
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Agouna-Deciat, B. Olivier, The Effect of Two Staphylococcal Global Regulators (*agr* and *sar*) on Acid Phosphatase Production in *Staphylococcus aureus*. Master of Science (Molecular Biology and Immunology), May 2003, 75 pp., 3 tables, 14 illustrations, 16 titles.

Staphylococcus aureus produces an extensive number of cell-surface associated proteins, extracellular proteins and enzymes that contribute to its virulence. The key to better preventive or curative approaches resides in identifying and targeting the very genes and their products that play major roles in the survival of the bacteria within the host and the establishment of diseases. Two well known regulatory loci, the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sar*), control the expression of most of *S. aureus* genes that encode for its virulence factors. Other virulence gene regulators have recently been isolated. Over 40 proteins and enzymes produced by *S. aureus* have been identified and several of them have been linked to staphylococcal pathogenesis. In this study, we attempt to determine the role of *agr* and *sar* in the regulation of the production of a secreted staphylococcal acid phosphatase (Sap) suspected to contribute to virulence.

The Effects of Two Staphylococcal Global Regulators (*agr* and *sar*) on Acid Phosphatase production in *Staphylococcus aureus*.

B. Olivier Agouna-Deciat, B.S.

APPROVED:

Major Professor

Committee Member

Committee Member

University Member

Chair, Department of Molecular Biology and Immunology

Dean, Graduate School of Biomedical Sciences

THE EFFECTS OF TWO STAPHYLOCOCCAL GLOBAL REGULATORS (*agr* AND *sar*) ON ACID PHOSPHATASE PRODUCTION IN *Staphylococcus aureus*

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas
Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Bahrka Olivier Agouna-Deciat. B. S.

Fort Worth, Texas

May 2003

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF ILLUSTRATIONS

Page

INTRODUCTION

Staphylococcus aureus is a gram-positive coccus and a major cause of nosocomial infections (15). The ability of this bacterium to produce a wide array of virulence factors makes it highly pathogenic to humans as well as other warm-blooded animals (104). The diseases caused by this bacterium range from simple skin infections like boils to more serious and life-threatening infections such as endocarditis, osteomyelitis, pneumonia, meningitis, and septicemia (32). The most common entry point into the human host is usually a break in the skin or mucous membrane from where the organism can reach the blood stream (61). Once in the bloodstream, metastatic infections like those mentioned above are often fatal if not treated with an appropriate antibiotic (11, 66, and 106). The formation of abscesses with massive infiltration of polymorphonuclear (PMN) leukocytes and intense tissue damage is a typical histological characteristic of lesions caused by *S. aureus*. The skin and mucosal surfaces are the primary line of host defense against *S. aureus* invasion, but whenever breached, PMN must play the major role by clearing out the bacteria (70). When *S. aureus* gain access to the bloodstream and underlining tissues, monocytes and macrophages are activated and release cytokines (glycoproteins) involved in the regulation of the host immune system. Cytokines released include TNFα, IL-1α/β (Interleukine-1 α/β), IL-6, IL-8, IL-10, IL-12, interferon α (IFNα), and colony stimulating factors (CSF). Other cell types such as endothelial cells, fibroblasts and lymphocytes can also produce most of these cytokines. This cascade of cytokine release contributes to the activation of PMNs circulating in the blood stream. Activated PMNs

adhere to activated endothelial cells and move in-between them to reach the site of infection. Once at the site of infection, PMNs are to internalize invading bacteria and kill them in a process called phagocytosis (102). However, phagocytosis generally occurs after proper opsonization of the bacterium, which consists of antibodies and activated C3 (part of the complement system) coating the microorganism (102). In the case of *S. aureus*, opsonization is rendered difficult due to, on one hand, the ability of many strains to produce a capsule; and on the other hand, the production and localization of protein A to the bacterial cell wall (101). The capsule prevents the peptidoglycan molecule located in the cell wall from reacting with host antibody and C3b (14, 81, and 83); protein A can bind the Fc portion of IgG in human serum and to specific antibody thus preventing them to react with Fc receptors on PMNs and thereby preventing phagocytosis (82, 101, and 97). Phagocytosis is the cellular process by which PMNs and macrophages engulf bacteria via pseudopode extension. The internalized microorganism is located in a vesicle called phagosome. ATPases present in the phagosome membrane pump proton into the phagosome causing its pH to decrease to approximately 5. The acidified phagosome containing the bacterium then fuses with one or more lysosomes that contain inactive bactericidal lysosomal granules. The lysosomal granules are activated by the low pH of the phagolysosome. The lysosomal granules are composed of bactericidal proteins including proteases and lysozyme with the ability to directly destroy surface proteins of bacteria; defensins that kills the bacteria by creating pores into their cytoplasmic membrane; and third, myeloperoxidase that activates an NADPH oxidase located in the phagosome membrane. The reaction between the myeloperoxidase and the

NADPH oxydase under low pH produces highly reactive superoxide radicals that are capable of inactivating essential bacterial surface proteins and damaging nucleic acids. This last mechanism by which bacterial are killed within the phagolysosome is called oxidative burst.

