (Gill et al, 2005), which is one of the most abundant types of transcriptional regulators in prokaryotic kingdom (Momany e Neidle, 2012). The protein is implicated in the regulation a diverse set of genes, including those involved in virulence, cell division, metabolism, quorum sensing, responses to oxidative stress, adhesion, and motility (Maddocks and Oyston, 2008).

In silico analysis demonstrated the presence of seven LysR regulators in *S. aureus* of which three still remains with unkown functions (Ibarra et al, 2013). CidR, CcPE, HutR, and GtlC have been characterized and are involved in antibiotic tolerance and carbon metabolism, histidine catabolism, and glutamate metabolism, respectively (Yang et al., 2005; Ravcheev et al., 2011; Hartman et al, 2013). LTTR family members have a helix-turn-helix motif as the DNA-binding domain, domains involved in coinducer recognition and/or response and a domain required for both DNA binding and co-inducer response (Schell, 1993). LysR regulators bind to promoter regions but can also bind to the downstream (Maddocks and Oyston, 2008).

In this study we studied the role of the putative LysR in staphylococcal pathogenesis. Bovine strains overexpressing LysR showed a reduced capacity to invade MAC-T cells and lower virulence in a mastitis murine model. Transcriptome analysis suggested that LysR reduces the colonization process by probably downregulating sortase A and some proteins related to the biosynthesis of teichoic acids.

2. MATERIAL AND METHODS

2.1. Bacterial strains and growth conditions

Two different *Staphylococcus aureus* strains (RN4220 and RF122) were used in this study. The *S. aureus* RN4220 is a restriction-defective strain and was used as a cloning intermediate. *Escherichia coli* DH5α was also used as an intermediate host in transformation experiments. Cultures were grown in Brain-Heart infusion (BHI - Becton Dickinson, Mississauga, ON, Canada) for 16 h at 35 °C with shaking. Stock cultures were maintained in BHI with 20% glycerol at -80 ° C.

Tryptone-soy broth (TSB) (TSB - Becton Dickinson Mississauga, ON, Canada) or milk was used for bacterial growth. Milk was collected and plated in TSA to check contamination. Mueller–Hinton agar (Becton Dickinson, Mississauga, ON, Canada) was used in the hemolysis assay. For oxygen-limited cultivation, the bacteria were grown at 35 °C without shaking in 15 ml-tube completely filled with TSB or milk.

2.2. Growth curve

Bacteria were first grown in TSB or milk in an orbital shaker (225 rpm) at 35 °C for 16 h. The culture was then diluted in fresh media and grown in an orbital shaker (225 rpm) at 35 °C. For every 60 min during 10 h, 200 µL of the cultures were serially diluted and 10 µL of each dilution were plated on TSA. The colonies were counted after 24 h at 35 °C.

2.3. Plasmid construction

The gene SAB2209 (*S. aureus* COL SACOL2325) was amplified by polymerase chain reaction (PCR) from the total DNA of *S. aureus* RF122 using F-NNNNNGGATCCCATAAAATTCGCTCCTGTTCT (*Bam*HI site underlined) and R-NNNNNGGTACCGGGCAGAATGTGAGTATTTCG (*Kpn*I site underlined) as primers, in 50 µL volume reaction. An 1262 bp size amplicon was generated in a reaction that used 94°C in the denaturation step for 5 min followed by 30 cycles of 45 s at 94 °C, 45 s at 60 °C, 90 s at 72 °C, and a final extension at

72 °C for 10 min. The products were double digested with high fidelity restriction enzymes (New England Biolabs, Pickering, ON, Canada). The digested products were purified using QIAquick PCR Purification Kit (Qiagen, Toronto, ON, Canada) and linked to digested vectors pCN36 (containing Pblaz promoter) and pCN43. Ligation was carried out for overnight at 16 °C with T4 DNA ligase (New England Biolabs, Pickering, ON, Canada). The QIAfilter Plasmid Midi kit (QIAGEN, Toronto, ON, Canada) was used for mini-prep plasmid DNA extraction with a first step bacterial lysis with lysostaphin (200 mg/mL for 1 h at room temperature). All plasmid constructions were propagated in *Escherichia coli* DH5α as the intermediate host.

The SACOL2325 gene was cloned into pCN43, a high copy plasmid, and pCN36-PblaZ, a low copy plasmid (Supporting data Fig. 1S).

2.4. Preparation of electrocompetent *S. aureus* and electroporation of clinical strains

Electrocompetent *S. aureus* cells were prepared using a previously described protocol (Löfblom et al, 2007). A 30 mL culture of *S. aureus* was grown in TSB overnight at 37 °C and 225 rpm. The cells were diluted into 500-mL BM2 medium (1% Casein hydrolysate, 2.5% Yeast extract, 0.5% Glucose, 2.5% NaCl, 0.1% K2HPO4, pH 7.5) to an $OD_{578\ nm}$ 0.5 and grown at 35 °C and 225 rpm until an OD_{578nm} of 0.6. The cells were incubated on ice for 15 min to stop growth, harvested by centrifugation (3000 g, 10 min and 4 °C), and washed with 500, 250 and 80 mL of ice-cold sterile water. Subsequent washes were performed with 10 and 5-mL of ice-cold 10% glycerol. The cell pellet was resuspended in 2 mL of ice-cold10% glycerol (around $4x10^{10}$ cells/mL). Finally, 60 µL aliquots were immediately transferred for storage at -80 °C. The electrocompetent cells were thawed on ice for min, and incubated at room temperature for 30 min. Four µg/mL of the plasmid were added to the cells and incubated 5 min at room temperature. The cells were transferred in 2 mm gap electroporation cuvettes and electroporated at 25 µF and 2.0 kV. Immediately after the procedure, 1 ml of BM2 was added and the cells were transferred into a 14 mL tube and incubated at 2 h at 35 °C. Finally, the cells were diluted and plated on BM2 agar with erythromycin 20 µg/mL and incubated at 35 °C. All the

plasmids were first transformed into *S. aureus* RN4220 and then into *S. aureus* RF122, the final host.

2.5. Hemolysis and antibiotic susceptibility

Bacterial suspensions corresponding to 0.5 McFarland scale were prepared for each strain and were spotted (2 µL) onto MH agar with 5% sheep blood supplemented or not with antibiotics. Hemolysis was assessed after 24 h of incubation at 35 °C followed by an overnight incubation at 4 °C.

The minimal inhibitory concentrations (MICs) for all strains were determined by a broth microdilution technique, following the recommendations of the Clinical and Laboratory Standards Institute guidelines (CLSI, 2006). *S. aureus* RF122 was used as a reference strain in all MIC tests.

2.6. Biofilm formation

Biofilm formation was assessed in polystyrene 96-well tissue culture microplates as previously described (Vasudevan et al., 2003), with modifications. Strains were grown on TSB agar containing 0.25% glucose (TSBg) with or without 10 µg/mL erythromycin and incubated at 35 ˚C for 18 h to 24 h. These cultures were used to adjust an appropriate volume of TSBg to 0.5 Mcfarland for transfer into wells containing half volume of the same medium with or without erythromycin or tetracycline (final concentration 10 µg/mL). The plates were incubated at 35 °C for 24 h. The medium was discarded and the wells were gently washed three times with 200 µL of sterile PBS (pH 7.4), dried and then stained with crystal violet for 30 min. The plate was washed three times with 200 µL of sterile distilled water, air dried, 200 µL of commercial ethanol were added to each well and plates were incubated at room temperature for 1 h with frequent agitation. The absorbance of the wells was then measured at OD_{560nm} using a plate reader (Bio-Tek, Winooski, United States). The biofilm formation was performed in triplicate.

2.7. Tolerance to oxidative stress

The response to oxidative stress was determined using a disk diffusion assay. Overnight cultures were inoculated into fresh TSB broth and grown under agitation at 35 °C until exponential phase. For the experiment 200 µL of cultures

were spread on TSA agar with or without antibiotics. A 6 mm-diameter sterile filter disk was put on the center of the plate, to which 10 µL of 1% H_2O_2 was added. The plates were incubated at 35 °C overnight and inhibition zones were measured. The oxidative stress tolerance experiments were performed in triplicate.

2.8. RNA extraction

Overnight cultures were used to inoculate TSB at an $OD_{600\ nm}$ of 0.1 and let grow for 7 hours at 35 °C under low oxygen conditions. Bacteria were collected, treated with RNAprotect according to the manufacturer's recommendations (QIAGEN, ON, Canada) and storaged at -80 °C until use. Total RNA extraction was performed as previously described (Klein et al., 2012). DNase treatment was done twice with the DNA-free kit (Applied Biosystems/Ambion, CA, USA). The RNA concentration in samples was determined and samples were stored at -80 °C until use.

2.9. qPCR

Overexpression of the SAB2209 gene in *S. aureus* RF122 was confirmed using real-time qPCR. For this, 1 µg of total DNAse-treated RNA was used to construct a cDNA library using 0.5 mM deoxynucleotide phosphate, 50 ng of random hexamers and 200 U of Invitrogen Superscript II reverse transcriptase, according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). RT-qPCR was performed using Stratagene MX3000P Real-Time PCR instrument (Stratagene, LaJolla, CA USA). The amplification conditions consisted of 10 min at 95 °C, followed by 35 cycles of 1 min at 60 °C, 1 min at 72 °C and finished with a dissociation ramp from 55 °C to 95 °C. The amplification reactions were performed in 20 µl containing 1 µL of cDNA, master mix of 6 mM Tris-HCl, pH 8.3, 25 mM KCl, 4 mM $MgCl_2$, 75 mM trehalose, 0.1% (v/v) tween 20, 0.1 mg/ml nonacetylated BSA, 0.07x SYBR green (Invitrogen, Carlsbad, CA, USA), 125 nM dNTPs, 0.5 U JumpStart Taq DNA Polymerase (Sigma, Oakville, Canada) and 100 nM of the primers (F-CATCTCGGCTTAGGTTACGC and R-GTTCGGAGTGTTTGCGTTTT), that generated a 142 bp amplicon. The relative expression ratios were calculated by using the cycle threshold (Ct) of the *gyr*B (F-GGTGCTGGGCAAATACAAGT and R-TCCCACACTAAATGGTGCAA).

2.10. Bacterial invasion and persistence in MAC-T cells

Bacterial invasion and persistence in bovine mammary epithelial cell line (MAC-T) was carried out as previously described (Brouillette et al., 2004), with modifications. The cells were grown in high-glucose Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), insulin (5 mg/mL), hydrocortisone (5 mg/mL), penicillin (100 U/mL), and streptomycin sulfate (100 mg/mL). The MAC-T cells were seeded (8 x 10^5 cells per well) in 24-well culture plates (Costar) and incubated at 37°C under 6% CO_2. Upon reaching confluency, the cells were washed once with sterile PBS and incubated overnight (37°C under 5% CO_2) in invasion medium (DMEM/1 % FBS without antibiotics). Then they were washed with DMEM salt solution (Gibco-BRL) and incubated with 1 x$\cdot 10^6$ DMEM-washed bacteria grown overnight on TSB. Invasiveness was determined by exposing the monolayers to bacteria for 3 h. Then, they were washed and incubated an additional 30 min in invasion medium containing 20 lysostaphin µg/mL and and gentamicin 50 µg/mL. Following extensive washing, the monolayers were lysed with sterile distilled water. The lysate was serially diluted and plated on TSA for cfu/mL count. Persistence was determined using the same procedure, except that the monolayers were incubated in invasion medium containing 20 lysostaphin µg/mL and and gentamicin 50 µg/mL and assayed for intracellular bacteria at 12 h and 24 h.

2.11. Mastitis mouse model

Mastitis mouse was carried out as described by Brouillette et al (2003), with modifications. Briefly, the 13- to 14-day-old pups were removed from lactating CD1 mice (Charles River, St.-Constant, Canada) 1 h before inoculation. The lactating mice were anesthetized by using ketamine and xylazine at 87 and 13 mg/kg of body weight, as an analgesic. Using a 100-µL glass syringe and a 33-gauge blunt needle, the mammary glands (fourth on the right [R4] and fourth on the left [L4]) were inoculated with 100 µL of bacterial suspension containing 10^2 cfu of *S. aureus*. To allow precise injection into the mammary ducts, the near ends of the teats were aseptically removed before inoculation. After 8, 14, and 20 h of infection, the mammary glands of each mouse were aseptically removed, weighed, and homogenized in PBS at a final volume of 3 ml. To evaluate the CFU content, the homogenate was serially diluted and plated onto TSB (Becton

Dickinson, Mississauga, ON, Canada), with or without erythromycin 10 µg/ml. The guidelines of the Canadian Council on Animal Care were respected during all the procedures (Olfert et al., 1993).

2.12. Directional RNA-seq

Three hundred nanograms of total RNA was fragmented by magnesium catalyzed hydrolysis (40 mM Tris-Acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc) for 7 minutes at 95°C, and purified with the RNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's recommendations (general procedure protocol). The resulting RNA molecules were treated with antarctic phosphatase (New England Biolabs) before inactivation of the enzyme and purification using the RNA Clean & Concentrator-5 kit (Zymo Research). A DNA-RNA hybrid adaptor (5'-Phos/rArGrArUrCrGrGrArArGAGCACACGTCT/AmMO-3') was ligated to the 3' end of fragments (T4 RNA ligase, Enzymatics) and purified with RNA clean XP beads (Beckman Coulter genomics) to remove unligated adaptors. 5′ RNA extremities were phosphorylated with T4 PNK (Enzymatics) before ligating a DNA-RNA hybrid adaptor (5′-ACACGACGrCrUrCrUrUrCrCrGrArUrCrU-3′) to the 5′ end of RNA fragments. Ligation products were purified with RNA clean XP beads (Beckman Coulter genomics) and immediately used for reverse transcription reaction using a DNA oligo annealing to the 3’ of RNA molecules oligo (5′-AGACGTGTGCTCTTCCGATCT-3′). The resulting cDNA was purified with AMPure XP beads (Beckman Coulter genomics) and amplified by qPCR with VeraSeq 2.0 DNA polymerase (Enzymatics) using oligos (5'-CACGACGCTCTTCCGATCT-3' and 5′-AGACGTGTGCTCTTCCGATCT-3′). The reaction was stopped towards the end of the exponential amplification phase as monitored on a CFX connect qPCR instrument (Bio-Rad). The amplified library was purified with AMPure XP beads (Beckman Coulter genomics) and quantified using a NanoDrop 2000 (Thermo Scientific). Amplified ribosomal cDNA was depleted using the Trimmer cDNA normalization kit (Evrogen) according to the manufacturer's recommendations. The normalized library was then purified with AMPure XP beads (Beckman Coulter genomics) and amplified with VeraSeq 2.0 DNA polymerase using full length Illumina oligos (unique

barcoded reverse oligo for each sample). The resulting library was quantified and the fragment size distribution was determined using a Bioanalyzer 2100 (Agilent).

2.12.1 Illumina sequencing

Illumina sequencing was performed on a Illumina HiSeq 2000 Sequencing system at the Plateau de Biologie Moléculaire et génomique fonctionnelle of the Institut de Recherche Cliniques de Montréal. Approximately 7.5 million paired-end reads of 50 bp were obtained for each library. Sample multiplexing relied on a 6 bp barcode located within the reverse read adaptor.

2.12.2. Data analysis

Reads were aligned against the reference genome (*S. aureus* RF122, genbank AJ938182.1) using BWA. Data was next analyzed using Cufflinks' Cuffdiff module.

2.13. Statistical analysis

Statistical analyses were carried out with the GraphPad Prism software (v.6.02). For *in vitro* tests, one-way analysis of variance [ANOVA] and t test were performed to determine differences between pCN43 and pCN43-2209.* $P < 0.001$. For *in vivo* tests, Mann-Withney analysis was performed at each time point to determine if bacterial CFUs were statistically different between pCN43 and pCN43-2209. **$P< 0.01$.

3. RESULTS

Previous studies identified the upregulation of the gene SACOL2325 during intramammary infections and *in vitro* cultivation in milk (Allard et al. 2013). This gene was annotated as a LysR-type regulator of 1151bp and codes a putative protein of 294 amino acids (Gill et al., 2005) (FIG. 1) and is equivalent to the gene SAB2209 in *S. aureus* RF122. As expected for protein belonging to the LysR family, SACOL2209 has N-terminal DNA binding helix-turn-helix motif and a C-terminal LTTR substrate binding domain, which is structurally homologous to the type 2 periplasmic binding proteins.

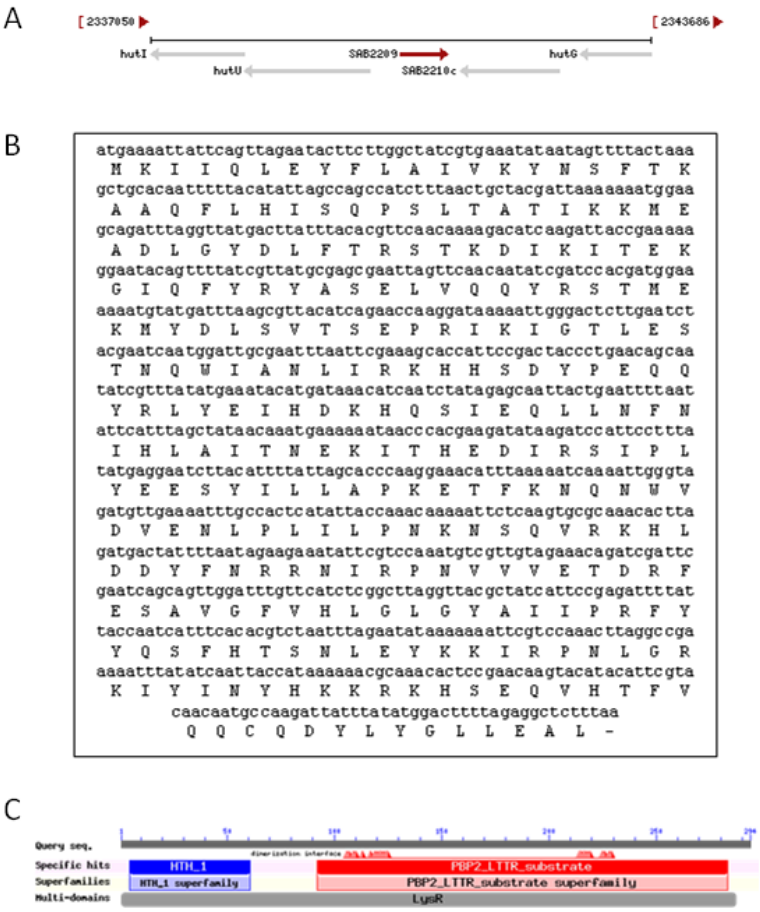

Figure 1. Genomic context organization, predicted amino acid sequence, and domains of the SAB2209 gene of *Staphylococcus aureus* RF122. SAB2209 genomic context in RF122 (A). Nucleotide sequence and predicted amino acid sequence (B). A schematic representation of the domain structure found in SAB2209 protein (C). Helix-turn-helix domain (HTH_1). LysR-type transcriptional regulator member of the type 2 periplasmic binding protein superfamily (PBP2_LTTR-2).

The alignment of the regulatory proteins from *Bacillus subtilis* (AlsR, GltR, Yofa, GltC, CysL and CitR), *Escherichia coli* (CynR and MetR), *Pseudomonas putida* (CatR), and *Caulobacter crescentus* (IlvR) is shown in Figure 2. SACOL2209 showed 26% identity with CynR from *Escherichia coli* and 25% to GltC of *Bacillus subtilis*.

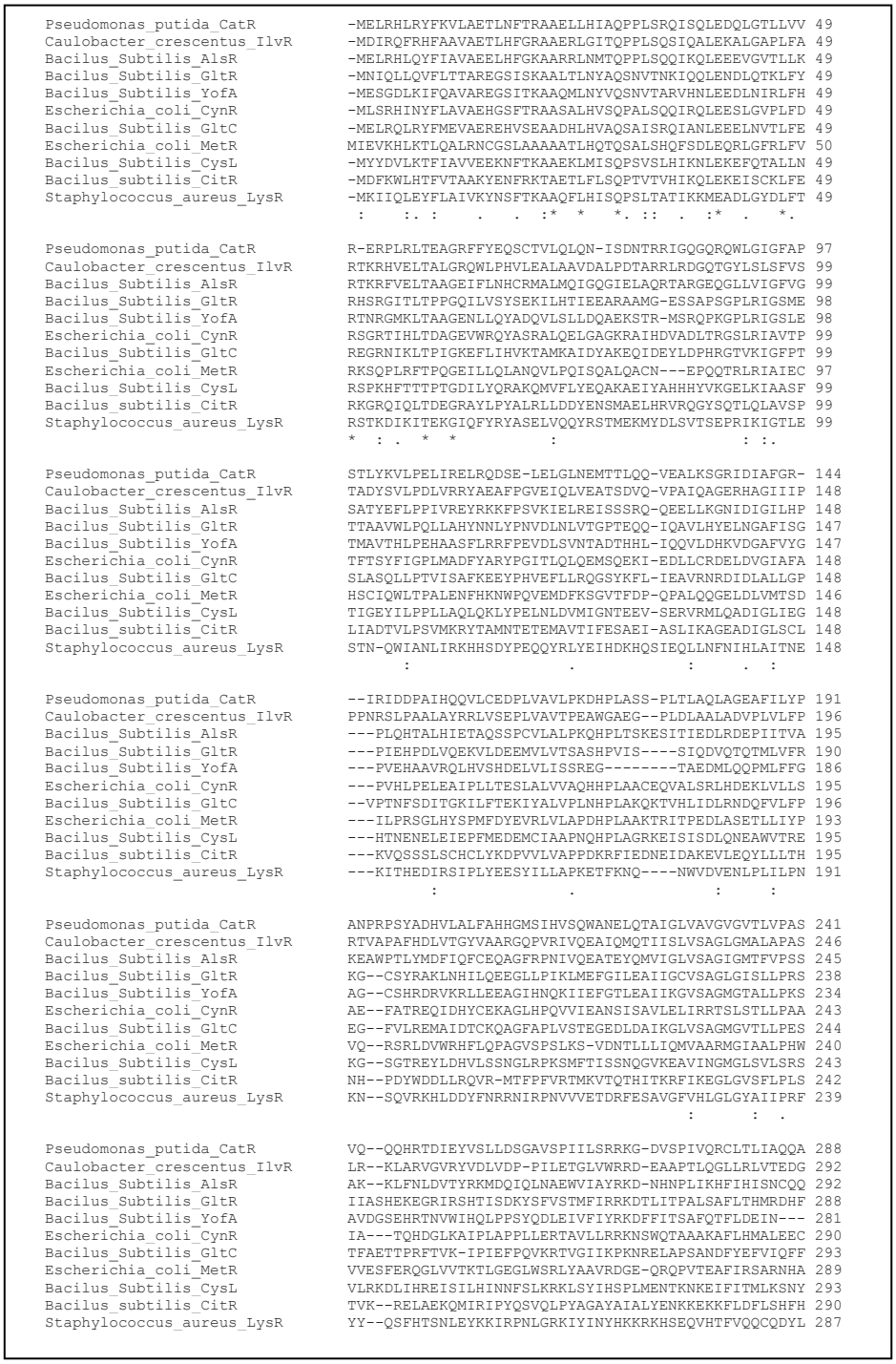

Figure 2. Amino acid sequence alignment of LysR-type transcriptional regulators of bacterial species. Identical amino acids are illustrated by stars; points indicate conservative changes. Alignment was done by ClustalW.

To determine the role of the putative LysR on virulence of *S. aureus*, the SAB2209 (SACOL2325) gene was amplified and overexpressed in *S. aureus* RF122, what was confirmed by RT-qPCR (FIG. 3).

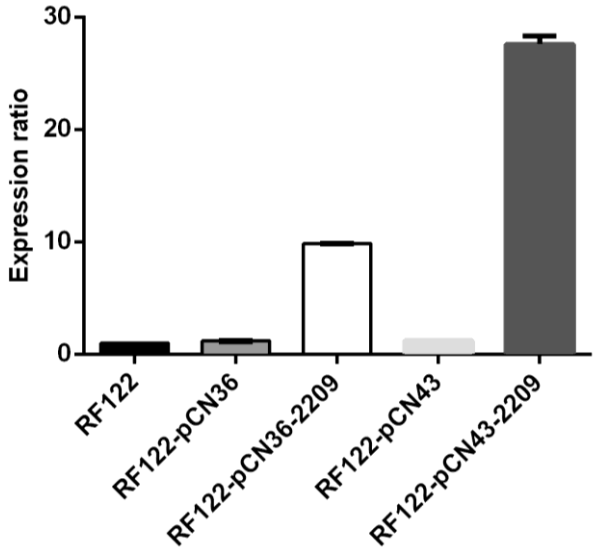

Figura 3 Relative expression of SAB2209 overexpressed in pCN43 and pCN36_PblaZ. Expression values were calculated using the $2^{-\Delta Ct}$ method with *gyr*B used as an endogenous control.

Then growth of the transformants was evaluated in broth and fresh milk. No difference was seen when the transformants were grown in these media under different aerobic conditions (Figure 4A and 4B). However, low levels of oxygen reduced bacterial growth. Hemolytic activity didn't change after transformation (Figure 4C). LysR overexpression interfered with oxidative stress response (Figure 4D) and biofilm formation (Figura 4E). The negative effect caused a 30% of reduction on biofilm in cells overexpressing SAB2209. Since no difference was observed when LysR was overexpressed from pCN36PblaZ, a low copy plasmid, or from pCN43, a high copy plasmid, pCN43 was chosen for subsequently assays. The ability of pathogens to resist a stress condition might hamper their virulence and though their ability to cause infections. To address if LysR transformants were sensitive to oxidative stress, a disk diffusion assay was done and the growth inhibition zones surrounding the discs were measured. The sensibility to oxidative stress increased 17% in cells overexpressing SAB2209 when compared with

RF122 carrying empty plasmids (Figure 4D). Antibiotic susceptibility showed a slight change for gentamicin and fusidic acid (Table 1).

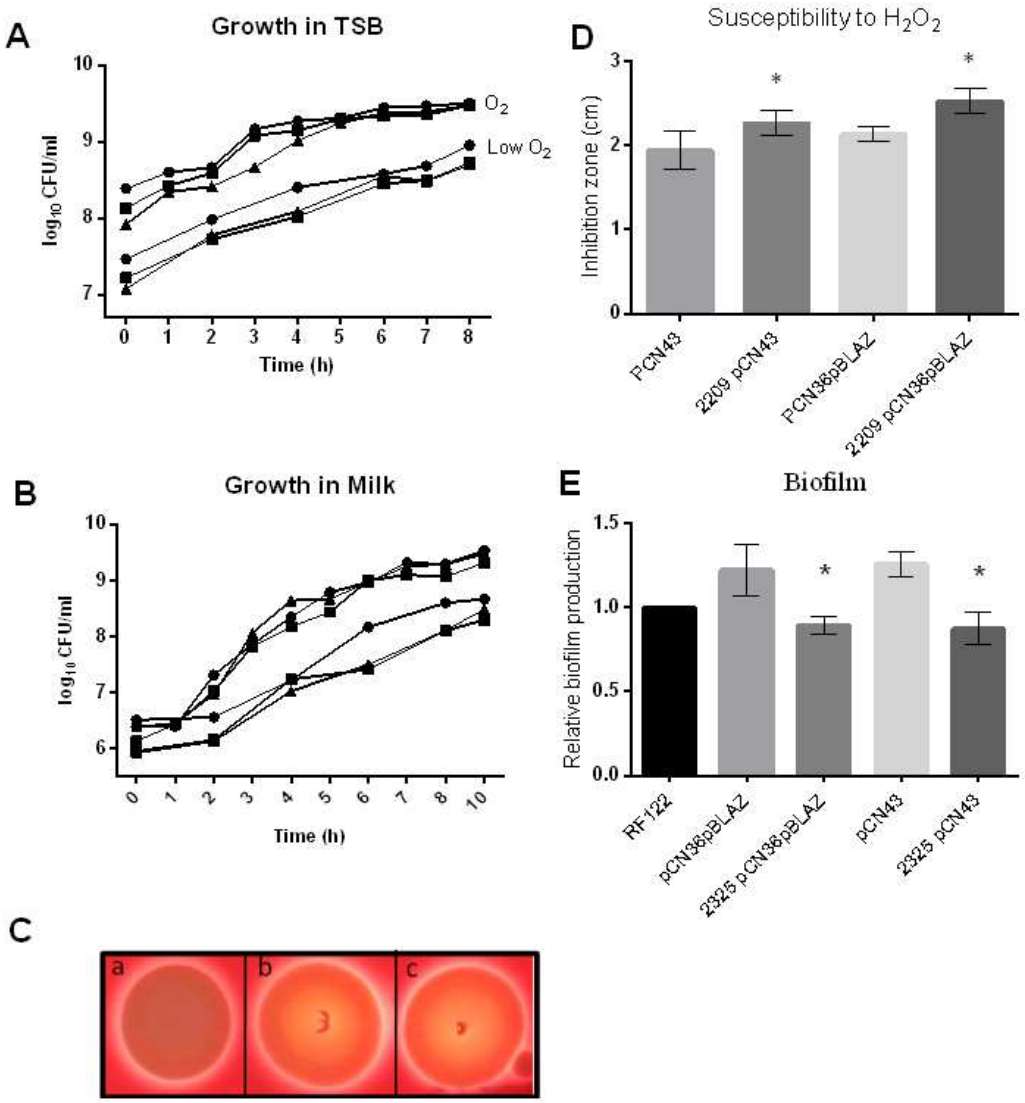

Figure 4. Effect of superexpression of SAB2209 on *Staphylococcus aureus* RF122. Bacterial growth, hemolytic activity, biofilm formation, and response to oxidative stress were assayed. Growth curves were done in TSB (A) and milk (B) for RF122 (black circle), RF122 containing pCN43 (black square), RF122 with pCN43-2209 (black triangle). Hemolytic activities (C). Oxidative stress (D). Biofilm formation (E). All experiments were conducted in triplicate.

Table 1. Effect of overexpression of the gene SAB2209 on antibiotic susceptibility of *Staphylococcus aureus* RF122.

Strain	MIC[a] (µg/mL)												
	AMP	ST	OXA	TET	CHL	GEN	NOR	NT	VAN	HQN	CLI	NIS	AF
RF122	>128	16	>128	1	16	1	1	32	2	>128	0,25	>128	1
pCN43	128	16	128	0,5	16	4	1	32	2	>128	>128	>128	1
2325pCN43	>128	16	>128	1	16	2	2	32	2	>128	>128	>128	0,25

[a] Minimum inhibitory concentration of ampicillin (AMP); streptomycin (ST); oxacillin (OXA); tetracycline (TET); chloramphenicol (CHL); gentamicin (GEN); norfloxacin (NOR); nitrofurantoin (NT); vancomycin (VAN); 4-Hydroxy-2-heptylquinoline-*N*-oxide (HQNO); clindamycin (CLI); nisin (NIS); fusidic acid (AF).

The possible role of LysR in the colonization of bovine mammary epithelial cells (MAC-T) was evaluated. In the adhesion assays cells were incubated with *S. aureus* for 3 h before extracellular bacteria was killed. To determine persistence, the same assay was used, except that monolayers were incubated in medium containing lysostaphin after 12 and 24 h when the intracellular bacteria were plated. The CFU determination revealed a reduction in the bacterial count demonstrating that adhesion and persistence were affected (Figure 5).

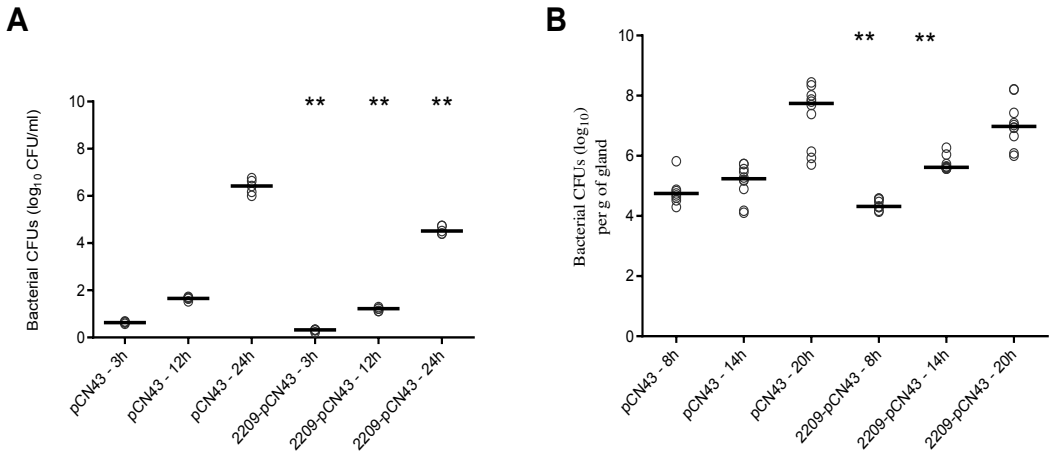

Figure 5. LysR overexpression effect on *Staphylococcus aureus* invasion MAC-T cells and infection in murine model. Adhesion and persistence of *S. aureus* carrying pCN43 and pCN43SAB2209 in MAC-T cell (A). Intramammary infection in murine model (B).

There was a 51.7% reduction in the adhesive property of LysR overexpressed *S. aureus* while a lower reduction in persistence was detected after 12 (27.3%) and 24 h (28.7%) (Figure 5A). Similar results were seen *in vivo* as LysR transformants showed impaired virulence in the first hours of infection (Figure 5B). However, after 14 h the number of bacteria recovered from the mammary glands started to equal the control level, and no difference were seen after 20 h. These data suggested a role of LysR in virulence by downregulating the first steps of colonization in epithelial cells.

RNAseq analysis was used to measure transcriptome changes caused by LysR overexpression in *S. aureus* cultivated TSB in low oxygen levels, because RNA extracted from bacteria grown in milk didn't have the required quality. Significant alteration in the expression of 927 genes (2-fold change) was seen (Supporting data 2S). This represented 34.8% (927/2663) of *S. aureus* RF122 ORFs (GenBank: AJ938182); A total of 358 genes were down-regulated and 569 were up-regulated (Supporting data 2S). The most changed categories included those involved in cell envelope, transport and binding proteins, protein fate and purines, pyrimidines, nucleosides, and nucleotides metabolism (Figure 6). In addition, genes encoding hypothetical proteins represented 13.5 % (125/927) of those significantly altered.

Due to the reduced colonization seen in Figure 5, the expression of genes that could explain the observed phenotype was checked (Table 3). Among the down-regulated genes, we can highlight *tag*B (-7.3) and *tag*X (-11.2) related to teichoic acids synthesis, and *pbp*4 (-7.3) and *pbp*F (-6.0), penicillin-binding proteins, which are involved in peptidoglycan biosynthesis and cell growth (Table 3). Two genes essencial to capsule production, capE and capF, were down-regulated -6.2-fold and -5.0. But other capsule-related genes, *cap*K and *cap*D, were up-regulated 5.4-fold and 6.3-fold (table 3).

Among the adhesin-coding genes *fnb*A (fibronectin-binding protein) and *ebp*S (cell surface elastin binding protein) were up-regulated 4.5 and 3.3-fold, respectively. However, the overexpression of LysR promoted reduced expression of the *srt*A transcript (-3-fold), a sortase A protein required for cell-wall anchoring of LPXTG-containing proteins, like FnBPA, EbpS, and Isd protein family. Others cell wall-anchored proteins *isd*H and *isd*F were also more expressed in cells with 2209-pCN43 (Table 3). Proteins envolved in transport and

binding were also up-regulated like seven iron-related proteins *hts*C (3.9-fold), *fhu*B (4.3-fold), SAB0905 (4.3-fold), *isd*F (4.7-fold), sirA (6.0-fold), *isd*H (8.3-fold), and *hts*B (11.6-fold) (Table 3). Genes implicated in nitrogenous bases metabolism were highly up-regulated.

Another up-regulated genes group was implicated in purines, pyrimidines, nucleosides, and nucleotides metabolism (Supporting data 2S). Purine biosynthesis was up-regulated is several steps, genes like *pur*F, *pur*K, *pur*H, *pur*M and *pur*N increased at least 8-fold each one (Table 3). Transcription of adenosine synthase A (*ads*A) also showed significant alteration 8.8-fold (Table 3).

LysR overexpression was observed to up-regulate *sig*B (2.7-fold), but *asp*23, known indicator of SigB activity doesn't change. Maybe because the transcription of *rsb*W, anti-sigma B factor, was strongly stimulated (8.4-fold) (Table 3).

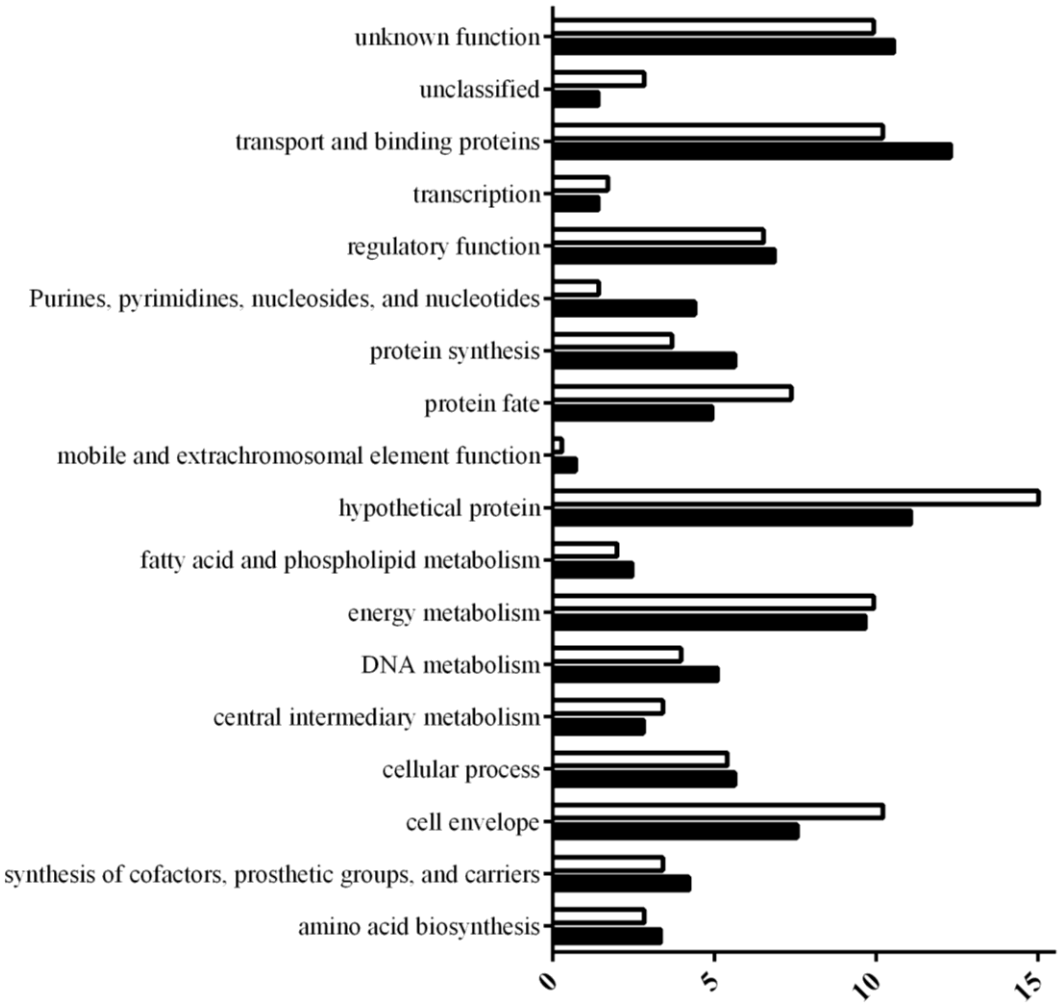

Figura 6. Functional categories of genes with ≥ 2-fold up-regulation (black bar) or down-regulation (white bar) in *Staphylococcus aureus* RF122 pCN43-SAB2209

Table 3. Relative expression of genes important for virulence in *Staphylococcus aureus*.

COL	RF122	Gene	Role	log2 fold (2325/pCN43)
SACOL0696	SAB0589	*tag*B	cell envelope	-7.3
SACOL0697	SAB0590	*tag*X	cell envelope	-11.2
SACOL0699	SAB0592c	*pbp*4	cell envelope	-7.2
SACOL0140	SAB0094	*cap*E	cell envelope	-6.2
SACOL1609	SAB1424c	*pbp*F	cell envelope	-6.0
SACOL2564	SAB2424c	*feo*B (ferrous iron transport protein B)	transport and binding proteins	-5.5
SACOL0141	SAB0095	*cap*F	cell envelope	-5.0
SACOL0860	SAB0748	*nuc* (thermonuclease)	DNA metabolism	-3.3
SACOL2539	SAB2402c	*srt*A (sortase A)	protein fate	-3.0
SACOL2054	SAB1949c	*sig*B	transcription	2.7
SACOL1522	SAB1343c	*ebp*S (elastin binding protein)	cell envelope	3.3
SACOL2167	SAB2058c	ferrichrome ABC transporter (*hts*C)	transport and binding proteins	3.9
SACOL0705	SAB0597	*fhu*B (ferrichrome transport permease)	transport and binding proteins	4.3
SACOL1045	SAB0905	probable iron transport protein	transport and binding proteins	4.4
SACOL2511	SAB2375c	*fnb*A (fibronectin-binding protein)	cell envelope	4.5
SACOL1144	SAB0998	*isd*F	transport and binding proteins	4.7
-	SAB0100	*cap*8K	cell envelope	5.5
SACOL0099	SAB0054c	*sir*A (iron-regulated lipoprotein)	transport and binding proteins	6.0
SACOL0139	SAB0093	*cap*D	cell envelope	6.3
SACOL1781	SAB1590c	*isd*H (iron-regulated surface protein)	regulatory function	8.3
SACOL2055	SAB1950c	*rsb*W (anti-sigma B factor)	transcription	8.4
SACOL1079	SAB0937	*pur*F	Purines, pyrimidines, nucleosides, and nucleotides	8.5
SACOL1074	SAB0932	*pur*K	Purines, pyrimidines, nucleosides, and nucleotides	8.6
SACOL0024	SAB0023	*ads*A (adenosine synthase)	Purines, pyrimidines, nucleosides, and nucleotides	8.8
SACOL1082	SAB0940	*pur*H	Purines, pyrimidines, nucleosides, and nucleotides	8.9
SACOL0150	SAB0104	*cap*O	cell envelope	9.2
SACOL1082	SAB0939	*pur*N	Purines, pyrimidines, nucleosides, and nucleotides	10.3
SACOL1080	SAB0938	*pur*M	Purines, pyrimidines, nucleosides, and nucleotides	10.4
SACOL2325	SAB2209	LysR	regulatory function	11.1
SACOL2166	SAB2057c	ferrichrome ABC transporter	transport and binding proteins	11.6
SACOL1073	SAB0931	purE	Purines, pyrimidines, nucleosides, and nucleotides	12.3

4. DISCUSSION

Staphylococcus aureus is a common cause of bovine mastitis. Research focused on the comprehension of its pathogenesis will certainly impact the development of new strategies to treat or prevent staphylococcal infections (Mulhbacher et al., 2010; Ster et al., 2013).

The transcriptional profile of four strains of *S. aureus* during cow mastitis was estabilished and identified several genes that might be related to the bacterial virulence (Allard et al. 2013). A LysR-type transcriptional regulator (SAB2209), was shown to be 5-fold more expressed during intramammary infection suggesting a role in bacterial pathogenesis. The several attemps done to construct a defective LysR strain failed and for this reason the inverse approach, the overexpression, was pursuited. From the assayes done here we suggest that LysR may negatively regulate biofilm production and colonization, precisely the invasion step.

S. aureus can express a broad range of virulence factors that play a fundamental role in adhesion, dissemination of the disease, and immune evasion. The adhesion to host components, such as matrix and plasma proteins (e. g. fibronectin and fibrinogen), is an early and essential step during microbial pathogenesis and biofilm formation (Otto, 2008; Sinha and Fraunholz, 2010). Several adhesins have been described as relevant for host colonization like ClfA, ClfB, FnBPA, FnBPB, Efb, Cna, SdrC, SdrD and SdrE (Foster et al., 2014). The invasion is mediated via fibronectin bridging between host integrins and staphylococcal surface proteins, FnBPA and FnBPB. FnBP mutants showed reduced virulence due to failure in initial ahesion (Dziewanowska et al., 1999; Brouillette et al., 2003). However, this could not explain the results found here since transcriptomics revealed a 4.5-fold regulation of *fnb*A. Genes encoding well-known virulence factors such as *spa*, *clf*A, *sdr*C, and *ebh* contain premature stop codons and thus are pseudogenes in the epidemic bovine strain RF122 (Herron-Olson et al., 2007).