In hospital settings, patients with open wounds due to surgery or burns are at high risk for contracting *S. aureus* (103). Acquired immunodeficiency syndrome (AIDS), cancers and other diseases that compromise the immune system are also factors contributing to severe forms of staphylococcal infections (63, 62, 95, 28, and 60). Hospital acquired *S. aureus* infections are usually more severe and hard to treat due to the multi-drug resistance of the infecting strain (94 and 8). Over the years, *S. aureus* has gain resistance to most conventional antibiotic treatments, making it even harder to fight the infection once established (17). Methicillin-resistant *S. aureus* (MRSA) have caused serious public health problems in hospital settings since the 1960's (5, and 78) and recently reports are raising concerns for outbreaks in the community caused by MRSA (47, 16, and 13). For many years vancomycin was the only efficient anti-microbial agent against *S. aureus*; but unfortunately in 1996, the first clinical isolate of *S. aureus* with intermediate resistance to vancomycin was reported from Japan (48). Later on, eight other clinical cases of infection caused by vancomycin-intermediate *S. aureus* (VISA) were reported in the United States (96 and 34). The first clinical case of vancomycin resistant *S. aureus* (VRSA) in the United States was reported by the Center for Diseases Control (CDC) in July 2002 (18). The present anti-microbial resistance of *S. aureus* is by

its self a sound appeal for more adapted research leading to discovery of better ways to combat this pathogen.

S. aureus great pathogenic potential is due to its ability to produce a substantial number of extracellular and cell surface-associated proteins contributing to its virulence. These proteins or virulence factors can be classified according to their presumed roles in the infective process; thus there are factors involved in the adhesion of the bacteria to the host cells or extracellular matrices, those that contribute to the evasion of the host immune system by preventing or reducing the activity of phagocytes and/or specific antibodies, those that mediate the invasion of host tissues by either attacking the cells or destroying elements of the extracellular matrices, and last but not least, factors called superantigens that are capable of inducing an exaggerated and pathologic response of the host immune system (84). The coordinated interactions of these different groups of virulence agents make *S. aureus* a very potent pathogen hard to combat. Over 40 distinct extracellular and cell surface-associated proteins have been identified and somewhat characterized (2). Many of these proteins have been found to contribute to the virulence of *S. aureus*. However, for many others, a direct role in virulence is yet to be defined. Among the well-studied virulence factors are the hemolysins (α,β, and δ) with their respective genes *hla*, *hlb*, and *hld*. These secreted toxins contribute to pathogenesis by facilitating the invasion of host tissue. The exfoliative toxins A and B (EtaA and EtaB), and the toxic shock syndrome toxin-1 (TSST-1) are also well known and are respectively directly responsible in the development of the scalded skin syndrome and the staphylococcal toxic shock syndrome. Proteases, lipase and coagulase produced by *S.*

aureus participate in the destruction of host tissues and thus contributing to invasion and the spreading of the infection; whereas, the clumping factors A and B (*clfA* and *B* products), collagen binding protein (*cna* product), and fibronectin binding proteins A and B (*fnbA* and *fnbB* products) are cell surface proteins that contribute to the adhesion of the bacterium to the host cells, thereby are important for the establishment of many staphylococcal infections. With the exception of a couple of toxins (TSST-1, EtaA/B), the virulence of *S. aureus* is usually due to the combined actions of several virulence factors (50 and 61). Many others factors produced by *S. aureus* are yet to be characterized.

Two well-characterized regulators of virulence factors in *S. aureus* are the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sar*) (2). These regulators control expression of *S. aureus* genes that primarily encode for extracellular and cell wall-associated proteins in a concerted and temporal fashion (21, 56, and 75). A number of these proteins are clearly involved in the disease process and thus considered to be virulence factors. The *agr/sar* regulatory system works as a toggle switch between the exponential and post-exponential phases of growth; the cell surface proteins are synthesized during the exponential phase while the secreted toxins are synthesized during the post-exponential phase (51 and 56). Other virulence gene regulators have recently been characterized; not only their interaction with expression of virulence genes but also their interaction with the *agr/sar* system as either repressors or co-activators (25, 26, 64, 68, 93, and 98). Several others regulatory loci have been identified but very little is known about them as of yet.

The *agr* locus, which was identify by transposon mutagenesis using Tn*551*, plays a major role in the expression of most known accessory genes of *S. aureus* (85). We mention accessory genes by opposition to housekeeping genes that are necessary for the normal physiologic functions of the microorganism (29 and 30). In the case of *S. aureus*, most of those accessory genes can be categorized as virulence factors due to their different roles in the infective process (84). The transposon mutant of *agr* revealed a phenotype with decreased production of most of the extracellular virulence factors while the expression of the cell wall-associated proteins remained normal (80). Results from these studies concluded that the *agr* locus, when expressed, activates the expression of extracellular virulence factors in *S. aureus* during the post-exponential phase of growth while it represses the cell wall-associated proteins produced during the exponential phase of growth (80). The *agr* locus has been cloned and its sequence determined (73 and 80). The locus contains two divergent transcripts, RNAII and RNAIII, which are expressed from promoters P2 and P3, respectively (56 and 77). RNAII codes for four open reading frames (AgrA, AgrC, AgrB, and AgrD), and RNAIII codes for δ-hemolysin (77 and 52). RNAIII has been shown to be the effector molecule in the regulation by *agr* (75). RNAIII regulation occurs at either the transcriptional level or the translational level, or both (75, 99, and 71). The Agr proteins synthesized from RNAII are indispensable for the expression of RNAIII from its promoter (77). Open reading frames encoded by the RNAII operon also regulate transcription from the P2 promoter in a feedback mechanism (77). The AgrA and AgrC molecules are thought to perform as a two-component signal transduction system (53, 73, 79, 80, and 85). Two-component signal transduction