FnBP is a cell wall-anchored (CWA) protein that, in similarity to other adhesins, contains a sorting signal LPXTG (Foster et al., 2014). The sortase enzyme (SrtA) cleaves the C-terminal cell wall-sorting signal (LPXTG) and catalyzes a covalent linkage to the peptidoglycan (Marraffini et al., 2006). In *S. aureus*, mutation in the *srtA* gene results in defective anchoring of several

proteins, including a number of surface-associated adherence factors infections (Mazmanian et al., 2000; Weidenmaier et al., 2008). For this reason, ΔsrtA bacteria are defective in the establishment of infections. In this study, a 3-fold down-regulation of *srtA* was seen in LysR transformants what may decrease sortase activity and weaken the adherence of *S. aureus* in host cells.

Genes involved in teichoic acid biosynthesis (*tagX*, *tagB*) were highly down-regulated (> 7 fold). Wall teichoic acid (WTA) polymers have previously been implicated in *S. aureus* attachment to nasal epithelial cells (Weidenmaier et al., 2004). Mutants ΔtagO are considerably less adherent to human endothelial cells and this attenuates virulence in a rabbit model (Weidenmaier et al., 2005). Besides being essential for cell viability, WTA contributes to adhesion and biofilm formation in *S. aureus* (Weidenmaier et al., 2005; Weidenmaier et al., 2008; Chan et al., 2013).

Biofilm formation was reduced in *S. aureus* RF122 pCN43 and may be related to the negative expression of *tagX*, *tagB*, and *srtA*. Biofilm development is a two-step process involving an initial attachment and a subsequent maturation phase, which are physiologically different from each other and require phase-specific factors (Otto, 2008). The attachment requires adhesins, like FnbA, to bind in host surface, while maturation requires production of polysaccharide intercellular adhesin (PIA) with other polymers such as teichoic acids and proteins to form an extracellular matrix (Otto, 2008).

Pathogens have developed specific iron acquisition systems to capture iron from environment and support bacterial growth during infection (Andrews et al., 2003). The mammary gland is an environment with very low concentrations of iron (10^{-18} M) and the metal is usually bound to the host's proteins. The iron-transport systems in *S. aureus* include SirABC, FhuABG, HtsABC, and Isd proteins (Dale et al., 2004; Skaar and Schneewind, 2004; Skaar and Schneewind, 2003; Sebulsky and Heinrichs, 2001). Iron-related proteins such as FhuB, SirA, IsdF, isdH, HtsC, HtsB, and SAB0905 showed up-regulation in cells overexpressing LysR, that may act as an activator in the control of these genes.

Capsule production was also affected by LysR, as revealed by *capE*, and *capF* down-regulation (-6.2-fold and -5.0-fold, respectively). These two enzymes seem to be essential for capsule production. Natural *cap*5E mutation in *S. aureus* NCTC 8325-4 and gene-specific mutation of *cap*5F in *S. aureus* Newman, renders

the strain CP5-negative (Wann et al., 1999; Kneidinger et al., 2003). To colonize the respiratory epithelium *Streptococcus pneumoniae* reduces the capsule production and promotes expression of certain surface-associated proteins and carbohydrate-containing cell wall structures. This strategy allows the intimate contact of pneumococci with host cells is associated with a reduction of capsular material, thereby unmasking surface-exposed adhesins and intensifying the host-pathogen interaction. The positive effect of capsule reduction on adhesion was not observed in this study, probably due to failure of other components, such as FnbA and sortase A, responsible for adherence affected by LysR.

Transcriptomics data revealed that LysR stimulated purine biosynthesis in *S. aureus* RF122 and up-regulated adenosine sythase (AdsA). *S. aureus* exploits the immunomodulatory attributes of signaling molecule adenosine to survive within neutrophils and escape host immune responses (Thammavongsa et al., 2009; Kim et al., 2012). This immune evasion strategy uses AdsA to convert adenosine monophosphate (AMP) into adenosine, a potent immunosuppressive signaling molecule. Studies have been demonstrated that during the infection wild-type *S. aureus* and adsA mutants are phagocytosed by neutrophils, however wild-type survives within neutrophils whereas adsA mutants are killed (Thammavongsa et al., 2009). Thus, up-regulate adenosine sythase might be an advantage to persist inside the host cells.

LysR up-regulated *sig*B expression (2.7-fold), which encodes σ^B, an alternative sigma factor involved in the response to environmental stresses such as during stationary phase, heat exposure and change in osmotic pressure (Pané-Farré et al., 2006; Hecker et al., 2007). However, expression of the gene *asp23*, an direct indicator of the σ^B activity, was not altered. This could be due to the up-regulation (8.4-fold) of *rsb*W, an anti-σ^B protein. Under non-stress conditions σ^B is held in an inactive complex by its antagonist RsbW. Following stress, RsbW is antagonized by the anti-anti-sigma factor RsbV, releasing σ^B free to interact with core RNA polymerase (Petersohn et al., 2001; Hecker et al., 2007).

Attempts to identify potential LysR box on intergenic regions of the differentially expressed genes failed. LTTR box consists of the sequence T-N_{11}-A, but can vary in both base pair composition and length (Maddocks and Oyston, 2008). There are multiple binding sites within the intergenic region between an LTTR and its associated gene/operon (or upstream of distant LTTR-regulated

genes). Broadly they bind at 235 to +20 bp (regulatory binding site, RBS, and autoregulatory site) and 240 to 220 bp (activation binding site, ABS) (Belitsky et al., 1995; Lochowska et al., 2001; Porrúa et al., 2007). However, binding sites as far away as 2218 bp with respect to the promoter region as well as internal binding sites (+350 bp; IBS), have been identified (Wilson et al., 1995; Viswanathan et al., 2007). A different strategy will be necessary to show an interaction between LysR and genes described above.

5. CONCLUSION

Here we described changes caused by the overexpression of a new transcriptional regulator that belongs to the LysR family in *S. aureus*. The results suggest that SAB2209 (SACOL2325) influences initial steps in *S. aureus* colonization and immune evasion, by altering the expression of genes associated with cell wall function and evasive strategies. Also, the alterations in the transcriptome of *S. aureus* induced by LysR may allow bacteria to adapt to changing conditions in the udder environment and increase bacterial survival inside host cells.

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APÊNDICE

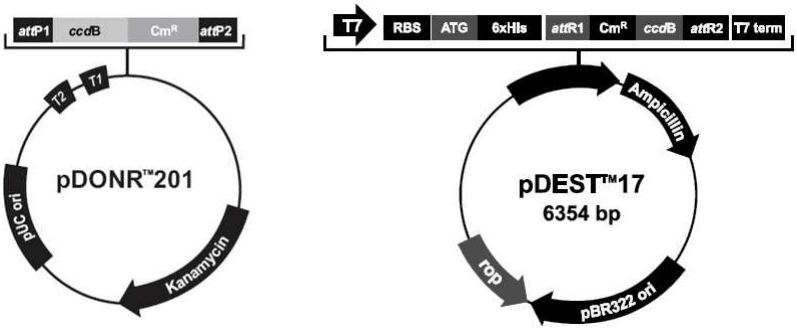

Figura 1A. Mapas físicos do vetor de entrada pDONR 201 e do vetor de expressão pDEST 17 (Invitrogen). No vetor de entrada pDONOR201 estão esquematizados os sítios *att*P1 e *att*P2, que recombinam com sítios attB1 e attB2, na reação BP; gene de resistência a canamicina (Knr); origem de replicação (pUC ori). No vetor de destino pDEST17 estão esquematizados os sítios *att*R1 e *att*R2, que recombin am com sítios *att*L1 e *att*L2, na reação LR; gene de resistência à ampicilina (Apr); origem de replicação (pBR322 ori) (Fonte: Invitrogen).

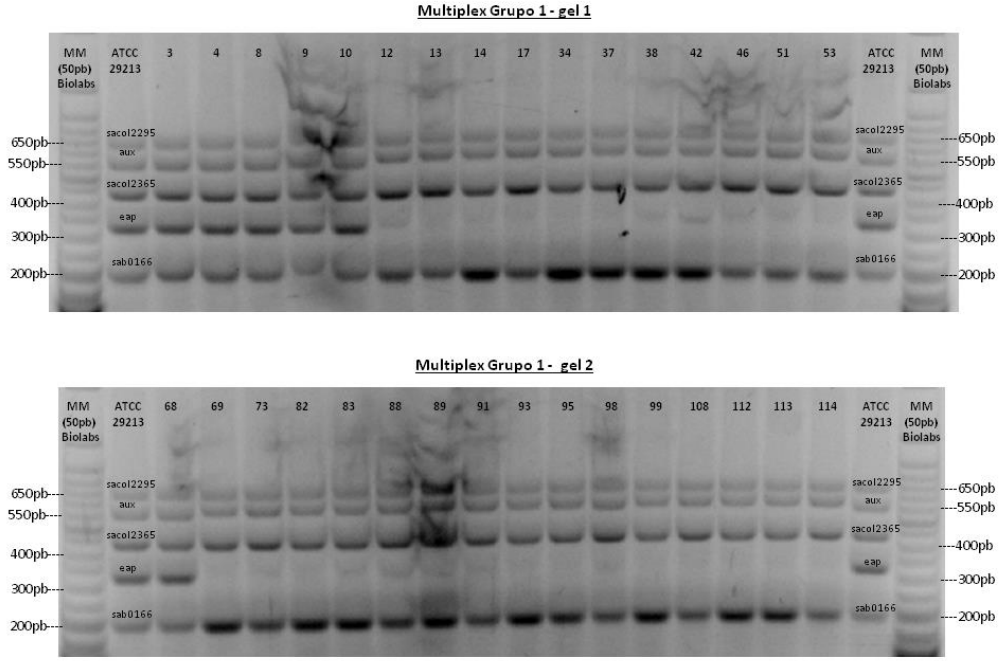

Figura 2A. Avaliação da presença dos genes do grupo1 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%.

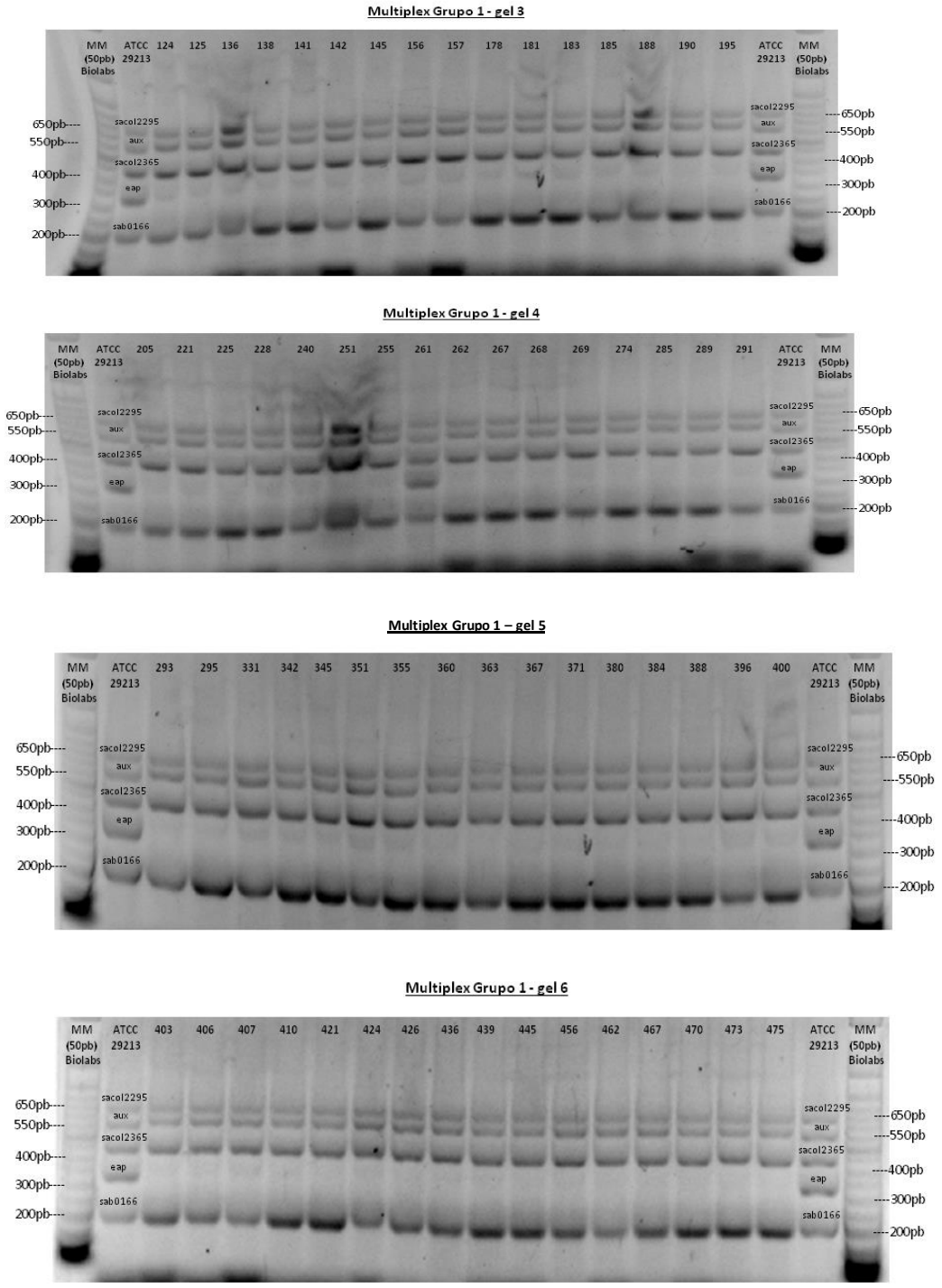

Figura 3A. Avaliação da presença dos genes do grupo1 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%.

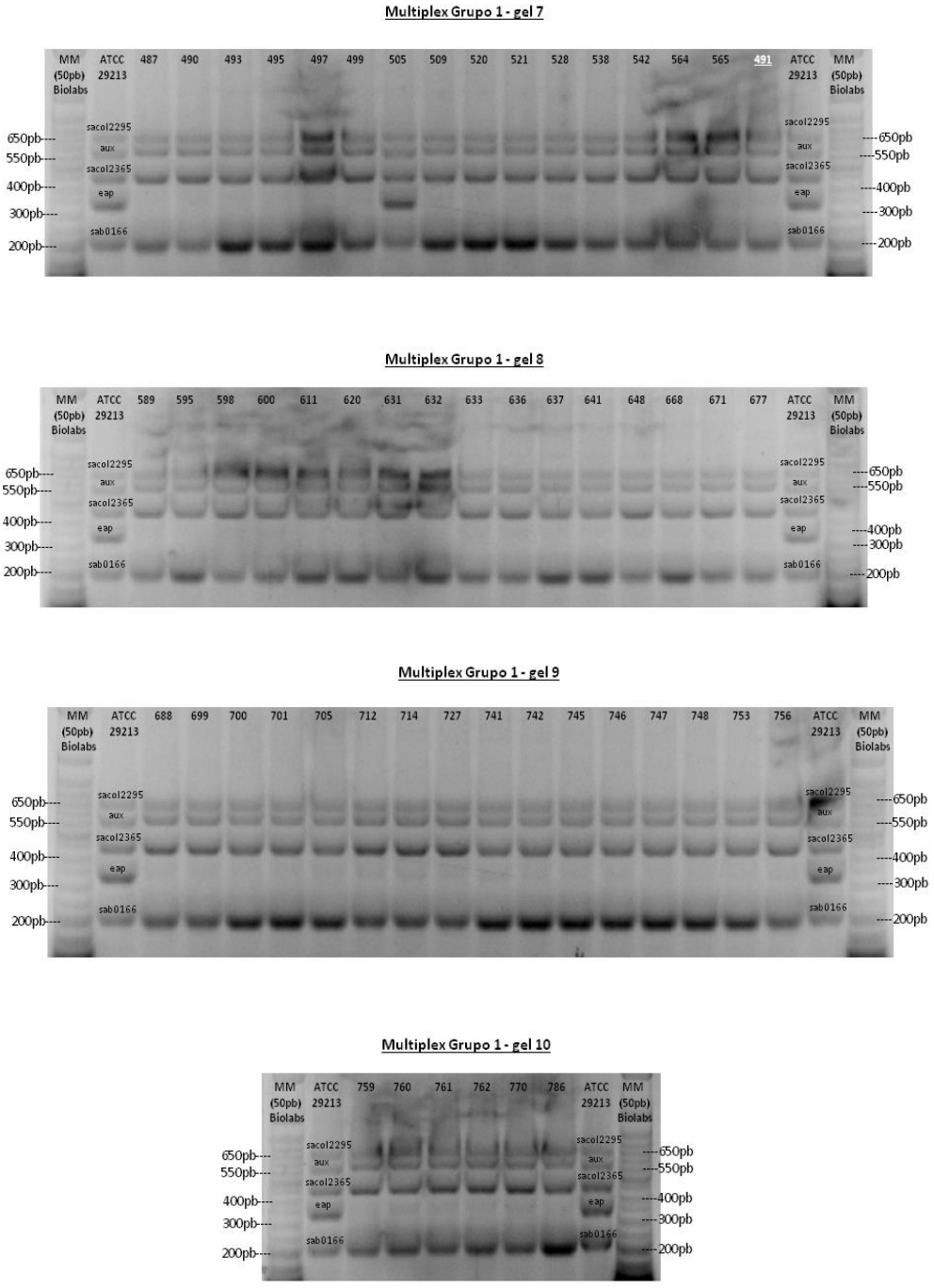

Figura 4A. Avaliação da presença dos genes do grupo1 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%.

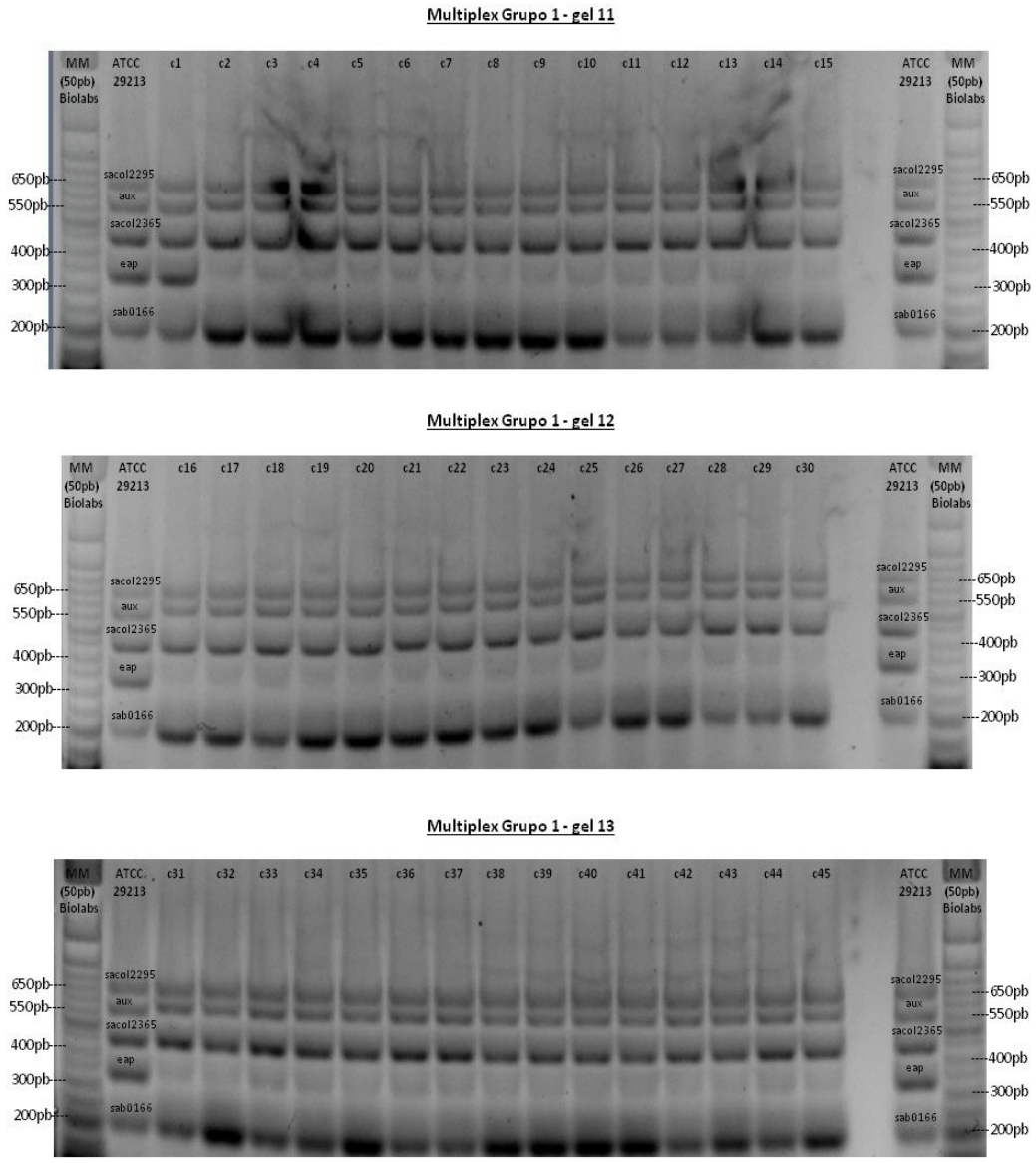

Figura 5A. Avaliação da presença dos genes do grupo1 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%

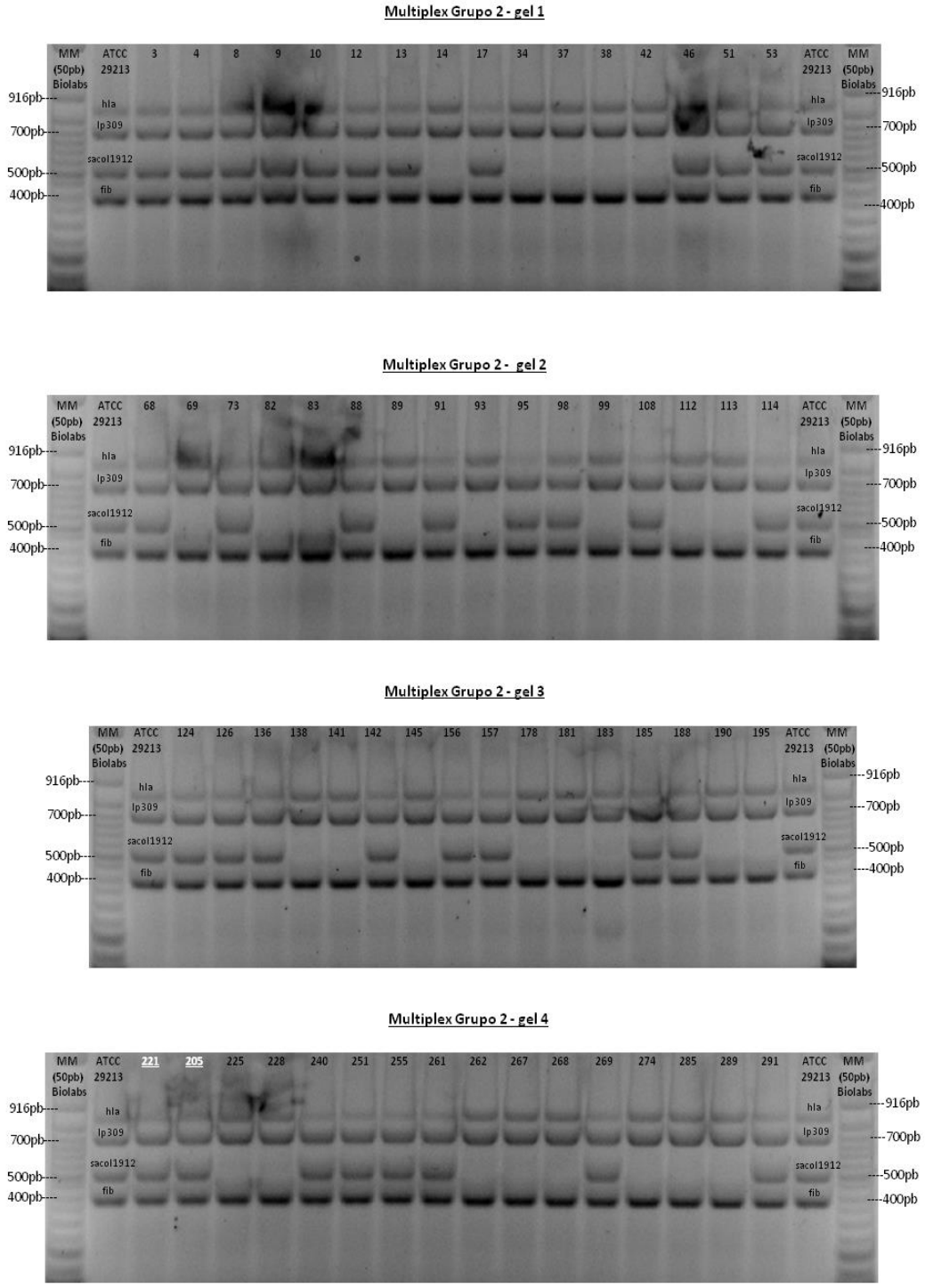

Figura 6A. Avaliação da presença dos genes do grupo 2 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%.