systems in cells involve a complex composed of a sensor molecule usually located on the membrane and a molecule acting as responder located inside the cell. The sensor reacts with a specific ligand that could be an environmental factor or an extracellular stimulating molecule; and that reaction activates the sensor, which in turn activates the responder. In the Agr system, AgrC constitute the membrane bound sensor that is autophosphorylated upon binding of autoinducing peptide (AIP) molecules (54, 59, 67, and 76). AIP are AgrD molecules processed by the membrane-bound AgrB and translocated outside of the cell (2). Phosphorylated AgrC induces the phosphorylation of AgrA, the responder, which in turn activates P2 and P3 leading to the expression of RNAII and RNAIII (2).

A second well-described regulatory locus, *sar* was also found by transposon mutagenesis (21). The *sar* locus was shown by Southern hybridization and sequencing to be distinct from the *agr* locus (21 and 25). Insertion of Tn*917TV1* into the *S. aureus* chromosome resulted in a mutant that presented with a pleiotrophic affect on the expression of several virulence factors similar to the *agr* mutation (21). However, the *sar* mutation resulted in an increase in α-toxin production and protease activity while the expression of most cell wall-associated protein was decreased (21); which is contrary to what is seen in the *agr* mutant. The *sar* locus codes for three overlapping transcripts (*sarA*, *sarB*, and *sarC*) each with its own promoters (6). All three overlapping transcript have been shown to encode SarA (6) which is a regulatory DNA binding protein that up regulates transcription of RNAII and RNAIII (*agr* transcripts) by binding to their respective promoters P2 and P3 (22, 46, and 72). This interaction with *agr* promoters

region is one of the way by which *sar* regulate certain virulence factors as demonstrated for α- and β-toxin (22 and 24) and protein A (23). However, SarA has also been shown to directly bind to a conserved region, homologous to the one on *agr* promoters, upstream of the –35 promoter regions of several virulence factor genes including α-toxin gene (*hla*), protein A gene (*spa*), fibronectin binding protein genes (*fnb*) and enterotoxin C gene (*sec*) (27). Through this direct mechanism of action, *sar* has been shown to up-regulate the production of α-toxin (24 and 27), fibronectin binding protein A (107), and repress protein A (23) independently of *agr*. It has also been shown that *sar* inhibits collagen-binding protein independently of *agr* (38 and 9). SarB and SarC are also involved in the *agr*-dependent regulation by modulating RNAII transcription during the stationary phase of growth (22). Indeed, *sarB* has been shown to be important for expression of cell wall-associated proteins (107). The fact that the *agr/sar* double mutant is more limited in the synthesis of many virulence factors confirms that *sar* regulatory mechanisms are both *agr*-dependent and *agr*-independent (12).

A few other gene regulators have recently been identified and characterized. Among them are a number of loci that have been characterized as *sar* homologues based on sequence identities of their products and similar function of the latter (2 and 20). The products of these loci form a family of six highly basic DNA-binding proteins with conserved primary sequence motif (2 and 20). These *sar* homologues include *sarR*, *sarS*, *sarT*, *sarU*, *sarV*, and *rot*. Most of these have been characterized and identified as being repressors of specific accessory genes primarily those that encode for virulence factors (68, 98, 64, 2, and 93). However, recent studies are unveiling their abilities to up-

regulate some of those virulence factors as well (2 and 90). The up-regulation could happen by direct interaction of the regulator with the promoter region of a particular gene or one could still argue that they still act essentially as repressors and that any apparent up-regulation could be the result of the repression of another repressor. Following this hypothesis, it is logical that the expression of the gene being repressed would increase due to the fact that another repressor is blocking the activity of its repressor. Other recently identified and characterized regulators of *S. aureus* virulence include *sae* (42, 41, and 40), *srrAB* (108), and *arlSR* (33).

As complicated and confusing the regulation of virulence factors in *S. aureus* may appear, we are probably still far from putting the last peace of that puzzle into place. There sure still is plenty of room for more discoveries as the list of specific factors produced by this organism that are associated with virulence continues to grow. We should emphasize the fact that the abundance and diversity of *S. aureus* virulence determinant are key elements in the versatility of that pathogen.

In this study, we are looking at the possible regulatory role of either *agr* or *sar* on the expression and production of a secreted acid phosphatase that was recently characterized by Du Plessis *et al* (31) in the background of *S. aureus* strain 154, and whether or not this enzyme plays a role in virulence. Our interest in studying the Staphylococcal acid phosphatase (Sap) is based on reports suggesting the contribution of acid phosphatase to microbial pathogenesis by promoting the survival of intracellular pathogens within hosts' phagocytic cells (86, 89, 4, 19, and 87). The molecular weight of the acid phosphatase found in *S. aureus* strain 154 was estimated to be approximately 30

kDa based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/zymographic analysis. The staphylococcal acid phosphatase gene (*sap*) has been identified and sequenced; the size of the gene was found to be 891 bp (31). Other bacteria known to produce acid phosphatase include *Coxiella burnetti*, the causative agent of Q fever in humans, an acid phosphatase was found to contribute to virulence by enabling the bacterium to avoid being killed after internalization by phagocytic cells and also allowing it to survive within the phagolysosome (4). Reilly *et al* (87) has also reported that *Francisella tularensis*, which causes the fatal human disease, tularemia, produces an acid phosphatase capable of suppressing the respiratory burst induced in activated neutrophils. Again in another bacterium, *Bordetella bronchiseptica*, a natural pathogen of the animal respiratory tract, an acid phosphatase is found to be contributing to the intracellular survival of that organism (19). All of the previously mentioned microorganisms have in common the fact that they are obligate or facultative intracellular pathogens and thus they are oriented towards developing means of survival within host phagocytic cells. Yet, bacteria such as the equine pathogen *Streptococcus equi*, not classified as intracellular, have also been found to produce acid phosphatase (44). Sap as described by Du Plessis *et al* (31) showed optimum activity at pH 5.0 and 37°C, which corresponds to the optimum activity pHs of the acid phosphatases produced by the previously mentioned intracellular bacteria. We should also recall that the pH within phagocytic cells, macrophages in particular, is lowered to that range in order to carry out their bactericidal activity on internalized pathogenic bacteria. Acid phosphatases are considered ubiquitous class of enzymes that catalyze the hydrolysis of

phosphomonoesters under acidic pH conditions (87). The primary activity of acid phosphatases is to contribute to the mobilization of phosphate, but some acid phosphatases can also participate in essential biological functions in the cell such as metabolism regulation, energy conservation and signal transduction (87).

As of now, *S. aureus* is classified as an extracellular pathogen even though there is evidence of it being internalized by certain non-professional phagocytic cells including epithelial cells, endothelial cells, and osteoblasts (1, 61, 100, 7, and 49). Yet, there have been reports of *S. aureus* ability to survive within polymorphonuclear cells and monocytes of the human immune system (88, 69, and 55). More recently, Gresham *et al.* (43) demonstrated that not only *S. aureus* could survive within neutrophils but that this was in fact also a major contribution to virulence and persistence of the infection. Thus, based on previous studies suggesting that acid phosphatase produced by other bacteria contribute to survival within host phagocytic cells, we hypothesize that along with other factors, Sap also plays a role in *S. aureus* virulence by enabling the bacterium to survive within the phagolysosome of professional phagocytic cells and avoid being killed during phagocytosis. In this study, we have begun the characterization of Sap gene (*sap*) and we also investigated the role of *agr* and *sar* in the regulation of *sap* expression. Our data show that *sap* is conserved among the strains of *S. aureus* included in our study and absent in the strains of *Staphylococcus epidermidis* analyzed. We also confirmed that *sap* product is secreted and that mechanisms controlling its expression as well as secretion are strain-dependent. Finally we also demonstrated that *agr* and *sar* partially affect the

production of Sap at the transcriptional, post-transcriptional and secretion levels. The effects of *agr* and *sar* were also shown to be strain-dependent.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

We routinely used the appropriate solid or liquid media and for the mutants the corresponding selective antibiotics were added each time. *S. aureus* wild type strains RN6390 (NTH83 and NTH207) and UAMS-1 (NTH13) were routinely grown on tryptic soy agar (TSA) or in tryptic soy broth (TSB). For the different *S. aureus* RN6390 and UAMS-1 mutants, we added the appropriate selective antibiotic. To grow the RN6390 mutants: kanamycin was added to a final concentration of 50 μg/ml for the *sar* mutant (NTH208); tetracycline to a final concentration of 3 μg/ml for the *agr* mutant (NTH209); for the *agr/sar* double mutant both kanamycin and tetracycline were added at a final concentration of 50 μg/ml and 3 μg/ml respectively. For the UAMS-1 mutants: kanamycin and neomycin were both added to a final concentration of 50 μg/ml each for UAMS-1 *sar*-mutant (NTH332); tetracycline was added to a final concentration of 5 μg/ml for the UAMS-1 *agr*-mutant (NTH333); all three, kanamycin, neomycin and tetracycline were added to a final concentration of 50, 50 and 5 μg/ml, respectively for the UAMS-1 *agr/sar* double mutant (NTH334). Bacterial cells were routinely streaked onto media from frozen (-85°C) culture stocks and after an overnight incubation at 37°C, were used to inoculate broth. *E. coli* strains HB101 or JM109 used in cloning experiments were routinely cultured on Luria-Bertani (LB) agar plates or in LB broth containing 100 μg/ml of carbenicillin when plasmids containing

carbenicillin resistance genes were present. Plates were routinely incubated overnight at 37°C. Inoculated TSB or LB cultures were incubated at 37°C with constant agitation of 180 rotations per minute (rpm) for *S. aureus* cells or 225 rpm for *E. coli* cells by using a G-24 Environmental Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, NJ).