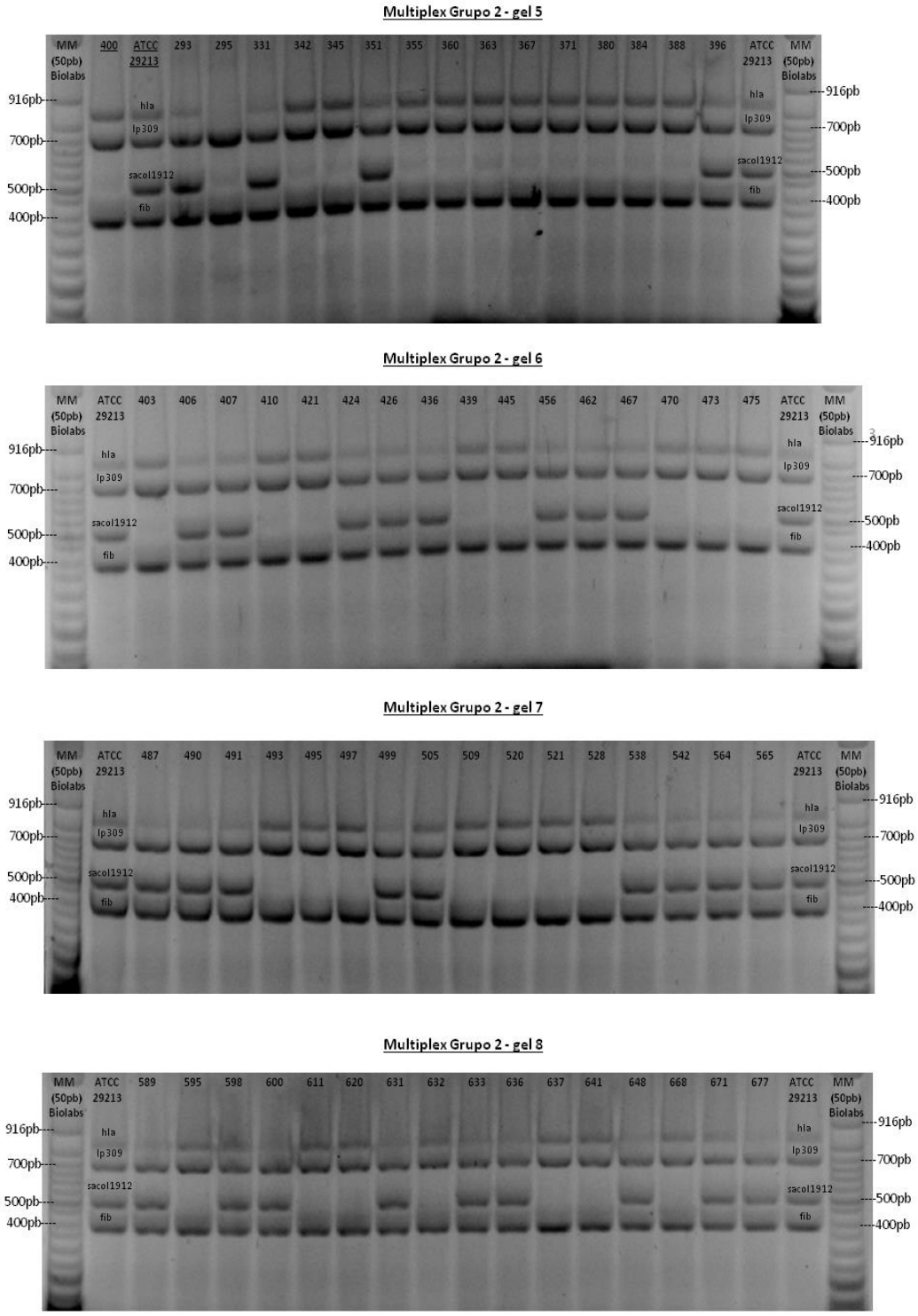

Figura 7A. Avaliação da presença dos genes do grupo 2 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%.

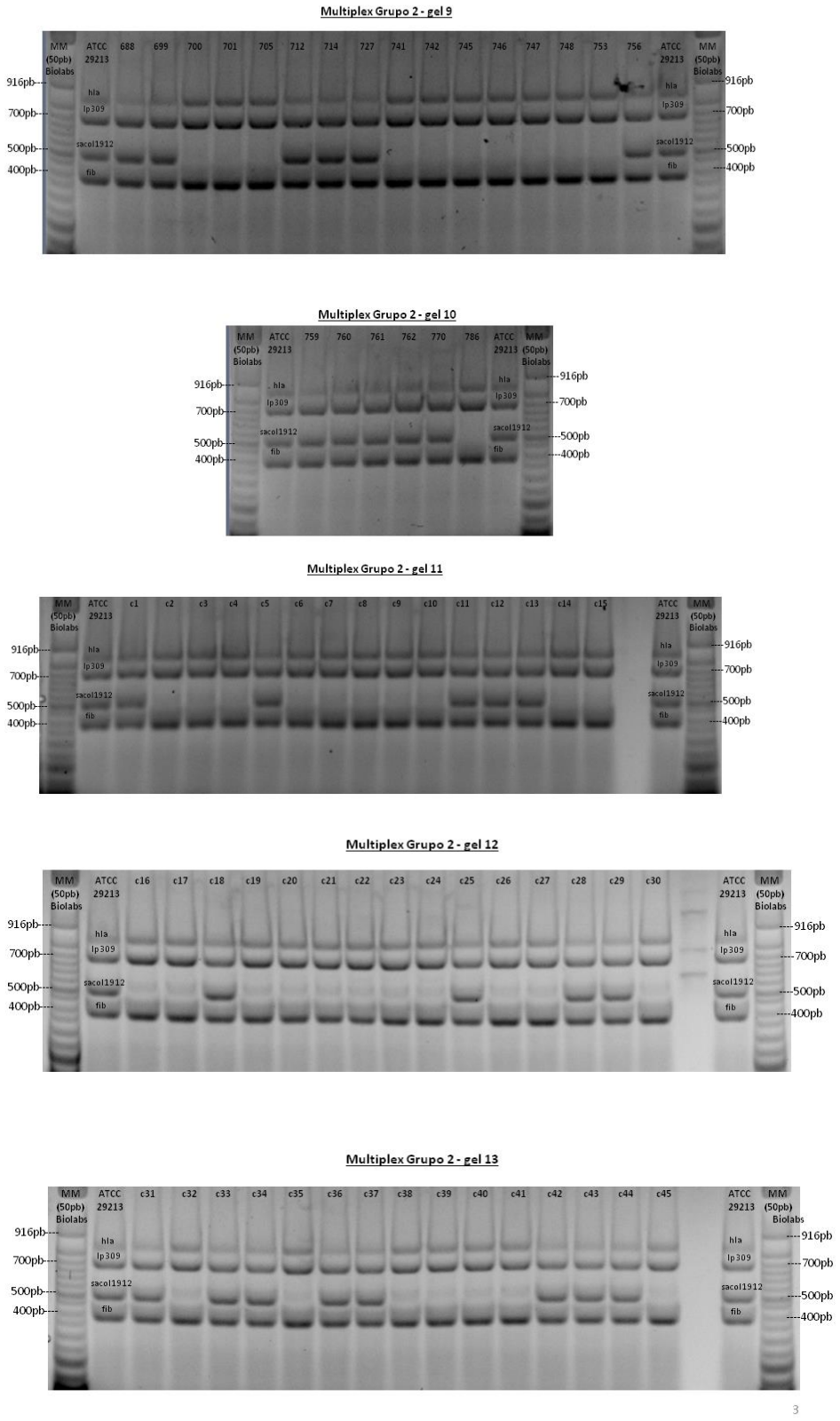

Figura 8A. Avaliação da presença dos genes do grupo 2 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%.

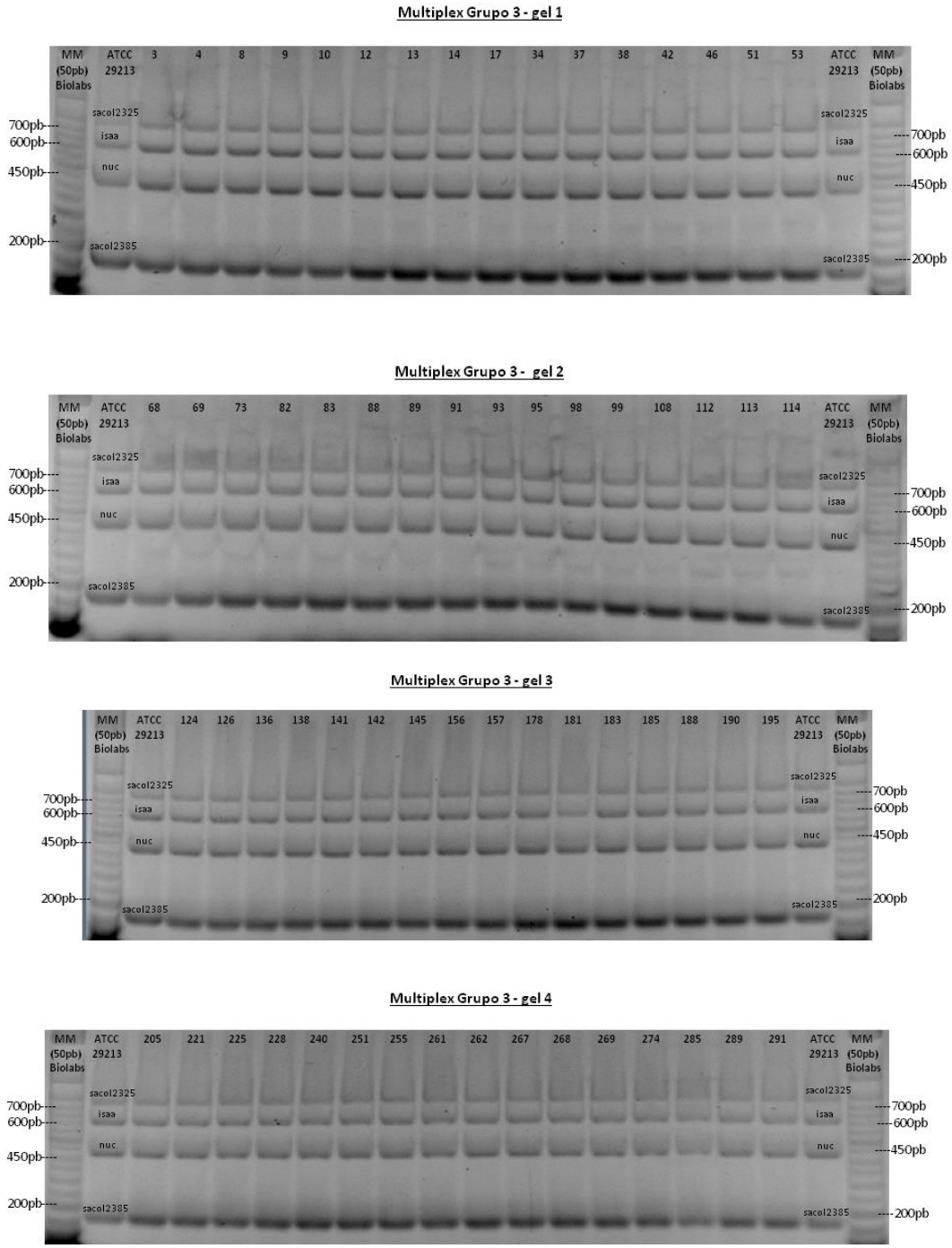

Figura 9A. Avaliação da presença dos genes do grupo 3 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%.

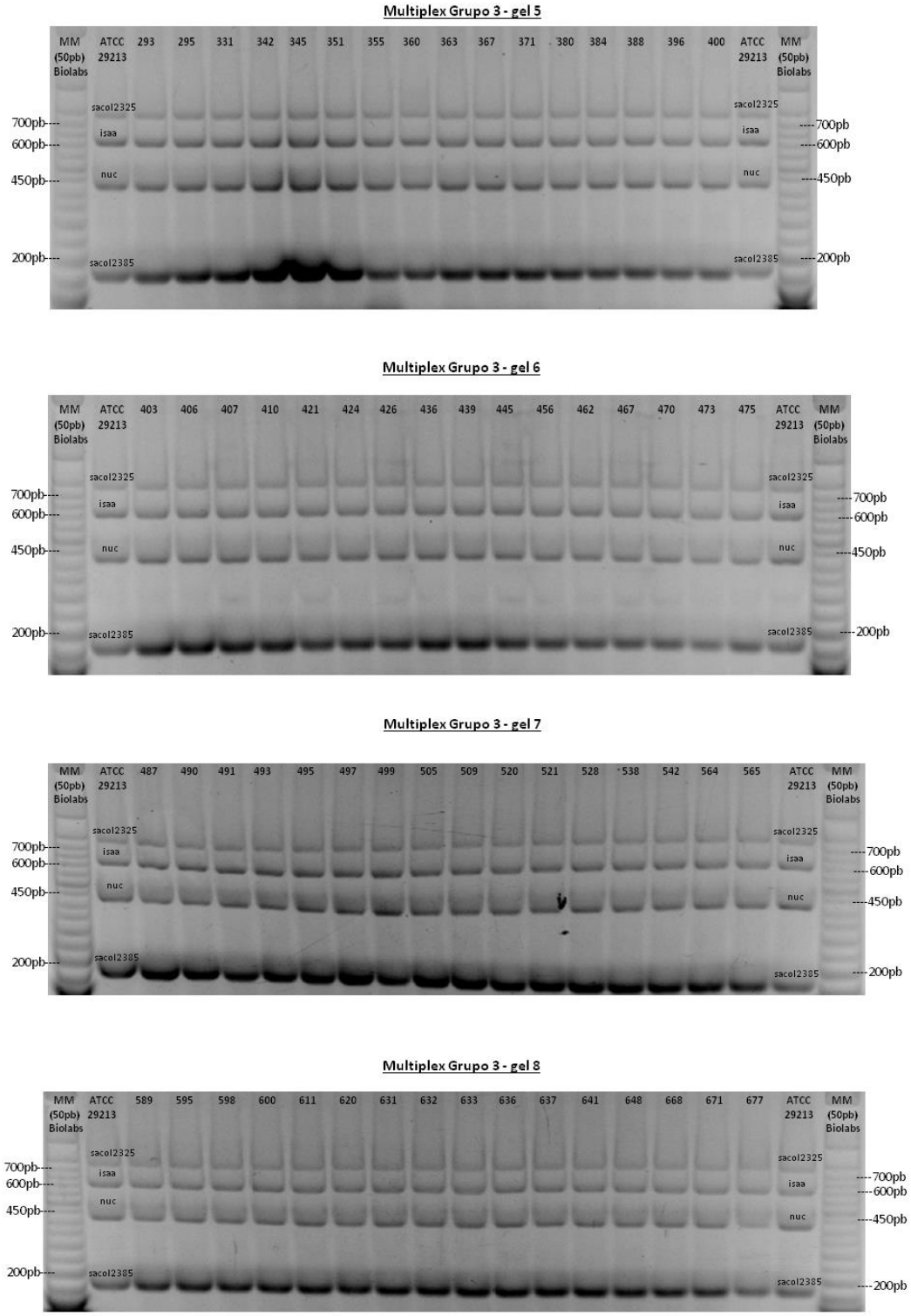

Figura 10A. Avaliação da presença dos genes do grupo 3 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%.

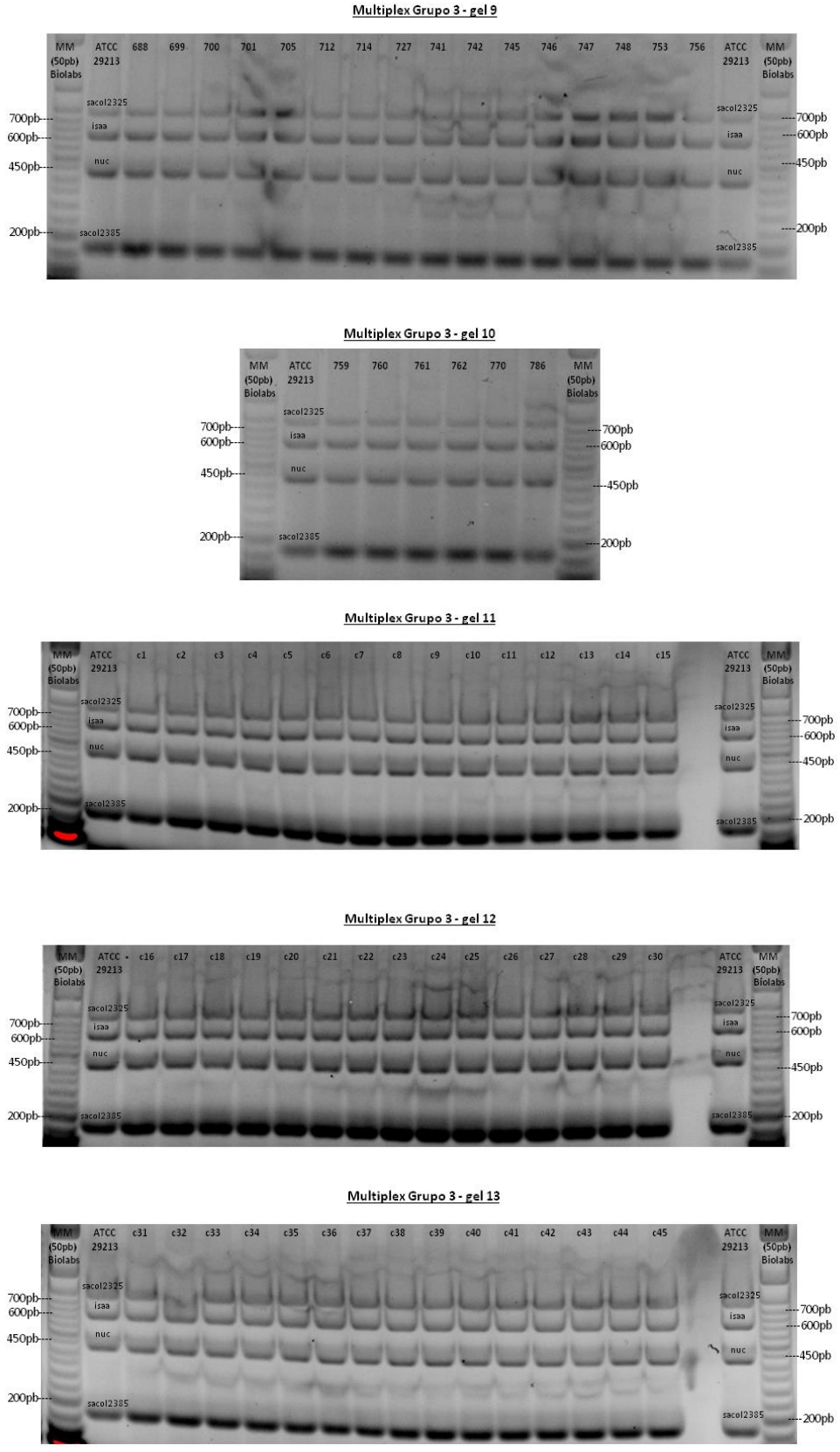

Figura 11A. Avaliação da presença dos genes do grupo 3 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%.