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics	Source[a]
S. aureus		
RN6390 (NTH83)	Prototypic strain	A. Cheung, Darmouth
RN6390 (NTH207)	Prototypic strain	M. S. Smeltzer, UAMS
RN6390 (NTH208)	*sar::kan.* Selected with kanamycin (50 μg/ml)	M. S. Smeltzer, UAMS
RN6390 (NTH209)	Δ*agr::tmn* mutant. Selected with tetracycline (3 μg/ml)	M. S. Smeltzer, UAMS
RN6390 (NTH210)	*agr*/*sar* double mutant. Selected with kanamycin (50 μg/ml) and tetracycline (3 μg /ml)	M. S. Smeltzer, UAMS
UAMS-1 (NTH13)	Osteomyelitis	M. S. Smeltzer, UAMS
UAMS-1 (NTH332)	*sar::kan.* Selected with kanamycin (50 μg/ml) and neomycin (50 μg/ml)	Jon Blevins, UAMS
UAMS-1 (NTH333)	*agr*-null. Selected with tetracycline (5 μg/ml)	Jon Blevins, UAMS
UAMS-1 (NTH334)	*agr*/*sar* double mutant. Selected with kanamycin (50 μg/ml), neomycin (50 μg/ml) and tetracycline (5 μg/ml)	Jon Blevins, UAMS
HAC1	Blood	OMCT

HAC2	Skin abscess	OMCT
HAC3	Peritoneal fluid	OMCT
HAC4	Respiratory secretion	OMCT
HAC6	Surgical wound	OMCT
UAB1	Sputum	UAB
S. epidermidis ATCC12228		ATCC
HAC33	Blood	OMCT
E. coli JM109		Promega
HB101		T. Romeo, Emory University School of Medicine

[a] UAMS, University of Arkansas for Medical Sciences; OMCT, Osteopathic Medical Center of Texas; UAB, University of Alabama at Birmingham; ATCC, American Type Culture Collection, Manassas, VA; Promega, Madison, WI.

Isolation and preliminary characterization of the staphylococcal acid phosphatase gene (*sap*) from *S. aureus* RN6390.

PCR amplification of *sap*.

The purpose of this experiment was to amplify a full-length *sap* gene to be used in subsequent cloning and characterization experiments. The published sequence of the staphylococcal acid phosphatase (*sap*) gene was obtained from the NCBI database

(Accession #: AY061973). The sequence was used to Blast against the entire *S. aureus* N315 chromosome to obtain the flanking sequence of up to a 1000 bases up and down-stream of *sap*. The entire region was analyzed using the DNAMAN computer software (DNAMAN, Lynnon Biosoft, Vaudreuil, Quebec). Primer sequences retained were Blast against the remaining chromosome in order to limit incidence of non-specific annealing. Primers were analyzed for hairpin formation and also to make sure that they will not anneal to each other. Site for restriction endonuclease cleavage were added to the forward and reverse primers in order to facilitate subsequent cloning into appropriate plasmid vectors. A *Kpn*I site was added to the 5'-end of the forward primer and a *Xba*I site was added to the 5'-end of the reverse primer. The choice of these restriction sites was based on the fact that they are present in the right orientation in the multiple cloning sites of plasmids pCR2.1 (Invitrogen, Carlsbad, CA) and pCL10 (92). The forward primer (SaAPF) was predicted to anneal 164 bp upstream of the beginning of the gene and the reverse primer (SaAPR) was predicted to anneal 158 bp downstream of the end of the gene.

TABLE 2. Primers sequences

Name	Sequence	Sense	TM
SaAPF	GCGC**GGTACC**TCTCATGTGATAGGTCTCC	Forward	66.7°C
SaAPR	GCGC**TCTAGA**CTTATCCTTCATTATACCCGAAC	Reverse	63.1°C

Sequences are written from 5' to 3'. The inserted restriction sites, *Kpn*I for SaAPF and *Xba*I for SaAPR, are bolded and underlined.

The PCR was done using the Promega PCR system kit (Promega, Madison, WI) and the SaAPF/SaAPR primers. *S. aureus* RN6390 chromosomal DNA was used as template. We set up the reactions with three different dilutions: 1:25, 1:50, and 1:100 of the chromosomal DNA stock. The reactions were performed in triplicate. The reactions were in microcentrifuge tubes using the following scheme:

Component	**Volume**
PCR master mix (2X)	12.5 µl
SaAPF (40 µM)	0.6 µl
SaAPR (40 µM)	0.6 µl
DNA template	5 µl (1:25, 1:50, 1:100)
Nuclease-free water	6.3 µl
Total:	25 µl

The reaction were run in a PTC-100 programmable thermocycler (M. J. Research, Inc., Waltham, MA) with the following parameters:

1: Denaturing for 2min at 95°C
2: Denaturing for 30sec at 95°C
3: Annealing for 30 sec starting at 45°C and increasing 0.5°C at every cycle.
4: Elongating for 2 min at 72°C
5: Go to step 2 34 more times
6: Elongating (final) for 5 min at 72°C
7: hold at 4°C.

Samples of each amplification were resolved by electrophoresis using 0.8% agarose gels. The PCR products were extracted using the QIAGEN QIAquick Gel Extraction kit (Quiagen Inc., Valencia, CA). Verification that we had effectively isolated the desired PCR product was done by agarose gel electrophoresis. The eluted purified PCR product was stored at - 20°C until needed.

Chromosomal DNA isolation from *S. aureus*.