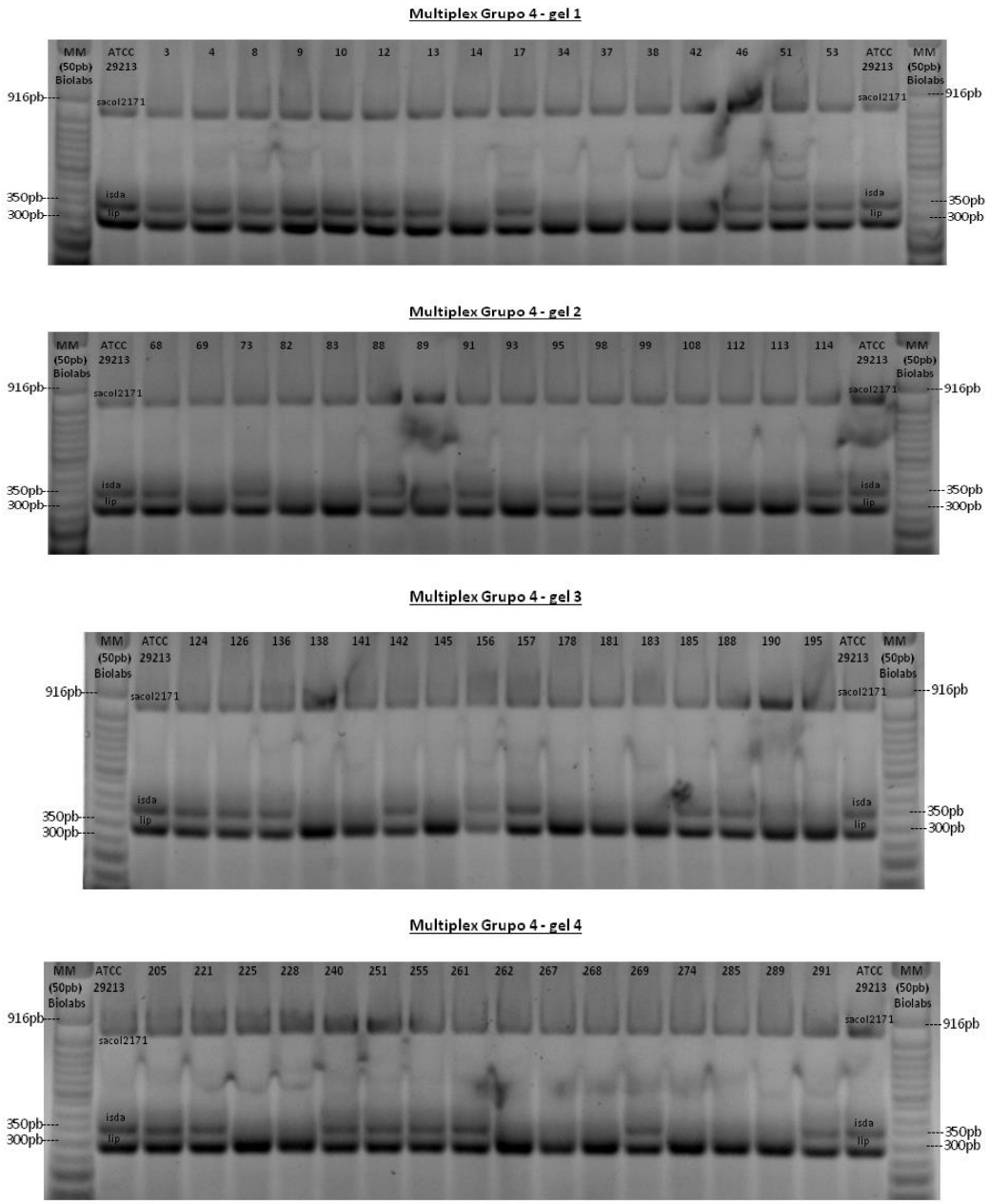

Figura 12A. Avaliação da presença dos genes do grupo 4 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%.

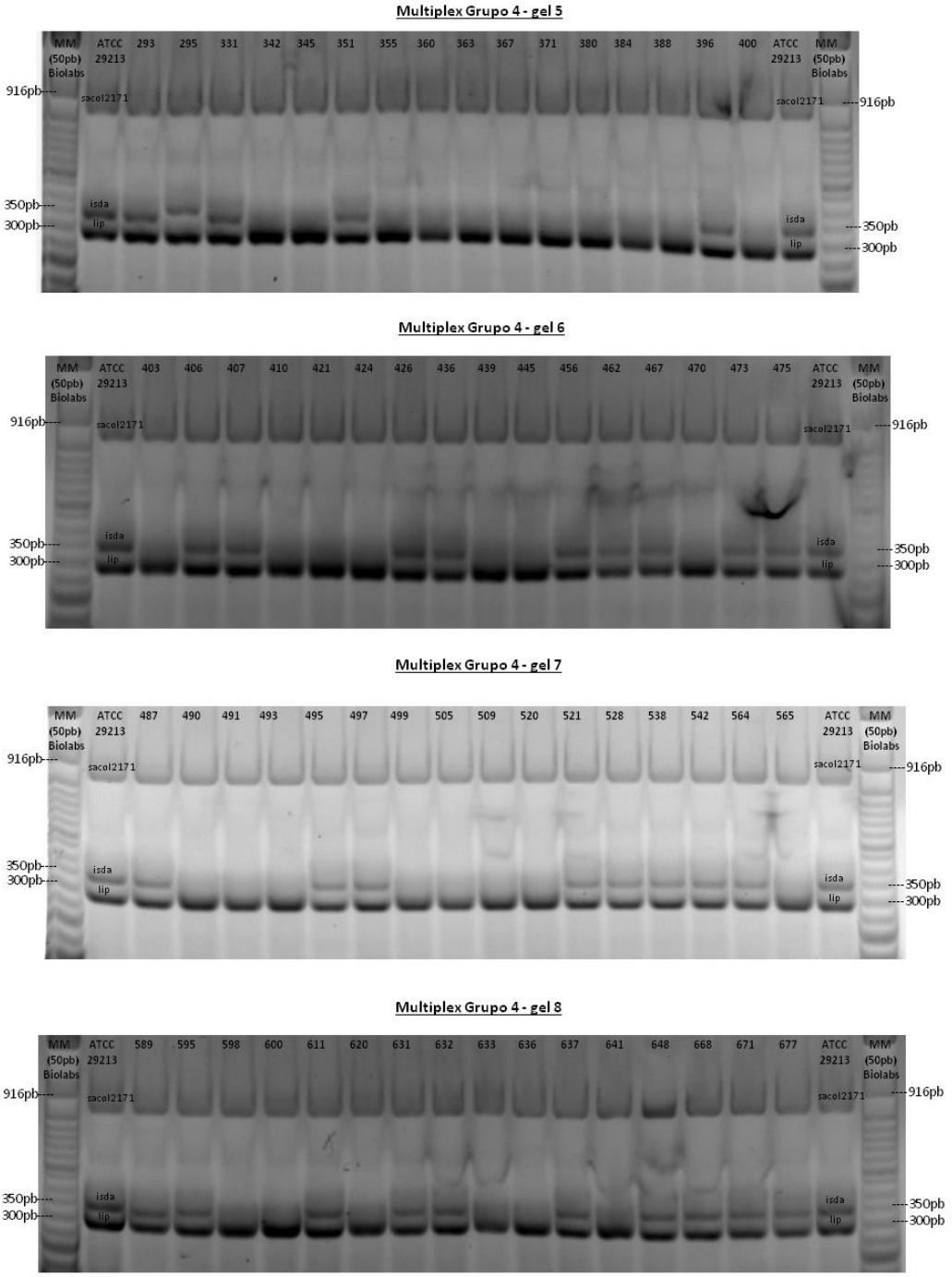

Figura 13A. Avaliação da presença dos genes do grupo 4 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%.

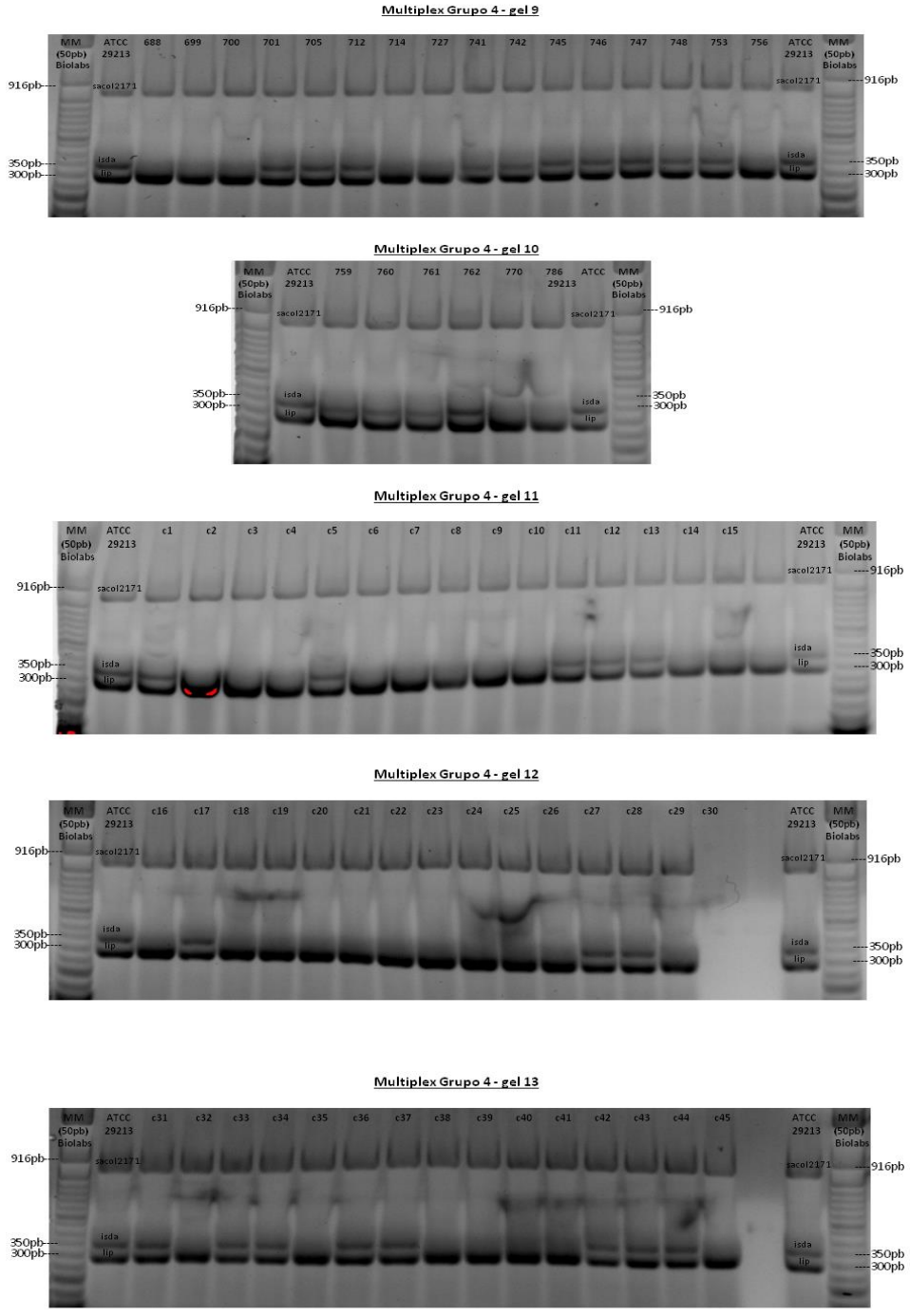

Figura 14A. Avaliação da presença dos genes do grupo 4 em isolados de *Staphylococcus aureus*, por meio de análise eletroforética, em gel de agarose 1,5%.

SUPPORTING DATA

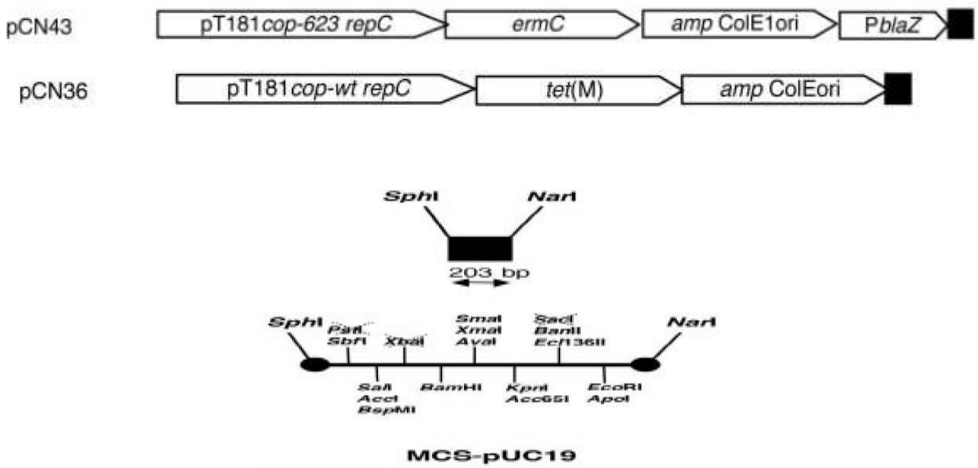

FIG. 1S. Cassette restriction maps of shuttle vector pCN series. The gene designations include pT181 *cop-wt repC*, replicon for replication in *Staphylococcus*. Each pT181 cassette contains the single-stranded origin of replication, double-stranded origin of replication, the copy control system, and the *repC* gene encoding the replication protein RepC of the pT181 rolling-circle replicon. Antibiotic resistance modules consist *ermC* (ribosomal methylase-encoding gene of pE194 for erythromycin resistance). Additional modules include *amp* ColE1 ori (*bla* gene conferring ampicillin resistance and ColE1ori for replication in *E. coli*), P*blaZ* constitutive -lactamase promoter module. The MCS from pUC19 is symbolized by a black box.

Supporting data 2S. Expression of all genes in *Staphylococcus aureus* analyzed by RNAseq.

ORF COL	ORF RF122	Gene name/ function	pCN43	pCN43-2209	log2 fold 2209/pCN43
0001	0001	dnaA	24,3556	253,5	3,37966
0004	0004	recF (DNA replication and repair protein)	566,153	4177,12	2,88325
0007	0007c	conserved hypothetical protein	427,863	3,69873	-6,85398
0009	0009	serS (seryl-tRNA synthetase)	0,91297	7,06441	2,95193
0011	0011	probable membrane protein	28908,8	19,0233	-10,5695
0013	0013	hypothetical protein	1116,35	94,5595	-3,56142
0022	0014	conserved hypothetical protein	301,273	1498,24	2,31413
0015	0015	rplI (50S ribosomal protein L9)	2,63948	571,985	7,75958
0018	0017	purA (adenylosuccinate synthetase)	21,3995	340,238	3,9909
0019	0018	yycF (two-component response regulator)	17,2123	111,399	2,69423
0020	0019	yycG (two-component sensor histidine kinase)	177,062	7,0205	-4,65654
0021	0020	yycH	137,799	815	2,56423
0023	0022	yycJ (metallo-beta-lactamase)	129,903	0,82870	-7,29236
0024	0023	probable 5 nucleotidase	7,11657	3135,28	8,7832
	0024	conserved hypothetical protein	1734,79	30,7326	-5,81885
0061	0028c	conserved hypothetical protein	96,3929	1,05649	-6,51158
0064	0031	probable hydroxyacylglutathione hydrolase	1,16132	181,482	7,28791
0065	0032	probable sulfide-quinone reductase	30,5946	957,696	4,96822
0067	0033c	probable nitrogen regulation protein	30,0962	811,26	4,75251
0084	0041	probable transriptional regulator	194,517	0,97689	-7,63747
0085	0042	aminoacylase	3,01317	495,669	7,36195

0088	0044c	probable membrane protein	81,248	1610,47	4,30901
0089	0045c	myosin-crossreactive streptococcal antigen-like protein	22,5032	797,464	5,14722
0092	0048	hypothetical protein	9,66453	0,34170	-4,82187
0097	0052c	sirC (siderophore transport protein	2485,02	1,29456	-10,9066
0099	0054c	sirA (iron-regulated lipoprotein)	0,030535	2,03999	6,06195
0103	0058	probable membrane transport protein	3,3155	0,58013	-2,51477
0111	0066	acetoin (diacetyl) reductase	7945,93	863,494	-3,20196
0118	0072	sodM (superoxide dismutase [Mn/Fe])	1,61324	0,1778	-3,18163
0119	0073	surface protein	47,8	233,297	2,28709
0120	0074c	probable transriptional regulator	172,384	3,12509	-5,78558
0122	0076	capsular polysaccharide synthesis enzyme CapC	0,22715	35,5654	7,29069
0124	0078	drm (phosphopentomutase)	13,352	65,0746	2,28504
0127	0083c	transport system protein	0,04377	0,87622	4,32296
0130	0086	cpdB	60,0461	500,495	3,05921
0136	0090	capA	2,64026	266,678	6,65828
0139	0093	capD (capsular polysaccharide synthesis enzyme	6,27204	509,099	6,34287
0140	0094	capE	153,805	2,08188	-6,20707
0141	0095	capF	22,1858	0,67125	-5,04663
	0100	cap8K	0,255254	11,4024	5,48126
0150	0104	capO	1,98562	1153,94	9,18277
0154	0108	aldA (aldehyde dehdydrogenase)	54,538	10,4991	-2,377
0155	0109	probable cation-efflux system membrane protein	649,305	156,23	-2,05523
0157	0111	hypothetical protein	5,24606	0,26028	-4,33305
	0113	probable lipoprotein	41,4005	0,79277	-5,7066
0161	0116	conserved hypothetical protein	1199,62	47,1462	-4,6693
0165	0120	4-phosphopantetheinyl transferase superfamily protein	0,176412	6,01599	5,09178
0166	0121c	probable integral membrane protein	744,506	13,161	-5,82194
0167	0122c	probable acetylglutamate kinase	0,044041	0,88183	4,32358
0171	0126c	probable branched-chain amino acid transport system carrier protein	25,5961	1,31148	-4,28666
0175	0129c	glcA (phosphotransferase system enzyme II glucose-specific factor IIA)	19,7661	2657,4	7,07084
0176	0130	conserved hypothetical protein	327,449	1989,44	2,60302
0177	0131	probable glucokinase regulator-related protein	590,755	8586,3	3,8614
0179	0133	probable transcriptional regulator RpiR family	162,919	2607,79	4,0006
0180	0135	type I restriction enzyme restriction chain	4,29136	72,6033	4,08053
	0144c	0144c	0,734189	187,296	7,99495
0185	0145	oligopeptide ABC transporter permease protein	0,756837	190,29	7,974
0187	0147	RGD-containing lipoprotein	2,2349	0,33260	-2,74834
0197	0157	probable NADH-dependent dehydrogenase	0,028636	0,81734	4,83502
0199	0159c	probable membrane protein	2921,07	2,3842	-10,2588
0202	0162c	probable two-component sensor histidine kinase	1,89927	855,964	8,81596
0204	0164	formate acetyltransferase	538,156	10612,2	4,30156
0205	0165	formate acetyltransferase activating enzyme	65,403	5056,86	6,27274
0211	0170c	3-ketoacyl-CoA transferase	0,023899	1,2815	5,74473
0215	0174c	acetyl-CoA/acetoacetyl-CoA transferase	1,58361	1448,53	9,83716
0217	0176c	probable dipeptide-binding ABC transporter	2,98808	0,46414	-2,68656
0220	0179c	flavohemoprotein	139,592	15,6004	-3,16157
0236	0190	probable 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	29,1685	2,28253	-3,6757
	0191	0191	40,4596	2,1931	-4,20544

0239	0193	teichoic acid biosynthesis protein F	1,59176	21,2194	3,73669
0243	0197	probable glycosyl transferase	1216,26	242,815	-2,32453
0244	0198	scdA (cell division and morphogenesis-related protein)	24,2521	497,384	4,35818
0245	0199	lytS (two-component sensor histidine kinase)	25,5863	1226,19	5,58267
0247	0201	lrgA (holin-like protein A)	1,41488	15,7793	3,47928
0249	0203c	probable transcriptional regulator GntR family	2,89259	1792,1	9,27508
0250	0204	probsble PTS system transport protein	210,576	1,92601	-6,77258
0251	0205	bglA (6-phospho-beta-glucosidase)	2,94134	1495,16	8,98961
0252	0207c	probable methyltransferase	0,044845	41,6528	9,85923
0259	0212	hypothetical protein	7,34655	0,55846	-3,71754
0271	0223	conserved hypothetical protein	12,1198	1,83036	-2,72717
0276	0228	probable DNA segregation ATPase and related protein	7,58487	1,16606	-2,70148
	0233	0233	7,7295	0,31787	-4,60386
0301	0242c	probable formate/nitrite transport protein	2,37242	870,174	8,5188
0306	0246	probable ATP-binding ABC transporter	11,3456	5012,16	8,78716
0307	0247	probable perfringolysin O regulatory protein	20,0663	1697,09	6,40215
0310	0250	probable nucleoside transporter permease	0,046442	7,21824	7,28006
0311	0251c	probable transport protein	1,63781	1784,78	10,0898
0312	0252c	N-acetylneuraminate lyase subunit	0,440002	155,51	8,46528
0314	0254c	probable transcription regulator	7,16886	29,765	2,0538
0316	0256c	probable membrane protein	0,548281	0,06256	-3,13155
2694	0257	geh (glycerol ester hydrolase)	2,55142	1300,14	8,99315
0384	0261	hypothetical phage-related protein	125,119	745,552	2,57501
0387	0264	hypothetical phage-related protein	18,0167	84,0778	2,22239
	0267	0267	4,93096	182,852	5,21266
0391	0271c	conserved hypothetical protein	0,640714	3798,2	12,5333
0397	0276	conserved hypothetical protein	1,31594	2251,3	10,7404
0407	0286c	glycerol-3-phosphate transporter	9,50687	1,63433	-2,54027
0408	0287	probable glyoxylase family protein	122,901	1,4354	-6,4199
0410	0289	probable NADH-dependent FMN reductase	3423,95	3,77739	-9,82406
0416	0295	conserved hypothetical protein	0,562574	213,15	8,56561
0421	0300	hypothetical protein	0,049683	1,31629	4,72756
0426	0304	th1 (acetyl-CoA acetyltransferase)	1,3269	1045,27	9,62161
0428	0306c	metE1 (5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase)	3,46241	403,3	6,86393
0435	0314	GTP-binding protein	103,732	4568,32	5,46074
0438	0317	ssb (single-strand DNA-binding protein)	2405,18	70,1387	-5,09979
0325	0325	conserved hypothetical protein	182168	14859,3	-3,61583
0448	0327c	hypothetical protein	1,98943	8,91515	2,16391
0449	0328c	hypothetical protein	1,3835	1351,77	9,93231
0452	0331c	ahpC (alkyl hydroperoxide reductase subunit C)	814,686	3575,4	2,13379
	0334c	hypothetical protein	481,546	5,22891	-6,52502
0455	0335c	hypothetical protein	52,6916	1977,65	5,23007
0456	0336c	hypothetical protein	49,7429	3577,9	6,16848
0457	0337c	hypothetical protein	477,872	3605,29	2,91542
0459	0339	pbuX (xanthine permease)	222,66	1160,41	2,38172
0460	0340	guaB	1,67459	353,563	7,72202
0461	0341	guaA	2107,4	331,736	-2,66736
0887	0364c	sel (staphylococcal enterotoxin L)	233,998	1523,41	2,70273
	0367c	hypothetical protein	355,385	37,1772	-3,2569
0470	0378	set9	1421,53	330,956	-2,10273
0494	0402	NADH dehydrogenase subunit 5	0,22179	1,47882	2,73718

0499	0407	conserved hypothetical protein	17,7432	1,36748	-3,69767
0501	0408	probable sodium-dependent transporter	0,020621	0,19350	3,23013
0507	0414	probable autolysin	32,4121	1683,15	5,69848
0511	0418c	probable membrane protein	3,5049	635,307	7,50194
0512	0419c	probable membrane protein	1790,81	1,27067	-10,4608
0514	0421	gltA (glutamate synthase large subunit)	1,56644	7,69248	2,29596
0516	0423	PTS system trehalose-specific IIBC component	329,795	3149,85	3,25565
020	0427	dnaX (DNA polymerase III gamma and tau subunits)	512,231	119,157	-2,10393
0523	0430	probable lysine decarboxylase	17,5474	479,941	4,77353
0526	0433	holB (probable DNA polymerase III delta prime subunit)	1,45758	1186,91	9,66943
0527	0434	conserved hypothetical protein	62,5567	4,8992	-3,67454
0439	0439	metG (methionyl-tRNA synthetase)	24,0152	630,158	4,7137
0534	0440	probable sec-independent hydrolase	392,812	3789,99	3,27028
0536	0442	dimethyladenosine transferase	4,46284	0,29270	-3,93045
0538	0444	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	28,8467	1355,51	5,55429
0541	0447	spoVG (stage V sporulation protein G)	932,626	5841,87	2,64706
0545	0450	prs (50S ribosomal protein L25 general stress protein)	12,0045	118,534	3,30366
0549	0455	probable tetrapyrrole methylase	8538,49	1823,16	-2,22754
0552	0457	polyribonucleotide nucleotidyltransferase	7,85584	66,6883	3,0856
0554	0459	hpt (probable hypoxanthine-guanine phosphoribosyltransferase)	0,081596	5,87901	6,17092
0556	0461	probable heat shock chaperonin protein 33	9,41886	183,25	4,28212
0557	0462	cysteine synthase	70,0117	1539,1	4,45835
0563	0468c	transcriptional regulator of GntR family	1,29079	1529,75	10,2108
0564	0469	probable pyridoxine biosynthesis protein	878,16	55,8907	-3,9738
0565	0470	probable pyridoxine biosynthesis protein	337,554	10,7087	-4,97827
0566	0471c	nupC (pyrimidine nucleoside transport protein)	25,5599	2705,44	6,72584
0567	0472	probable transcriptional repressor of stress genes	17,2627	6621,04	8,58326
0568	0473	conserved hypothetical protein	29,5468	1117,53	5,24117
0569	0474	ATP guanido phosphotransferase family protein	2577,99	2,32705	-10,1135
0572	0476	radA (DNA repair protein)	30,734	1,76792	-4,11971
0574	0478	gltX (glutamyl-tRNA synthetase)	7,89391	719,657	6,51043
0575	0479	cysE (Serine acetyltransferase)	2131,68	25,8593	-6,36516
0576	0480	cysS (cysteinyl-tRNA synthetase [])	144,205	1,94808	-6,20993
0582	0487	nusG (transcription antitermination protein)	71,0783	5,90581	-3,5892
0583	0488	rplK (50S ribosomal protein L11)	111,333	1473,47	3,72626
0584	0489	rplA (50S ribosomal protein L1)	1158,87	181,466	-2,67494
0586	0491	rplL (50S ribosomal protein L7/L12)	1083,22	107,264	-3,33608
0591	0496	rpsL (30S ribosomal protein S12)	36,2613	14338,8	8,62728
0592	0497	rpsG (30S ribosomal protein S7)	9,62645	136,923	3,83022
0595	0500c	N-acyl-L-amino acid amidohydrolase	34,6686	210,33	2,60095
0596	0501	kbl (probable 2-amino-3-ketobutyrate coenzyme A ligase)	5,82482	3286,1	9,13995
0599	0504	probable L-threonine 3-dehydrogenase	1,06574	797,27	9,54706
0600	0505	branched-chain amino acid aminotransferase	29,698	6,82406	-2,12167
0603	0507c	deoxypurine kinase subunit	41,2442	228,468	2,46973
0607	0511	conserved hypothetical protein	1,56443	144,061	6,52489
0613	0516c	conserved hypothetical protein	10,3131	70,0363	2,76362
0614	0517c	conserved hypothetical protein	1,87132	16,8281	3,16874
0615	0518c	conserved hypothetical protein	1904,08	7,26464	-8,03399
0616	0519	glucosamine-6-phosphate deaminase	49,0197	8,21866	-2,57639

0618	0521	probable 6-phospho-3-hexuloisomerase	275,662	7,17518	-5,26374
0620	0523	proP (proline/betaine transporter)	23,4954	113,512	2,2724
	0524	probable membrane protein	359422	10563,3	-5,08855
0622	0526	acetyl-CoA-acetyltransferase	1006,69	1,6029	-9,29472
0625	0529c	hypothetical protein	7082,12	218,002	-5,02177
0627	0531	ung (uracil-DNA glycosylase)	0,056984	0,29203	2,35747
0630	0534	probable membrane-embedded amino acid transporter	328,672	2440,68	2,89256
0637	0535c	probable membrane protein	0,120174	38,6361	8,32868
0634	0538	pta (phosphotransacetylase)	7,86521	63,7524	3,01892
0635	0539	lipoate-protein ligase A protein	2817,51	7,56696	-8,54049
0638	0542	mvaK2 (phosphomevalonate kinase)	1,39717	280,618	7,64996
0640	0544c	probable mercury(II) reductase	0,377528	68,6351	7,50622
0655	0552	probable oxidoreductase	15,5878	1,38352	-3,494
0658	0555	conserved hypothetical protein	126,946	1,92313	-6,04462
0659	0556	conserved hypothetical protein	3,45664	0,27848	-3,63368
0663	0559	arginyl-tRNA synthetase	1,38354	17,9556	3,698
0668	0565	probable hydrolase	824,89	96,4176	-3,09683
0682	0576	probable Na+/H+ antiporter protein	2,66786	670,512	7,97343
0687	0580	sodium-hydrogen exhange family protein	119,593	647,398	2,43652
0691	0584	mntR (iron dependent repressor)	336,979	4033,07	3,58115
0695	0588	tagG (teichoic acid translocation permease protein)	1303,51	9654,4	2,88878
0696	0589	tagB	2358,99	14,7847	-7,31792
0697	0590	teichoic acid biosynthesis enzyme	1478,52	0,63916	-11,1757
0699	0592c	pbp4	77,8326	0,52898	-7,20101
0701	0594	nucleoside transport protein	18,7481	1,55447	-3,59224
0703	0595	probable membrane protein	101,167	0,90574	-6,80343
0704	0596	fhuA (ferrichromeABC transporter ATP binding protein)	911,203	1,66746	-9,09398
0705	0597	fhuB (ferrichrome transport permease)	0,030037	0,58785	4,29063
0710	0602	conserved hypothetical protein	2935,11	311,098	-3,23797
0720	0611	0611(VraG)	2,22979	0,34319	-2,69981
0721	0612	conserved hypothetical protein	0,063354	3,93088	5,95526
0722	0613	low-affinity inorganic phosphate transporter	253,528	1287,69	2,34457
0723	0614c	secretory antigen SsaA-like protein	357,525	57,5719	-2,63461
0727	0618	conserved hypothetical protein	25,4571	677,708	4,73452
0728	0619	conserved hypothetical protein	870,699	3,62819	-7,90678
0735	0624c	probable membrane protein	31,2274	852,099	4,77014
0737	0626	probable lipoprotein	4,07493	101,038	4,63198
0738	0627	hypothetical protein	155,214	925,567	2,57608
0742	0631c	conserved hypothetical protein	1,96974	1664,18	9,72259
0744	0633	probable ATP-binding ABC transporter protein	6,61719	674,849	6,6722
0745	0634	probable ATP-binding ABC transporter protein	5,60502	0,745492	-2,91045
0746	0635c	probable transcriptional regulator MarR family	8398,8	1713,41	-2,29332
0747	0636	probable cobalamin synthesis-related protein	2,78197	2856,9	10,0041
0748	0637	probable oxidoreductase	55,3343	9,96591	-2,4731
0750	0639	probable membrane transport protein	391,605	2,34041	-7,38649
0751	0640	probable DNA photolyase	22,7888	0,4932	-5,52998
0753	0643c	conserved hypothetical protein	6,42711	156,96	4,61008
0754	0644	norA (quinolone resistance protein)	5,17341	235,757	5,51004
0761	0650	N-acetylglucosamine-6-phosphate deacetylase	2,78456	11,7463	2,07668
0762	0651	probable magnesium and cobalt efflux protein	13,3424	2750,26	7,6874
0764	0653	probable bactoprenol glucosyl transferase or stress response regulator	554,592	8692,71	3,97031

0765	0654c	saeS	32,0413	4,07218	-2,97606
0766	0655c	saeR	9,95079	2,4681	-2,01141
0769	0658c	probable membrane protein	31,096	1,88433	-4,04461
0772	0661c	probable aluminum resistance protein	1,37497	0,161088	-3,09348
0773	0662	probable anthranilate/para-aminobenzoate synthase component II	1,52403	20,9897	3,78372
	0663	probable anthranilate/para-aminobenzoate synthase component I	0,0247834	0,638084	4,6863
0780	0670	ATP-dependent DNA helicase	3,42153	416,738	6,92836
0783	0672	glycine betaine/carnitine/choline ATP-binding ABC transport protein	21,9795	401,883	4,19255
0787	0675c	probable multidrug resistance protein	26,8669	1676,74	5,96368
0791	0681	ribonucleotide reductase function protein	35,2787	1469,29	5,38018
0792	0682	rir1 (ribonucelotide diphosphate reductase alpha chain)	988,347	5478,26	2,47063
	0687	sstC (ABC transporter ATP-binding protein)	6,74323	1,04754	-2,68643
0805	0694	probable glycerate kinase	46,9848	1,74369	-4,75198
0808	0697c	probable membrane protein	3878,71	0,699657	-12,4366
0809	0698c	probable membrane protein	21,7844	1,42406	-3,93521
0811	0700c	probable membrane protein	30,593	4,15899	-2,8789
0815	0704	conserved hypothetical protein	584,142	3392,15	2,53781
0816	0705	secA (preprotein translocase subunit)	4,3095	125,694	4,86626
0818	0706	prfB (peptide chain release factor 2)	9,89946	2194,89	7,79258
0829	0717	trxB (thioredoxin reductase)	1449,48	89,96	-4,01011
0832	0721	conserved hypothetical protein	30,5896	126,287	2,0456
0833	0722	clpP (ATP dependent clp protease proteolytic subunit)	1270,95	3,10004	-8,67941
0834	0724c	probable cell division inhibitor	5,85833	478,126	6,35076
0837	0727	gapR (glycolytic operon regulator)	1,71562	372,212	7,76125
0729	0729	pgk (phosphoglycerate kinase)	1,52875	25,5516	4,06299
0840	0730	tpiA (triosephosphate isomerase)	419,928	1699,57	2,01695
0842	0732	eno (enolase 2-phosphoglycerate dehydratase)	348,024	4954,94	3,83161
0845	0735	probable carboxylesterase precursor	4242,73	1001,29	-2,08313
0846	0736	rnr (ribonuclease R)	89,2764	681,824	2,93305
0847	0737	smpB (tmRNA and SsrA-binding protein)	0,112827	178,233	10,6254
0854	0742	probable membrane protein	2,08496	1204,24	9,17389
0860	0748	nuc (staphylococcal thermonuclease precursor)	34,7079	3,54706	-3,29057
0874	0761	conserved hypothetical protein	1864,02	13249,3	2,82943
0876	0763	conserved hypothetical protein	24,2353	0,782701	-4,95251
0877	0764	gcvH (glycine cleavage system H protein)	11651,8	454,057	-4,68154
	0765	0765	4,50364	142,732	4,98608
	0766	0766	190,134	0,509999	-8,54231
0882	0768	ATP-binding ABC transporter protein	3,13362	56,7825	4,17955
0884	0770	probable substrate-binding ABC transporter protein	1,67248	16,8104	3,3293
0914	0774	ATP-binding ABC transporter protein	51,4986	284,885	2,46778
0915	0775	probable cysteine desulfurase	3,30906	1467,02	8,79225
0921	0786	probable hemolysin	2,88657	253,089	6,45414
0924	0788	conserved hypothetical protein	0,465042	1367,95	11,5224
0925	0789	probable membrane protein	32,3365	1,70325	-4,2468
0926	0790	conserved hypothetical protein	1,64488	307,09	7,54454
0929	0793c	conserved hypothetical protein	841,777	7683,54	3,19026
0932	0796	glycerate dehydrogenase	139,773	4,0752	-5,10007
0935	0798	dltA (D-alanine-D-alanyl carrier protein ligase)	188,024	1200,14	2,67422
0807	0807	probable NADH dehydrogenase	575,746	54,7869	-3,39353
0945	0808	probable cytosol aminopeptidase	618,238	86,7134	-2,83384

0946	0809	probable transporter protein	2,55236	429,745	7,3955
0947	0810	conserved hypothetical protein	15625,2	5,41198	-11,4954
0950	0814c	mnhF (Na+/H+ antiporter subunit)	7,27515	51112	12,7784
0952	0816c	mnhD (Na+/H+ antiporter subunit)	84,5245	3,84209	-4,45941
0957	0821	peptidyl-prolyl cis-trans isomerase	38,3695	5693,22	7,21314
0958	0822	conserved hypothetical protein	114,322	23,8869	-2,25882
0959	0823	probable NADH-dependent flavin oxidoreductase	107,433	1,29829	-6,37069
0960	0824	rocD (ornithine aminotransferase)	3276,05	490,836	-2,73864
0961	0825	NAD-specific glutamate dehydrogenase	166,318	693,017	2,05895
0962	0827c	glpQ (glycerophosphoryl diester phosphodiesterase)	1,58965	0,0863525	-4,20233
0966	0830	glucose-6-phosphate isomerase A	1045,45	3,2679	-8,32155
0967	0832	probable membrane protein	1,73083	0,234666	-2,88278
0835	0835	ATP-dependent nuclease subunit B	505,307	70,6823	-2,83774
973	0837	fumarylacetoacetate hydrolase family protein	117,01	3919,85	5,0661
0975	0839c	coenzyme A disulfide reductase	133,499	27,1716	-2,29666
0976	0840c	probable hydrolase	109,009	7,73148	-3,81755
	0842	0842	0,214341	4,21071	4,29608
0985	0846	conserved hypothetical protein	35,5154	2,03948	-4,12217
0987	0848	fabH (3-oxoacyl-ACP synthase III)	1,13661	0,0952221	-3,57729
0988	0849	fabF (3-oxoacyl-ACP synthase II)	59,513	1805,55	4,92309
0992	0853	oppC (oligopeptide transport system permease protein	820,605	9,57975	-6,42056
0993	0854	oppD (oligopeptide transport system ATP-binding protein)	0,304179	1,32126	2,11892
0995	0856	oligopeptide binding protein	1,30264	53,1941	5,35176
1003	0865	probable genetic competence repressor	2175,65	465,074	-2,22591
1005	0869c	conserved hypothetical protein	2044,66	6,03004	-8,40548
1008	0870c	conserved hypothetical protein	709,891	0,35894	-10,9496
1010	0872	GTP pyrophosphokinase	746,453	30,7877	-4,59963
1011	0873	probable inorganic polyphosphate/ATP-NAD kinase	1,45485	133,313	6,5178
1012	0874	probable RNA pseudoridylate synthase	1,51511	0,107417	-3,81813
1016	0877	fabI (trans-2-enoyl-ACP reductase)	4,68386	19,0001	2,02024
1017	0878c	probable membrane protein	2,20617	14,9427	2,75983
1022	0883c	UDP-glucose diacylglycerol glucosyltransferase	0,602364	6,92594	3,5233
1026	0887	probable toxic anion resistance membrane protein	38,2115	2,30983	-4,04815
1030	0889	Na+ transporting ATP synthase	15,2393	197,02	3,69248
1045	0905	probable iron transport protein	9,5734	198,783	4,37602
1048	0907	conserved hypothetical protein	0,87515	4,21128	2,26666
1052	0910	2-oxoglutarate decarboxylase	45,2681	979,637	4,43568
1054	0912	menB (naphthoate synthase)	1680,2	10221,2	2,60486
1057	0915c	sspA (V8 protease)	2358,62	8,79494	-8,06706
	0918c	hypothetical protein	21,5422	0,241316	-6,48009
1068	0925c	qoxC (quinol oxidase polypeptide III)	71,9975	10638,5	7,20713
1069	0926c	qoxB (quinol oxidase polypeptide I)	2905,25	26710,8	3,20069
1070	0927c	qoxA	1177,38	16513,5	3,80999
1071	0928c	probable exported protein	6,48425	51,4422	2,98794
1073	0931	purE (probable phosphoribosylaminoimidazole carboxylase catalytic subunit)	1,46732	7730,27	12,3631
1074	0932	purK (phosphoribosylaminoimidazole carboxylase)	4,11663	1626,48	8,62607
1077	0935	purQ (phosphoribosylformylglycinamidine synthase I)	260,975	10,8831	-4,58375
1078	0936	purL (phosphoribosylformylglycinamidine	55,9828	3898,5	6,12179

		synthase I)			
1079	0937	purF (phosphoribosylpyrophosphate amidotransferase)	20,817	7901,34	8,56819
1080	0938	purM (phosphoribosylformylglycinamidine cyclo-ligase)	4,59641	6119,32	10,3786
1082	0939	purN (phosphoribosylformylglycinamidine formyltransferase)	9,78179	12259,2	10,2915
1082	0940	purH	1,29545	617,835	8,89762
1083	0941	purD (phosphoribosylamine-glycine ligase)	2,52584	196,808	6,28388
1091	0949	ptsH (histidine-containing phosphocarrier protein)	91,6488	803,095	3,13138
0950	0950	ptsI (phosphoenolpyruvate-protein phosphatase)	2766,82	12088,8	2,12737
1094	0952	cytochrome d ubiquinol oxidase subunit I	2,57512	210,159	6,35069
0700	0953	ATP binding cassette transporterA	207,762	1,53267	-7,08274
1103	0960	pdhB (pyruvate dehydrogenase E1 component beta subunit)	2853,88	12213,2	2,09745
1104	0961	pdhC (dihydrolipoamide S-acetyltransferase component of pyruvate dehydrogenase complex E2)	2261,63	131,187	-4,10767
1105	0962	pdhD (dihydrolipoamide dehydrogenase component of pyruvate dehydrogenase E3)	613,667	2966,39	2,27318
1107	0964	probable transcriptional regulator	0,995514	2184,14	11,0993
	0966	0966	57,0412	0,146568	-8,60429
112	0969	probable membrane protein	1146,15	5039,26	2,13642
1116	0973	probable inositol-1-monophosphatase	1092,74	4705,49	2,10639
1118	0974	probable GTP-binding elongation factor protein	1,99301	18,1818	3,18948
1123	0979	pyruvate carboxylase	4,23833	422,552	6,63949
1124	0980c	ctaA (probable heme O-oxygenase)	360,038	4785,91	3,73257
1125	0981	ctaB heme A IX farnesyltransferase)	103,281	892,021	3,11051
1130	0985c	probable exported protein	1063,25	5,80525	-7,5169
1136	0991	conserved hypothetical protein	6,05071	176,257	4,86443
1144	0998	isdF	0,0315821	0,820925	4,70007
1147	1001	rRNA methyltransferase	2,29666	15,8024	2,78253
1150	1004c	probable ribonuclease HIII	72,6602	14,4574	-2,32935
1151	1005	conserved hypothetical protein	41,3304	3,61585	-3,51479
1153	1007	DNA-dependent DNA polymerase beta chain	10,0223	1489,4	7,21537
1154	1008	DNA-dependent DNA polymerase family	4,62547	1,14072	-2,01966
1157	1010	uvrC (excinuclease ABC subunit C)	11,4242	86,0851	2,91367
1159	1012	sdhA (succinate dehydrogenase flavoprotein subunit)	915,463	4815,18	2,39502
1161	1014	murI (glutamate racemase)	9,31717	58,6919	2,6552
1164	1018	probable exported protein	1914,2	25,5831	-6,2254
1180	1032c	probable exotoxin	0,541858	4,65952	3,10419
1182	1034	carbamate kinase	32,1667	0,655044	-5,61783
1183	1035	probable membrane protein	279,937	1229,88	2,13535
1188	1039	probable Holliday junction resolvase	2,41201	3094,29	10,3252
1190	1041	conserved hypothetical protein	3,89428	0,792263	-2,2973
1194	1045	pbpA (penicillin-binding protein 1)	8,78884	1,3792	-2,67184
1196	1047	murD (UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase)	0,0613497	0,58596	3,25568
1201	1052	conserved hypothetical protein	166,058	6,19682	-4,74402
1202	1053	conserved hypothetical protein	65,1835	5778,85	6,47013
	1055	1055	0,472373	1273,09	11,3961
1205	1056	probable cell division-initiation protein	27,4452	1,3163	-4,382
1057	1057	ileS (isoleucyl-tRNA synthetase)	20,2801	1,72764	-3,55319
1207	1058	conserved hypothetical protein	8,58546	230,42	4,74623
	1059c	1059c	0,302013	3,16842	3,39108