Chromosomal DNA was isolated from *S. aureus* strains for later use in PCR and southern analysis experiments. The CTAB (Cetyl trimethyl ammonium bromide) chromosomal isolation procedure was used to isolate DNA from *S. aureus* (3). Briefly, cells were cultured overnight in 5 ml of TSB with appropriate antibiotic when needed as described earlier. Samples (1.5 ml each) were centrifuged and the supernatants were discarded. The cell pellets were suspended in 560 µl of TE buffer (10 mM Tris [pH 7.5] and 1 mM EDTA). To the suspended cells, we added 5 µl of lysostaphin (Applen and Barrett LTD, England. 10 mg/ml stock) and 5 µl of RNAse (Sigma-Aldrich, St. Louis, MO, 10 mg/ml stock), followed by 1 hour incubation at 37°C. Thirty µl containing 10 % SDS, 5 µl of RNAse (10 mg/ml), and 5 µl of proteinase K (Fisher Scientific, Pittsburgh, PA. 20 mg/ml stock) were added to each sample. The contents of each tube were mixed by vortexing for 15 min and then by shaking vigorously for another 15 min. The samples were incubated for another hour with intermittent shaking to facilitate mixing of the suspension. 100 µl of 5 M NaCl were added followed by thorough mixing (for at least 15 sec). After that 80 µl of pre-warmed (65°C) CTAB/NaCl (10% CTAB in 0.7 M NaCl) were added and samples were mixed thoroughly followed by 20 min incubation at 65°C. An equal volume of chloroform was added to each sample followed by vigorous shaking (at least 15 sec) and centrifugation for 5 min at 16,000 x *g*. The viscous upper phase of each sample was transferred to a fresh sterile tube. To the transferred viscous phase an equal volume of phenol/chloroform (25:25) was added and the content of the tube was mixed by shaking and briefly vortexing. The sample was centrifuged for 5 min at 6,000 x

g. The aqueous phase was transferred to another fresh tube and extracted again as before with the phenol/chloroform mixture. This step was repeated once again to obtain a neat interface. A last extraction with equal volume of chloroform was performed to remove residual phenol. The upper phase was transferred to a fresh tube and 500 µl of isopropanol was added to it to precipitate the DNA. The tube was inverted several times until DNA was visible. The sample was incubated overnight at -20°C to facilitate precipitation and increase yield. DNA was then collected by centrifugation at 16,000 x *g* for 15 min. The isopropanol was discarded and 1 ml of 70% ethanol was added to wash DNA followed by centrifugation at 16,000 x *g* for 10 min. Ethanol was removed and the DNA pellet was allowed to air-dry overnight. The pellet was resuspended in 100 µl of TE buffer and stored at 4°C until needed.

Cloning and verification of *sap* amplicon by restriction analysis and DNA sequencing.

These procedures were carried out in order to ensure that we had indeed isolated the right gene (*sap*). For this purpose we used the Promega pGEM-T Easy vector cloning kit (Promega, Madison, WI). Competent *E. coli* JM 109 cells provided with the kit were transformed with the ligation reaction mixtures. Cells were plated onto LB plates containing carbenicillin and 40 µl of X-Gal (40 mg/ml) and 40 µl of isopropylthio-β-galactoside (IPTG, 200 mg/ml) were added. Colonies containing the desired insert will remain white whereas colonies containing plasmids with no insert will turn blue. The plates were incubated overnight at 37°C. A selected number of white colonies were transferred to fresh LB/Carb plates to insure they remained white and were resistant to

carbenicillin. Plasmids were isolated using the mini-prep plasmid isolation kit from Bio-Rad (Bio-Rad, Hercules, CA). The plasmids were stored at -20°C until needed.

Various restriction enzyme purchased from Promega (Promega, Madison, WI) were used to cut the constructs either for verification of the desired insert or for removing insert in its entirety. *Kpn*I and *Xba*I were used to liberate the inserts from the plasmid. *Not*I was also used to liberate the inserts; its recognition sequences flank the multiple cloning site of the pGEM-T Easy cloning vector and it does not cut the *S. aureus* chromosome. *Hind*III was used to linearize the construct by cutting approximately in the middle of *sap* gene.

Plasmid DNA concentrations were determined using the Biotech Gene QuantII spectrophotometer (Pharmacia, Cambridge, England). Plasmids containing the desired insert were sequenced using the DNA Sequencing Laboratory in the Department of Microbiology and Immunology of the University of Arkansas for Medical Sciences and under the direction of Allen Gies. The sequencing was done using the M13 forward and reverse primers of the vector. The DNAMAN software was used to analyze the sequence data.

Examination of various staphylococcal species for the presence of *sap* by Southern analysis.

Southern analysis was used to study the distribution of *sap* among some *S. aureus* strains and other staphylococcal species (*S. epidermidis* strains).

The PCR product was cleaned using the QIAquick PCR Purification Kit from QIAGEN (Quiagen Inc., Valencia, CA). One µl of the resulted cleaner PCR product was resolved by agarose gel electrophoresis to verify the integrity and the yield of the desired product. To make the probe, 15 µl of the purified PCR product was heat denatured by boiling for 10 min. The denatured DNA was immediately chilled on ice. 2 µl of 10X Hexanucleotide mix (Roche Diagnostics, Mannheim, Germany) and 2 µl of 10X DIG label (Roche Diagnostics, Mannheim, Germany) were added to the sample. 1 µl of Klenow DNA polymerase I (Promega, Madison, WI) enzyme was added to the tube and the contents were mixed and incubated overnight at 37°C. Two µl of 200 mM EDTA (pH 8) were added to terminate the reaction. The DIG-labeled DNA, was precipitated by adding 1 µl of glycogen (Roche Diagnostics, Mannheim, Germany, 20 mg/ml), 0.1 volume of 4M LiCl and 2.5 volumes of ice-cold 100% ethanol. The contents of the tube were mixed well and incubated at -85°C for 30 min. The sample was then centrifuged at 4°C for 30 min at a speed of 16,000 x *g*. The DNA pellet was washed twice with 100 µl of ice-cold 70% ethanol and the pellet was allowed to air-dry overnight at room temperature. The pellet was suspended in 50 µl of TE buffer (pH 8.0) and stored at -20°C until needed.