1217	1069	pyrE (orotate phosphoribosyltransferase)	67,0294	6,10642	-3,4564
1220	1072c	probable fibronectin/fibrinogen-binding protein	2,85347	190,902	6,06397
1221	1073	gmk (guanylate kinase)	52,2499	5,51303	-3,24451
	1077	1077	0,38421	137,605	8,48443
126	1078c	probable membrane protein	9,77196	87739,8	13,1323
1229	1081	sun (probable RNA-binding protein)	0,714902	3,88089	2,44057
	1084	pknB (protein kinase)	3,79748	670,07	7,46313
1235	1086	ribulose-phosphate 3-epimerase	0,658624	3,62046	2,45865
1240	1090	conserved hypothetical protein	58,4757	2,3521	-4,63582
1243	1093	fatty acid/phospholipid synthesis protein	7,07706	4040,65	9,15722
1244	1094	fabD (malonyl CoA-acyl carrier protein transacylase)	432,226	1732,41	2,00292
1245	1095	fabG (3-oxoacyl-(acyl-carrier protein) reductase)	3,6605	132,143	5,17391
1248	1097	Ribonuclease III	1,6045	94,8926	5,8861
0245	1099	two component sensor histidine kinase	1074,85	0,932423	-10,1709
1253	1101	ffh (signal recognition particle protein)	18,0216	2282,47	6,98472
1255	1103	rimM (16S rRNA processing protein)	1,05395	5733,59	12,4094
1263	1110	succinyl-CoA synthetase alpha chain	28,1384	1494,57	5,73105
1268	1113	gid (tRNA (uracil-5-)-methyltransferase)	35,2169	0,650869	-5,75776
1269	1114	integrase/recombinase	3,24209	1068,85	8,36492
1270	1115	hslV (ATP-dependent protease heat shock protein)	0,0798283	2134,59	14,7067
1271	1116	hslU (ATP-dependent protease ATP-binding subunit of heat shock protein)	10,9799	48,0038	2,12829
1278	1118	rpsB (30S ribosomal protein S2)	260,644	3144,43	3,59264
1276	1119	tfs (Elongation factor Ts)	48,0501	2714,44	5,81997
1281	1124	probable zinc metalloprotease	62,1342	313,248	2,33384
1282	1125	proS (prolyl-tRNA synthetase)	923,408	118,919	-2,95699
1283	1126	polC (DNA polymerase III)	5,48899	544,45	6,63211
1284	1127	conserved hypothetical protein	4,66297	368,059	6,30254
1289	1132	rbfA (ribosome binding factor A)	4278,41	8,24536	-9,01928
1290	1133	truB (tRNA-pseudouridine 5S synthase)	3,72922	1912,6	9,00245
1293	1136	pnpA (polyribonucleotide nucleotidyltransferase)	15,2998	122,332	2,99921
1295	1138	DNA translocase SpoIII family	448,182	63,8016	-2,81242
1298	1141	probable protease (Zn) protein	2,21769	0,536171	-2,04829
1300	1143	probable membrane protein	0,937691	20,4489	4,44676
1300	1144	probable membrane protein	8,17331	165,569	4,34037
1302	1145	pgsA (phosphatidylglycerophosphate synthase)	18,4572	160,372	3,11917
1303	1146	cinA (competence-damage inducible protein)	0,560814	13,6095	4,60095
1304	1147	recA	190,484	3182,37	4,06236
1307	1150	conserved hypothetical protein	19,0661	1,1613	-4,0372
1309	1152	pyruvate ferredoxin oxidoreductase beta chain	0,0369699	365,124	13,2697
1320	1161	glpK (glycerol kinase)	10,1335	1596,41	7,29956
1325	1168c	glutathione peroxidase	97,3892	0,327491	-8,21616
1326	1169	GTP-binding protein	0,702943	89,7149	6,9958
1329	1172	glnA (glutamine synthetase)	144,548	6617,46	5,51666
1349	1178c	probable low specificity threonine aldolase	4,46173	0,644151	-2,79213
1351	1180	cardiolipin synthase	55,7812	7,95101	-2,81057
1358	1183c	conserved hypothetical protein	9,06732	212,167	4,54838
1360	1185c	probable aspartate kinase	0,019844	0,116735	2,55646
1362	1186	homoserine dehydrogenase	28,8215	1204,69	5,38537
1363	1187	thrC (threonine synthase)	2,63585	3547,97	10,3945
1364	1188	thrB (homoserine kinase)	1976,87	4,78634	-8,69008

1365	1189	conserved hypothetical protein	1066,65	3,66116	-8,18657
1367	1191c	amino acid permease	8,19382	359,393	5,45488
	1196	1196	346,515	1,16263	-8,21938
1375	1198	hypothetical protein	192,591	9,6777	-4,31473
1377	1200	transketolase	16,6641	415,09	4,63861
1380	1202	probable membrane protein	27,4891	2,85742	-3,26607
1383	1205c	mscL (large-conductance mechanosensitive channel)	1956,56	25,6963	-6,25061
1387	1209c	hypothetical protein	6382,98	23,1386	-8,10779
1389	1211	grlB (topoisomerase IV subunit B)	1052,15	119,738	-3,13538
1392	1213	amino acid carrier protein	10,6891	249,979	4,54759
1395	1215	probable membrane protein	99,7227	1553,26	3,96124
1396	1216	mprF (probable membrane protein)	58,056	3408,18	5,87541
1397	1217c	msrA1	37,5832	511,363	3,76619
1398	1218	peptide methionine sulfoxide reductase regulator	1685,7	0,691371	-11,2516
1401	1220c	prephenate dehydrogenase	1,54719	1617,69	10,0301
1402	1221	probable peptidase	961,325	19,9951	-5,5873
1403	1222	trpE (probable anthranilate synthase subunit)	0,217568	36,2563	7,38062
1411	1230	femB (methicillin resistance factor protein)	474,681	72,8388	-2,70418
	1231	hypothetical protein	1,22111	102,247	6,38772
1414	1234c	probable oligopeptide transporter ATPase	0,5719	19,9702	5,12594
1419	1240	oligoendopeptidase F	17,6941	2542,84	7,16703
1422	1243c	probable phosphate ABC transporter permease	1,21271	445,223	8,52015
1424	1245c	phosphate-binding lipoprotein	0,377223	535,791	10,472
1426	1246c	conserved hypothetical protein	8,91299	212,822	4,5776
1429	1249	asd (aspartate semialdehyde dehydrogenase)	1,49106	556,03	8,54268
1433	1253	peptidase	58,5642	0,643639	-6,50763
1435	1255	lysA (diaminopimelate decarboxylase)	5,1508	47,0981	3,1928
1439	1260	conserved hypothetical protein	13,9212	4030,25	8,17744
1444	1264c	conserved hypothetical protein	0,306038	0,036687	-3,06037
1447	1267c	conserved hypothetical protein	117,002	1,76132	-6,05373
1450	1270c	arlS (two-component sensor histidine kinase)	96,8051	0,156449	-9,27325
1451	1271c	arlR (two-component response regulator)	10,4813	0,331016	-4,98476
1453	1273c	murG	2,14521	823,068	8,58375
1455	1275c	carboxy-terminal processing proteinase	80,6143	11,2359	-2,84292
1397	1279c	msrA (peptide methionine sulfoxide reductase)	39,4693	2,26325	-4,12426
1460	1280c	conserved hypothetical protein	1594,81	32,0542	-5,63673
1461	1281c	dfrB (trimethoprim-sensitive dihydrofolate reductase)	82,8631	2,32836	-5,15334
1462	1282c	thyA (thymidylate synthase)	62,7017	3950,06	5,97723
1472	1290c	truncated cell surface fibronectin-binding protein	0,155475	1,23071	2,98474
1472	1291c	truncated cell surface fibronectin-binding protein	63,5226	1,64002	-5,27548
	1298	recombinase	1180,38	6,49442	-7,50583
1475	1301c	probable transmembrane efflux protein	871,166	1,04082	-9,70908
1477	1303c	tdcB (threonine dehydrase)	16,5367	0,316378	-5,70788
1479	1305c	probable 5-3 exonuclease	0,795903	3860,98	12,2441
1480	1306c	conserved hypothetical protein	16,5969	210,275	3,66329
1481	1307	probable membrane protein	0,461929	2,47056	2,4191
1309c	1309c	conserved hypothetical protein	7,49666	1,19609	-2,64792
1484	1310c	conserved hypothetical protein	305,968	9107,19	4,89555
1489	1313	recU (recombination protein U)	0,69175	1737,24	11,2943
1490	1314	pbp2	39,2895	203,832	2,37516

1493	1317c	DNA replication initiation protein	0,592428	0,147709	-2,00389
1495	1319c	dinG (probable ATP-dependent DNA helicase)	0,328253	250,703	9,57695
1497	1321c	papS (probable PolyA polymerase family protein)	1,62466	14,8997	3,19707
1498	1322c	glycosyltransferase	109,898	0,631691	-7,44273
1500	1324c	probable membrane protein	3931,47	18785,4	2,25647
1509	1331c	nucleoside-diphosphate kinase	66,1014	672,876	3,34759
1510	1332c	heptaprenyl diphosphate syntase component II	282,016	2329,52	3,04618
1511	1333c	menaquinone biosynthesis methyltransferase	36,0417	1089,29	4,91758
1513	1335c	hup (DNA-binding protein II)	50216	9387,82	-2,41928
1514	1336c	gpsA (glycerol-3-phosphate dehydrogenase)	4,30478	775,236	7,49255
1515	1337c	GTP-binding protein, Era/TrmE family	19,8367	1389,31	6,13005
1518	1340c	cmk (cytidylate kinase)	0,0565474	1,48431	4,71419
1522	1343c	ebpS (cell surface elastin binding protein)	245,397	2497,66	3,34739
1523	1344c	probable ATP-dependent DNA helicase	1,79176	478,652	8,06146
1524	1345c	conserved hypothetical protein	120,732	1,26782	-6,57331
1526	1347c	probable membrane protein	0,319313	1,97739	2,63055
1530	1348c	probable lipoprotein	1,14718	7,0997	2,62966
1533	1351c	hypothetical protein	32,0346	0,531061	-5,91461
1534	1352c	srrB (staphylococcal respiratory response protein B)	3,50776	217,505	5,95436
1535	1353c	srrA (glutamate synthase small subunit)	9,91224	273,823	4,78789
1536	1354c	rluB	58,3763	3918,74	6,06886
	1355c	1355c	29,6803	777,397	4,71107
1538	1356c	conserved hypothetical protein	157,157	0,357626	-8,77954
1542	1360c	probable ADP-ribose pyrophosphatase	2346,61	266,693	-3,13733
1543	1361	probable conserved oxidoreductase	167,374	1067,46	2,67304
1546	1364	probable pyrrolidine-5-carboxylate reductase	1,99731	184,506	6,52947
1548	1365c	probable metallo-beta-lactamase	47,3585	1,85005	-4,67799
1550	1367	probable transcriptional regulator AraC family	312,168	5334,52	4,09496
1551	1368c	malA (alpha-D-1,4-glucosidase)	714,996	25,4076	-4,81461
1555	1385c	probable peptidase T	336,533	3480,89	3,37064
1562	1390c	2-oxoisovalerate dehydrogenase alpha subunit	23,1529	1,57637	-3,87651
1563	1391c	dihydrolipoamide dehydrogenase	19,2816	470,65	4,60935
1564	1392c	DNA repair protein	3,46292	101,117	4,86789
1566	1394c	geranyltranstransferase	3,22836	14,722	2,1891
1571	1399c	accC (acetyl-CoA biotin carboxylase)	40,6737	181,105	2,15466
1588	1402c	proline dipeptidase	27,9224	1189,35	5,4126
1589	1403	probable lipoprotein	1574,61	349,124	-2,17318
1591	1405c	probable lipoate-protein ligase A	42,1177	1520,36	5,17384
1594	1408c	glycine dehydrogenase subunit 1	0,040674	157,065	11,915
1601	1416c	probable late competence protein	190,44	6,75507	-4,81722
1603	1418c	conserved hypothetical protein	27,5267	1,95528	-3,81539
1604	1419c	glkA (glucokinase)	393,13	5,46683	-6,16816
1606	1421c	probable membrane protein	166,966	929,958	2,47761
1607	1422c	conserved hypothetical protein	1493,25	1,74113	-9,74422
1609	1424c	pbpF	358,281	5,3968	-6,05284
1612	1427c	mreB (ABC transporter protein)	0,483162	2,41486	2,32136
1614	1429c	endonuclease IV	3,60865	1289,26	8,48087
1616	1431c	conserved hypothetical protein	16,8254	112,934	2,74676
1617	1432c	conserved hypothetical protein		0,155835	-3,326
1618	1433c	rpoD (RNA polymerase sigma factor)	1038,73	93,8203	-3,46877
1619	1434c	dnaG (DNA primase)	80,837	811,088	3,32677

1620	1435c	conserved hypothetical protein	23,2848	1,3606	-4,09707
1622	1437	glyS (glycyl-tRNA synthetase)	3080,11	713,146	-2,11071
1623	1438c	probable recombination protein	11,1208	3584,47	8,33235
1624	1439c	era (GTP-binding protein)	392,787	2257,66	2,52301
1628	1443c	phosphate starvation-induced protein	574,792	4858,25	3,07933
1630	1445c	probable exported protein	99,436	5822,46	5,87172
1631	1446c	probable membrane protein	292,506	5051,52	4,11018
1633	1448c	conserved hypothetical protein	7,3309	138,902	4,24393
1634	1449c	conserved hypothetical protein	0,045679	1825,67	15,2865
1635	1450c	probable methyltransferase	4,21145	330,037	6,29217
1636	1451c	dnaJ (chaperone protein)	15,0807	93,2362	2,62819
1638	1453c	grpE (heat shock molecular chaperone protein)	269,086	2,89943	-6,53616
1640	1455c	oxygen-independent coproporphyrinogen oxidase III	0,944432	0,071938 9	-3,7146
1650	1466c	probable nicotinate-nucleotide adenyltransferase	893,746	0,215614	-12,0172
1653	1469c	conserved hypothetical protein	0,026570	0,517123	4,28259
1556	1472	probable membrane protein	27379,4	31,3281	-9,77142
	1473c	1473c	9,86615	3227,79	8,35384
1659	1475c	probable membrane protein	1,90012	1283,74	9,40005
1663	1479c	conserved hypothetical protein	151,196	3,01568	-5,64779
1664	1480c	conserved hypothetical protein	1196,16	0,126163	-13,2108
1667	1483c	probable proteinase	0,088426	430,284	12,2485
1668	1484c	probable proteinase	430,481	0,770443	-9,12605
1672	1488c	conserved hypothetical protein	9241,27	8,93918	-10,0137
1673	1489c	alaS (alanyl-tRNA synthetase)	1,36262	56,8922	5,38377
1677	1493c	probable iron-sulfur cofactor synthesis protein	2,20555	3185,49	10,4962
	1495	hypothetical protein	72111	2912,97	-4,62965
1681	1496c	conserved hypothetical protein	2995,36	10,9237	-8,09913
1682	1497	probable ATPase	1618,14	13013,9	3,00765
1683	1498c	probsble dinucleotide utilizing enzyme	1300,11	9,42946	-7,10724
1685	1499c	aspS (aspartyl-tRNA synthetase)	0,843392	0,1502	-2,48932
1686	1500c	hisS (histidyl-tRNA synthetase)	4,0261	0,859698	-2,22748
1689	1503c	relA (GTP pyrophosphokinase)	239,334	36,0659	-2,73032
1698	1512c	probable DNA-binding protein	121,238	2227,62	4,19959
1701	1515c	conserved hypothetical protein	5,44338	39,1201	2,84533
1704	1518c	truncated probable rod shape-determining protein	3,00905	21,1226	2,81141
1708	1522c	probable leader peptidase protein	6,22524	1630,08	8,03259
1709	1523c	folC (folylpolyglutamate synthase)	23,8749	1288,99	5,7546
1710	1524c	valS (valyl-tRNA synthetase)	255,37	1440,98	2,49639
1711	1525	tag (DNA-3-methyladenine glycosylase)	41,8265	5,44954	-2,94021
1714	1527c	hemL	476,873	14,9633	-4,9941
1715	1528c	hemB	0,062627	21,1255	8,39798
1717	1530c	hemC (Porphobilinogen deaminase)	678,576	5,48084	-6,95197
1718	1531c	membrane uroporphyrinogen III methylase	6,21984	1074,24	7,43223
1721	1534c	clpX (ATP-dependent Clp protease ATP-binding subunit)	37,5145	736,409	4,29499
1722	1535c	tig (trigger factor prolyl isomerase)	5,03805	2627,15	9,02642
1727	1540c	infC (translation initiation factor IF-3)	630,921	4690,31	2,89415
1729	1542c	thrS (threonyl-tRNA synthetase 1)	13,8395	911,912	6,04204
1731	1543c	dnaI (primosomal protein)	1,82452	22,2734	3,60973
1732	1544c	dnaB (chromosome replication initiation and membrane attachment protein)	138,884	1340,96	3,27131
1733	1545c	conserved hypothetical UPF0168 protein	3111,67	8,214	-8,56539

1735	1547c	coaE (probable dephospho-CoA kinase)	7,81787	9389,25	10,23
1738	1550c	probable membrane protein	1,64104	13,0984	2,99671
1739	1551c	phoR	220,657	5766,77	4,70788
1740	1552c	phoP	45,8105	1645,94	5,16709
1742	1554c	citZ (citrate synthase II)	9829,27	1343,36	-2,87124
1745	1556c	pyk (pyruvate kinase)	1325,22	18,8531	-6,13528
1746	1557c	pfkA (6-phosphofructokinase)	32,293	218,242	2,75663
1747	1558c	accA (acetyl-CoA carboxylase carboxyl transferase subunit alpha)	4,88674	4604,17	9,87985
1748	1559c	accD	40,275	531,693	3,72263
1749	1560c	NAD-dependent malic enzyme	47,0116	6,25489	-2,90996
1750	1561c	dnaE (DNA polymerase III alpha subunit)	6,59637	181,719	4,78389
1751	1562c	conserved hypothetical protein	0,400768	142,278	8,47173
1752	1563c	probable DNA-binding protein	1526,21	118,533	-3,68659
1753	1564	conserved hypothetical protein	134,907	6402,76	5,56865
1756	1567	probable Xaa-Pro dipeptidase homolog	17,1097	1992,89	6,8639
1758	1568c	ald (alanine dehydrogenase)	1010,18	17988,7	4,1544
1760	1570c	ackA (acetate kinase)	147,088	944,145	2,68233
1761	1571c	conserved hypothetical protein	2,24847	600,971	8,06221
1762	1572c	probable thiol peroxidase	58,4418	982,915	4,07199
1763	1573c	probable membrane protein	5,28397	1,02372	-2,3678
1765	1575c	probable aminotransferase	0,281363	50,0978	7,47617
1767	1576c	conserved hypothetical protein	73,808	467,277	2,66243
1768	1577	conserved hypothetical protein	0,225653	384,932	10,7363
1769	1578	rpsD (30S ribosomal protein S4)	4504,02	35,5852	-6,98379
1770	1579c	conserved hypothetical protein	1007,08	1,20162	-9,71099
1771	1580	probable osmotic stress-related protein	3906,18	9,56678	-8,67351
1776	1585c	probable 1-acylglycerol-3-phosphate O-acyltransferase	3956,72	11,4082	-8,43809
1781	1590c	IsdH (iron-regulated surface protein)	0,99453	305,894	8,2648
1783	1593c	acsA (acetyl-CoA synthetase)	20,6117	3211,45	7,28362
1784	1594	acuA (acetoin utilization protein)	1740,16	1,96978	-9,78697
1785	1595	acuC (acetoin utilization protein)	48,5165	1,03692	-5,5481
1786	1596c	ccpA (catabolite control protein A)	2497,24	452,43	-2,46457
1787	1597c	chorismate mutase	855,157	35,6583	-4,58388
1790	1600c	murC	1,07539	416,897	8,59869
1791	1601c	SpoIIIE family cell division protein	4,53138	241,499	5,73592
1792	1602c	phenylalanyl-tRNA synthetase beta subunit	66,3455	2,15797	-4,94225
1795	1605c	probable endo-1,4-beta-glucanase	20,3895	1,91422	-3,41299
1796	1606	probable membrane protein	725,467	14,185	-5,67647
1797	1607c	conserved hypothetical protein	1,40739	22,7581	4,01528
1798	1608c	tRNA guanine methyltransferase	2,14279	1141,22	9,05687
1800	1610c	dat (D-alanine aminotransferase)	551,992	4881,81	3,1447
1801	1611c	Xaa-His dipeptidase homolog	542,67	2400,59	2,14524
1805	1615	probable exported protein	8,0954	1,68175	-2,26714
	1616c	1616c	4,20826	0,697676	-2,59259
1809	1619c	multidrug resistance protein B	0,271192	2,05907	2,92461
1812	1622c	rot	22,8193	2,08286	-3,45362
1814	1623c	conserved hypothetical protein	3,04533	79,2929	4,70252
1817	1625c	ribH (6,7-dimethyl-8-ribityllumazine synthase)	193,723	1,22321	-7,30718
1821	1629c	conserved hypothetical protein	3,39294	96,1678	4,82494
1825	1633c	probable exported protein	2,75722	795,656	8,17279
1829	1637c	probable membrane protein	0,126342	3,15829	4,64373

	1638	1638	0,676625	579,643	9,74259
1831	1639c	transaldolase	2001,85	15,1043	-7,05023
1835	1643c	aldo-keto reductase family protein	13,4685	120,51	3,16149
1840	1647c	conserved hypothetical protein	2008,05	3,06188	-9,35716
1841	1648c	conserved hypothetical protein	0,93462	4,01467	2,10283
1843	1650c	o-succinylbenzoic acid synthetase	0,060320	0,866794	3,84498
1844	1651c	O-succinylbenzoate-CoA ligase	0,596304	1006,79	10,7214
1848	1653	hypothetical protein	3727,16	28865	2,95317
1850	1657	conserved hypothetical protein	1,81641	0,210673	-3,10801
	1665c	1665c	0,006501	1,02492	7,30054
1867	1672c	serine proteinase	0,54544	4124,9	12,8847
1871	1676c	hypothetical protein	87,0503	504,788	2,53576
1873	1678c	probable transport protein	0,417162	22,4543	5,75024
1874	1679c	probable serine protease precursor	4,87816	0,840942	-2,53626
	1753c	phage anti-repressor protein	6,79703	62,6858	3,20516
	1761c	hypothetical protein	849,034	2,949	-8,16945
1889	1765c	hemE (uroporphyrinogen decarboxylase)	57,5937	1,4582	-5,30365
1891	1766	traP	4,3099	203,812	5,56344
1892	1767c	probable ABC transporter	10,5311	0,601048	-4,13103
1897	1774	probable peptidyl-prolyl cis-isomerase	0,700199	0,081695	-3,09943
1899	1776c	conserved hypothetical protein	26,1902	164,446	2,65051
1900	1777c	conserved hypothetical protein	10,8389	63,4631	2,54969
1902	1778c	conserved hypothetical protein	9,73946	681,002	6,12767
1904	1780c	DNA-binding protein	90,0692	3,16085	-4,83265
1905	1781c	probable two-component response regulator protein	2,21817	10,1101	2,18835
1906	1782c	probable two-component response system sensor histidine kinase	517,825	2,38078	-7,76489
1907	1783	probable RNA pseudouridylate synthase	25,6459	259,889	3,3411
1908	1784c	citG (fumarate hydratase class-II)	14,3135	317,195	4,46993
1914	1790c	iron-sulfur binding protein	999,913	0,588982	-10,7294
1916	1792c	substrate-binding glutamine ABC transporter	2,27305	67,1729	4,88518
1917	1793c	probable membrane protein	1,40275	51,3197	5,19319
1919	1794c	perR (peroxide operon regulator)	1907,03	13262,4	2,79794
1923	1798	probable membrane protein	196,792	1,32227	-7,21752
1924	1799c	ATP binding ABC transporter	552,853	0,585569	-9,88284
1926	1801c	probable membrane protein	0,321982	222,284	9,43121
1928	1803c	probable membrane protein	0,038922	0,900798	4,53251
1929	1804c	probable membrane protein	1,97262	90,6495	5,52211
1932	1806c	monofunctional glycosyltransferase	7,7727	474,347	5,93138
1935	1809	conserved hypothetical protein	2,02245	1551,7	9,58353
1936	1810c	conserved hypothetical protein	1454,1	0,227336	-12,643
1939	1813	probable protein tyrosine phosphatase	4,9967	287,756	5,84773
1940	1814	probable exported protein	50,9046	6098,67	6,90456
1942	1816c	two-component response regulator	4,69027	1603,72	8,41754
1943	1817c	vraS	1165,79	124,003	-3,23286
1946	1820c	methionyl aminopeptidase	24,7462	2391,79	6,59474
1951	1824c	probable UDP-N-acetylmuramyl tripeptide synthase	101,04	0,482276	-7,71085
1955	1827c	DNA polymerase IV	24,5613	973	5,30798
1956	1828c	probable_membrane_protein	56,4606	1081,07	4,25908
1957	1829c	RNA methyltransferase	1030,87	1,41621	-9,50761
1958	1830c	conserved hypothetical protein	1,58268	31,0023	4,29194
1961	1833c	gatA	253,139	2685,87	3,40739

1963	1835	putP (high affinity proline permease)	561,948	53,0458	-3,40513
1964	1838c	probable lipoprotein	0,432835	53,3892	6,94659
1967	1841c	PcrB family replication protein	18,7206	1,21274	-3,94829
1973	1848c	probable membrane protein	0,132401	106,308	9,64912
1974	1849c	probable NAD synthetase	0,447773	2,3855	2,41345
1975	1850c	nicotinate phosphoribosyltransferase	35,1708	826,202	4,55404
1980	1854c	conserved hypothetical protein	0,545204	3,04026	2,47933
1981	1855	bifunctional pyrazinamidase/nicotinamidase	15,8347	2,03051	-2,96318
1984	1857	aldehyde dehydrogenase	150,22	650,382	2,11421
1985	1858c	conserved hypothetical protein	10,5882	1204,89	6,8303
1986	1859	conserved hypothetical protein	1551,24	21891,8	3,8189
1987	1860c	probable exported protein	21,7624	1257,06	5,85207
1988	1861	conserved hypothetical protein	0,028636	840,384	14,8409
1989	1862c	probable exported protein	669,514	122,385	-2,45169
1991	1864c	probable membrane protein	1168,18	24,9104	-5,55137
1993	1866c	probable membrane protein	432,804	4030,05	3,21901
1995	1868c	probable membrane protein	62,375	441,99	2,82498
1997	1870c	GntR family transcriptional regulator	65,7737	11239,3	7,41683
2000	1872	aspartate transaminase	465,012	2381,03	2,35625
2006	1876c	probable leukocidin S subunit	2,37466	623,538	8,03662
	1877	probable succinyldiaminopimelate desuccinylase	696,882	18,6591	-5,22296
2017	1914c	groES (10 kDa heat shock chaperonin protein)	26,3807	5496,48	7,70288
2021	1919	conserved hypothetical protein	167,473	3637,4	4,4409
2023	1920	agrB	22,3526	2589,57	6,85613
2028	1924c	probable fructokinase	66,648	368,438	2,46679
2034	1929c	probable membrane protein	2,73378	0,624648	-2,12978
2035	1930c	conserved hypothetical protein	24,4523	161,361	2,72224
2036	1931	ATP-binding ABC transporter protein	0,45512	36,7142	6,33395
2038	1934c	O-sialoglycoprotein endopeptidase	6,87817	1073,26	7,28576
2041	1937c	conserved hypothetical protein	14,6072	2,58924	-2,49608
2043	1939	ilvB (acetolactate synthase large subunit)	0,618378	0,040890	-3,91866
2047	1943	leuB (3-isopropylmalate dehydrogenase)	1,34028	0,076114	-4,13821
2050	1946	ilvA (threonine dehydratase)	0,022106	0,495624	4,4867
2052	1947c	conserved hypothetical protein	1588,2	2,78617	-9,15489
2054	1949c	sigB	238,611	1576,96	2,72442
2055	1950c	rsbW (anti-sigma B factor)	3,84425	1317,85	8,42127
2057	1952c	rsbU (indirect positive regulator of sigma B)	1,04355	24,7014	4,56501
2060	1955c	alr (alanine racemase 1)	0,930747	63,2069	6,08555
2063	1958c	probable membrane protein	185,427	37,2284	-2,31637
2066	1960c	kdpC (potassium-transporting ATPase C chain)	11,6261	449,497	5,27287
2067	1961c	kdpB (potassium-transporting ATPase B chain)	1,37932	0,310434	-2,1516
2074	1967c	D-alanine-D-alanine ligase	4,37737	4048,08	9,85296
2075	1968	probable membrane protein	98,5986	1170,86	3,56986
2079	1972	cardiolipin synthetase	0,832622	685,415	9,6851
2080	1973	conserved hypothetical protein	24,8295	1,19632	-4,37538
2092	1984c	murA1	36,6966	3,76253	-3,28587
2094	1986c	atpC (ATP synthase epsilon chain)	195,697	13,9907	-3,80608
2096	1988c	atpG (ATP synthase gamma chain)	14,3624	3421,16	7,89604
2097	1989c	atpA (ATP synthase alpha chain)	17,5741	154,862	3,13946
2098	1990c	atpH (ATP synthase delta chain)	3,28251	390,992	6,89619
2101	1993c	atpB (ATP synthase A chain)	1396,51	237,306	-2,55701
2103	1995c	mnaA (UDP-GlcNAc 2-epimerase)	40,8586	5224,5	6,99851

2105	1997c	glyA (serine hydroxymethyltransferase)	338,55	1421,55	2,07003
2108	2000c	conserved hypothetical protein	120,46	2,03514	-5,88728
2109	2001c	conserved hypothetical protein	467,723	2,44645	-7,57882
2100	2002c	prfA (peptide chain release factor 1)	0,946676	520,931	9,10401
2111	2003c	thymidine kinase	6,54007	2448,61	8,54844
2112	2004c	rpmE (50S ribosomal protein L31 type B)	15,8752	133,079	3,06744
2113	2005c	rho	8,51376	88,9612	3,38531
2114	2006c	aldehyde dehydrogenase	682,787	3660,51	2,42254
2115	2007c	conserved hypothetical protein	0,301428	12,5869	5,38396
2116	2008c	murA2	2321,82	115,702	-4,32677
2125	2017c	probable amidase	9,15723	2,24627	-2,02738
2126	2018	luxS	2317,32	274,441	-3,07789
2127	2019c	probable membrane protein	11,5497	357,312	4,95126
2129	2021c	deoC2 (deoxyribose-phosphate aldolase)	2,7695	81,2913	4,8754
2131	2023c	general stress protein 20U	16,3655	3,51963	-2,21716
2133	2025c	hypothetical protein	18,1019	392,268	4,43763
2135	2027c	mannose-6-phosphate isomerase	1,67894	0,082011	-4,35558
2143	2032c	conserved hypothetical protein	4,91438	0,867649	-2,50182
2147	2037	probable transcription antiterminator	44,6165	1665,83	5,22252
2149	2039	mtlD (mannitol-1-phosphate 5-dehydrogenase)	24,2543	460,41	4,24661
2150	2040c	fmtB	1,9594	0,337237	-2,53857
2153	2043c	probable membrane protein	9,45082	3349,51	8,4693
2154	2044c	rocF (arginase)	157,631	16,1522	-3,28675
2156	2047c	ATP-binding protein, Mrp/Nbp35 family	6,52479	1597,11	7,93532
2159	2050c	probable drug resistance-involved transporter	9,40542	1,94256	-2,27553
2160	2051c	probable hemolysin	23,0891	2,81433	-3,03635
2161	2052c	conserved hypothetical protein	19,6334	1137,16	5,85598
2162	2053c	probable membrane protein	0,358112	12052,2	15,0385
2163	2054	hypothetical protein	851,113	57,8143	-3,87985
2164	2055c	probable membrane protein	29,4788	2431,34	6,36593
2166	2057c	ferrichrome ABC transporter	0,706977	2271,32	11,6496
2167	2058c	ferrichrome ABC transporter lipoprotein	3,91992	58,7429	3,90552
2171	2062	conserved hypothetical protein	58,2081	326,233	2,48661
2178	2068c	quinone oxidoreductase	521,378	4078,66	2,96769
2179	2069c	conserved exported hypothetical protein	0,839829	3,3987	2,01682
2180	2070c	lacG (6-phospho-beta-galactosidase)	1,06103	0,05139	-4,36761
2188	2077c	lacR	587,738	0,911045	-9,33344
2189	2078	probable regulatory protein	90,5855	1230,87	3,76426
2192	2080c	probable oxidoreductase	2,05251	37,4515	4,18956
2197	2085	probable exported protein	131,932	5060,65	5,26145
2199	2087c	alpha-acetolactate synthase	27,6182	347,339	3,65265
2208	2092c	tRNA pseudouridine synthase A	0,504166	2,10799	2,0639
2210	2094c	ATP-binding ABC transporter vraR	62,6553	889,743	3,82788
2309	2096c	rplQ (50S ribosomal protein L17)	6089,1	35,7091	-7,41379
2213	2097c	rpoA	692,311	4,76492	-7,18282
2234	2117c	rplV (50S ribosomal protein L22)	1,01641	27,827	4,77493
2236	2119c	rplB (50S ribosomal protein L2)	2306,68	222,814	-3,3719
2239	2122c	rplC (50S ribosomal protein L3)	9,50794	2807,67	8,20602
2241	2124	probable membrane protein	1463,59	0,661901	-11,1106
	2126c	2126c	0,513599	14,9765	4,86592
2245	2127	hypothetical protein	48,8088	971,663	4,31524
2246	2128	glcU (glucose uptake protein)	21,3502	0,976732	-4,45014

2248	2130c	probable membrane protein	2,26224	54,3576	4,58666
2251	2132	conserved hypothetical protein	11,1948	1,66187	-2,75194
2253	2134c	fmhB	12,9859	2,45223	-2,40477
2255	2136c	hypothetical protein	1,32414	150,147	6,82517
2261	2140c	moaA	2913,35	0,924393	-11,6219
2267	2146	moaC	7,71298	324,027	5,39268
2270	2149c	modC	1230,45	67,0007	-4,19886
2271	2150c	modB (molybdate transport permease protein)	23,6024	2,75579	-3,0984
2272	2151c	modA (molybdate-binding protein)	0,481756	139,014	8,17271
2276	2155c	inosine-uridine preferring nucleoside hydrolase	1,30274	54,5858	5,38891
2278	2157c	butyryl-CoA dehydrogenase homolog	490,433	73,6601	-2,7351
2285	2165	ureG (urease accessory protein)	0,715749	4,68397	2,71021
2286	2166	ureD (urease accessory protein)	59,1172	1,79418	-5,04218
2290	2170c	probable transcriptional regulator	1,82421	142,957	6,29217
2291	2172	probable secretory antigen precursor	56,167	4402,86	6,29257
2293	2174c	conserved hypothetical protein	1,92152	98,7342	5,68323
2295	2176	exported secretory antigen precursor	110,211	6918,5	5,97212
2296	2178	probable dehydrogenase	90,4428	1570,62	4,11818
2297	2181c	conserved hypothetical protein	1278,52	0,661732	-10,9159
2298	2183c	probable autolysin E	80,8561	1,01118	-6,32125
2306	2190c	probable membrane protein	522,839	0,948752	-9,10612
2309	2193c	amino acid permease	96,7616	0,562043	-7,42761
2310	2194	probable membrane protein	198,717	6,79812	-4,86943
2313	2197c	conserved hypothetical protein	173,78	1,23945	-7,13142
2316	2200c	PTS system arbutin-like IIBC component	0,370641	1,87884	2,34174
2317	2201	transcriptional regulator	1013,13	0,132685	-12,8985
2318	2202c	hypothetical protein	5,02573	180,406	5,16577
2319	2203c	probable peptidase	1787,6	3,52875	-8,98465
2325	2209	truncated hypothetical protein	83,0243	186353	11,1322
2327	2211c	hutG (formiminoglutamase)	819,533	8284,16	3,33748
2329	2213c	ribose 5-phosphate isomerase A	0,936392	1028,42	10,101
2330	2214	conserved hypothetical protein	1071,67	2,70503	-8,62999
2332	2215c	probable epimerase	0,960366	0,155337	-2,62818
2341	2225c	fni (isopentenyl-diphosphate delta-isomerase)	3,88398	55,8681	3,84642
2342	2226c	probable divalent cation transporter	1,95277	12,8862	2,72223
2347	2231c	probable multidrug resistance transporter	0,311049	181,289	9,18693
2352	2235c	tcaA	0,2421	0,979845	2,01695
2354	2237	probable membrane protein	103,271	0,726364	-7,15153
2360	2242	conserved hypothetical protein	6844,01	33118,4	2,27472
2361	2243	probable membrane protein	13224,6	53804,3	2,0245
2363	2245c	lldP (L-lactate permease)	2006,98	14425,3	2,8455
	2246c	hypothetical protein	1750,57	7540,07	2,10676
2364	2247	hypothetical protein	293,339	2,92509	-6,64795
2374	2255	conserved hypothetical protein	1,54431	814,927	9,04357
2375	2256	probable cationic membrane transport protein	7,25198	1192,58	7,36149
2376	2257c	scrA	3,01142	1447,51	8,90891
2377	2258	truncated conserved hypothetical protein	24,3288	13993,5	9,16787
2378	2260	hypothetical protein	10,709	569,989	5,73404
2379	2261	conserved hypothetical protein	16,2113	294,37	4,18256
2385	2266	hypothetical protein	2835,45	3,73231	-9,56929
2386	2267c	narT (nitrite extrusion protein)	7,0431	2055,19	8,18884
2391	2272c	conserved hypothetical protein	20,7345	812,765	5,29274

2392	2273c	narI (nitrate reductase gamma chain)	34,2693	2041,75	5,89674
2393	2274c	narJ (nitrate reductase delta chain)	3,29886	0,637906	-2,37055
2394	2275c	narH (nitrate reductase beta chain)	5,4149	969,329	7,48391
2395	2276c	narG (nitrate reductase alpha chain)	3,07645	22,5453	2,87349
2398	2280c	nasD (nitrite reductase)	8,46115	1,72107	-2,29755
2399	2281c	probable fumarate and nitrate reduction regulatory protein	652,008	0,115409	-12,4639
2403	2286c	probable zinc-binding lipoprotein	122,645	12,9698	-3,24127
2409	2291	fmhA	590,637	1,12539	-9,0357
2410	2292c	probable ABC transporter	79,7655	3649,47	5,51578
2411	2293c	ABC transporter permease	18,0288	752,842	5,38397
2413	2295c	multidrug resistanc-related transport system protein	0,44175	6,64984	3,91202
2419	2300	hlgA (gamma-hemolysin component A precursor)	13,909	118,663	3,09279
2436	2315	probable membrane protein	57,7576	10,8396	-2,41369
2437	2316	probable transport protein	50,0525	2,93789	-4,09059
2441	2320	amino acid ABC transporter	573,175	16,9216	-5,08204
2442	2321	sodium hydrogen exchanger family protein	173,011	893,394	2,36844
2445	2323	probable drug resistance-involved membrane protein	0,0179578	56,7926	11,6269
2449	2326c	transmembrane efflux protein	17,5847	1,70497	-3,3665
2450	2327c	opuCD	342,164	2570,37	2,90922
2451	2328c	opuCC	43,338	1288,46	4,89387
2453	2330c	opuCA	3,54117	14,9252	2,07545
2459	2333	probable carboxylesterase	686,868	9607,78	3,8061
2460	2334c	probable membrane protein	1,91516	78,6896	5,36064
2463	2338c	endo-1,4-beta-glucanase	230,243	4,82844	-5,57545
2469	2343c	glutamate synthase-ferredoxin large subunit	118,846	6,64425	-4,16084
2470	2344	probable membrane protein	7,33763	597,746	6,34808
2472	2346c	opp1F	0,045964	0,497979	3,43749
2478	2352c	hypothetical protein	1,89735	0,109929	-4,10935
2479	2353c	conserved hypothetical protein	4,58024	0,243023	-4,23626
2483	2356	probable transport protein	326,824	16,72	-4,28886
	2359c	2359c	34,3538	1,67989	-4,35403
2491	2363c	hypothetical protein	1153,66	1,88E+06	10,6741
2501	2371	phosphoglucomutase	2,17404	2106,82	9,92048
2508	2374c	gtaB	41,3768	4,37377	-3,24187
2511	2375c	fnbA (fibronectin-binding protein)	0,395452	9,27439	4,55168
2521	2385c	probable transport protein	36,7868	1335,11	5,18163
2522	2386	probable alkaline phosphatase	87,5023	3,07805	-4,82923
2527	2390	fructose-bisphosphatase	6,11618	407,209	6,05699
2528	2391	probable membrane protein	1,40255	0,177196	-2,98463
2534	2397c	probable NADH-dependentflavin reductase	785,832	2,23699	-8,45652
25238	2401	conserved hypothetical protein	5,33764	1996,73	8,54722
2539	2402c	srtA	37,5341	4,5875	-3,03242
2546	2406c	probable surface-exposed regulatory protein	3923,27	207,546	-4,24055
2548	2408	probable Holliday junction resolvase	0,557303	8,40025	3,9139
2553	2413c	pyruvate oxidase	18,5101	1175,87	5,98928
2554	2414c	probable membrane protein	666,925	3117,47	2,22478
2562	2422c	adaB	905,159	16,3902	-5,78726
2564	2424c	feoB (ferrous iron transport protein B)	235,528	5,04894	-5,54378
2572	2431	copA (copper-transporting ATPase)	759,761	6,09916	-6,96079
2574	2432c	probable D-specific D-2-hydroxyacid dehydrogenase	0,339186	0,0791052	-2,10023

2576	2434c	crtN (squalene synthase)	41,2755	481,204	3,54329
2577	2435c	squalene desaturase	986,045	6,21489	-7,30978
2578	2436c	hypothetical protein	226,15	6,26786	-5,17316
2579	2437c	probable phytoene dehydrogenase	85,5369	3932,43	5,52273
2584	2443c	isaA (immunodominant antigen A)	905,393	15976	4,14122
	2454	2454	364,062	1738,6	2,25567
2605	2463c	conserved hypothetical protein	6,02547	556,257	6,52853
2606	2464	dihydroorotate dehydrogenase	3,49274	1220,52	8,44892
2609	2467	conserved hypothetical protein	123,001	4574,62	5,21691
2610	2468c	hypothetical protein	0,069314	6,19	6,48064
2612	2469	conserved hypothetical protein	37,9029	1,35055	-4,81068
2615	2472c	panB	270,634	3,81387	-6,14894
2616	2473	conserved hypothetical protein	0,037344	1,72622	5,53057
	2477c	2477c	2,48697	2196,87	9,78684
2621	2478	probable membrane protein	1,82058	3191,88	10,7758
2622	2479	fda (fructose-bisphosphate aldolase class I)	311,675	2967,88	3,25132
2624	2482c	acetate-CoA ligase	598,165	3142,1	2,39311
2630	2488	conserved hypothetical protein	30,4522	1296,93	5,41241
2632	2489c	cudT (choline transporter)	1,78835	0,312609	-2,5162
2634	2490c	nrdG	4,16137	3910,92	9,87623
2635	2491c	nrdD	249,579	8942,47	5,1631
2636	2492c	probable magnesium citrate secondary transporter	2,02208	0,060806	-5,05548
2639	2494c	cysJ (sulfite reductase flavoprotein)	1,85007	381,321	7,68728
2645	2499c	probable two-component sensor histidine kinase	58,3211	1340,91	4,52305
2648	2502	phoB (alkaline phosphatase III precursor)	307,935	16,6388	-4,21
2653	2506c	probable transcriptional regulator	333,978	1495,27	2,16258
2655	2508c	arcD (arginine/ornithine antiporter)	114,829	681,237	2,56868
2657	2510c	arcA (arginine deiminase)	127,655	2,95226	-5,43428
2659	2512c	aur	0,367728	0,046805	-2,97389
2661	2514	probable exported protein	66,1993	4957,32	6,2266
2662	2515	probable antiterminator transcriptional regulator	1,41223	7,67806	2,44276
2665	2518c	probable membrane protein	7,61825	300,23	5,30046
2668	2521c	probable surface anchored protein	403,508	1,8438	-7,77377
2669	2522c	conserved hypothetical protein	127,588	0,056260	-11,1471
	2524c	2524c	84,0611	0,462607	-7,50551
2674	2527c	conserved hypothetical protein	0,809667	0,046678	-4,11649
	2529c	2529c	12,4271	121,023	3,28372
2680	2532	hypothetical protein	0,482642	14,2289	4,88173
2681	2533	hypothetical protein	2498,37	20,8587	-6,9042
2683	2535c	peptide methionine sulfoxide reductase	8,58578	266,162	4,95421
2690	2542	icaD	0,399813	11,5001	4,84617
2710	2562c	conserved hypothetical protein	1810,7	4,72102	-8,58323
2711	2563	conserved hypothetical protein	40,4131	2564,75	5,98785
2717	2569	hypothetical protein	0,112827	10346,3	16,4846
2718	2571	2-oxoglutarate-malate translocator	143,528	2,59946	-5,78698
2721	2574	probable high-affinity nickel-transport protein	0,865723	691,801	9,64224
2722	2575	hypothetical protein	0,281379	46,6619	7,37359
2723	2577c	probable membrane protein	43,2615	1126,78	4,70298
2732	2582c	probable membrane protein	12,0737	2761,68	7,83754
2735	2585c	conserved hypothetical protein	8,75043	1578,1	7,49462
2736	2586c	gidB (16S rRNA methyltransferase)	0,549069	1395,12	11,3111