Chromosomal DNA isolated from strains *S. aureus* and *S. epidermidis* were resolved by electrophoresis using 0.8% agarose gels containing ethidium bromide in 1X TBE buffer. When the electrophoresis was completed the resolved DNA was visualized using a UV trans-illuminator and a picture of the gel was immediately taken using the

alphaimager from Alpha Innotech Corporation. The gel was then incubated in 0.4 N NaOH-0.6 M NaCl for 30 min at room temperature with gentle agitation to denature the DNA. The gel was then washed in 1.5 M NaCl-0.5 M Tris-HCl, pH 7.5, for 30 min. Meanwhile, in preparation for the transfer, MagnaGraph transfer membrane 0.45 micron (Osmonics Inc.) was cut to match the size of the gel. The membrane was allowed to soak for 15 min in a container containing 10X sodium chloride/sodium citrate (SSC) solution. The gel was placed on filter paper face down and the membrane was cautiously placed on top of it making sure that no air bubbles were trapped between them. Two pieces of presoaked filter paper were placed on top of the membrane. The DNA was passively transferred to the membrane for 16-24 hours. The membrane was placed face up on a glass tray and UV fixed using the UV Gene Linker (Bio-Rad, Hercules, CA). The membrane was then immersed in 0.4 N NaOH for 30-60 sec to complete the denaturation of the cross-linked DNA. The membrane was promptly transferred to 0.2 M Tris-HCl pH 7.5-2X SSC solution to be neutralized. That same neutralization solution was used to facilitate the transfer of the membrane to a hybridization bottle. The neutralization solution was then poured off the bottle and replaced with the hybridization solution consisting of 20 ml of 1% SDS, 1 M NaCl, and 10% dextran sulfate (Fisher Biotechnology grade, Fisher Scientific, Pittsburgh, PA). The membrane was pre-heated in that solution for at least 15 min at 65°C with constant agitation using a Hybaid hybridization chamber (National Labnet Company, Woodridge, NJ). Next, the pre-heated membrane was pre-hybridized with 0.2 ml denatured salmon-sperm DNA (Sigma-Aldrich, St. Louis, MO, 10 mg/ml). Denaturation was carried out by heating the DNA at

95°C for 20 min. Pre-hybridization was carried out for 1 hr at 65°C with constant agitation. Ten µl of the digogxigenin *sap* probe in a total of 100 µl of water was added to the prehybridized membrane after having been denatured as described for salmon-sperm. Hybridization was routinely allowed to run overnight (18-24 hrs) at 65°C with constant agitation. When the hybridization was completed the membrane was processed with various solutions: twice with 50 ml 2X SSC at room temperature for 5 min each with constant agitation; twice with 50 ml 2X SSC and 1.0% SDS at 65°C for 30 min each with constant agitation; twice with 50 ml 0.1X SSC at room temperature for 30 min each with constant agitation; twice with 50 ml of blocking buffer (1X conjugate buffer, 0.1% Tween-20) at room temperature for 5 minute each with constant agitation; 30 min with 50 ml of blocking buffer at room temperature with constant agitation; with diluted (1:10,000) anti-digoxigenin-AP Fab fragments (Roche Diagnostics, Mannheim, Germany) 1:10,000 in 25 ml of conjugate buffer (1 g of sodium caseinate [US Biochemical], into 500 ml of 1X PBS dissolved by heating) at room temperature with constant agitation; once with Blocking buffer for five min at room temperature; 4 times with wash buffer (1X PBS, 0.3% Tween 20) for 5 min each at room temperature and finally twice with assay buffer (0.1 M diethanolamine [Fisher Scientific], and 1mM $MgCl_2$) for 5 min each at room temperature. The blot was transferred to a seal-a-meal bag and 5.5 µl of CDP-Star (Roche Diagnostics, Mannheim, Germany) in 1 ml of assay buffer was added. The bag was sealed and the chemiluminescent substrate was worked into the blot with gentle manipulation with gloved hands. Excess substrate and air bubbles were removed through another cut opening. The bag was sealed again and the

blot was then exposed to X-ray film (Fudji Medical, Stamford, CT) for various times until the appropriate exposure was achieved.

Expression of *sap* in *agr* and *sar* regulatory mutants of *S. aureus* strains RN6390 and UAMS-1.

These experiments were done to compare *sap* message levels in *S. aureus* RN6390, UAMS-1, and their respective isogenic regulatory mutants.

Isolation of total RNA.

S. aureus cultures were harvested at 3, 6, and 12 hrs of growth by pipetting 10 ml of cells in growth medium into 25-ml, screw capped Corex tube containing 10 ml of ice-cold acetone:ethanol (1:1). The cell suspensions were then stored overnight at -20°C. Cells were transferred to 25-ml Teflon tubes and centrifuged at 11.5 rpm 16,000 x *g* for 15 min. The cell pellets were suspended in 1 ml of TES (150 mM NaCl, 78 mM DiNaEDTA, and 100 mM Tris, pH 7.5) and centrifuged again as in the previous step. The cell pellets were suspended in 1 ml of HSTES (TES with 2.5 M NaCl added). Lysostaphin (Applen and Barrett LTD., England) was added to a final concentration of 50 µg/ml. The samples were incubated at 37°C for 30 min. Five ml of RNAzol B (Tet-Test, Inc., Friendswood, TX) was carefully added to each protoplast suspension slowly rocked to facilitate cell lysis. When the lysis was complete 600 µl of chloroform were added to each sample followed by a vigorous shaking for 15 seconds. Samples were then incubated on ice for 15 min before being centrifuged at 16,000 x *g*. Two, 600-µl portions of the aqueous phase were pipetted to 2 microcentrifuge tubes. 600 µl of isopropanol

was added to each tube and the contents were mixed by inversion. The RNA precipitation was facilitated by storing the tube overnight at -20°C. The following day, the samples were centrifuged for 15 min at 16,000 x *g* and 4°C. The supernatants were discarded and the pellets were washed twice with 1 ml of 70% ethanol made in Diethyl Pyrocarbonate (DEPC)-treated, deionized water. Tubes containing RNA pellets were inverted and all traces of ethanol were allowed to drain out for approximately 30-40 min. Each pellet was then suspended in 100 µl of DEPC-treated, deionized water and to facilitate complete suspension pellets were incubated at 65°C for 10-15 min. The RNA samples were stored at -85°C until needed.

To determine the concentration of RNA 5 µl portions each sample were diluted in 1 ml of DEPC-treated, deionized water and the absorbance was read at 260 and 280 nm using the UV/visible spectrophotometer LKB Ultrospec III (Pharmacia, Cambridge, England). The concentration was then calculated using the following formula:

RNA sample concentration = 40 x A_{260} x 201 (dilution factor).

The purity index was also calculated for each sample by taking the ratio of the A_{260}/A_{280} absorbance reading and high-quality RNA was used (purity index = 1.9 to 2).

Determination of steady-state RNA levels of *sap* by northern analysis.

Northern Analysis was essentially performed as described by Hart *et al.* (45). Briefly, total RNA was diluted in DEPC-treated, dionized water to a concentration of 1 µg/ml. A serial 2-fold dilution starting with 10 µl was performed as illustrated in Figure 1.

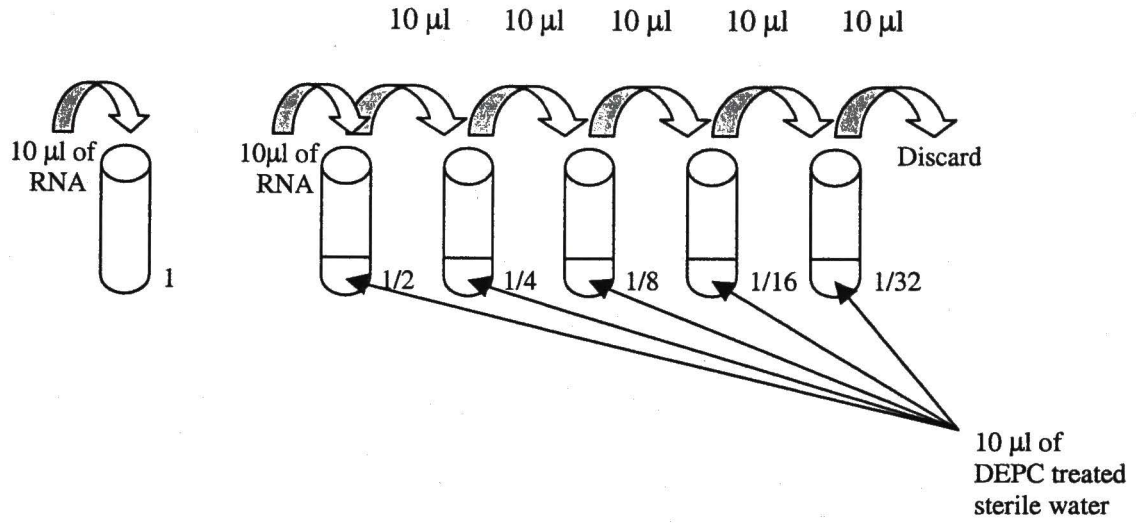


Figure 1. Serial 2-fold dilution of RNA samples.

RNA samples were denatured at 50°C for 1 hr in the presence of deionized glyoxal (Eastman Kodak Co., Rochester, N.Y.) and Dymethyl sulfoxide (Fisher Scientific, Pittsburgh, PA). RNA ladder (0.24-9.5 kb RNA, Invitrogen, Carlsbad, CA) were also prepared in the same manner. RNA samples were cooled on ice before adding RNA tracking dye containing 50% glycerol, 10 mM Na/Na_2PO_4, pH 7.0, and 0.4% bromophenol blue. The samples were then resolved by electrophoresis using a 1.2% agarose gel made with GTG agarose (FMC) in 10 mM Na/Na_2PO_4 (pH 7.0) and containing 10 mM of sodium iodoacetic acid (Sigma, St. louis, MO). The electrophoresis was done in a Model H5 horizontal system for submerged Gel Electrophoresis (BRL, Gaithersburg, MD) hooked up to a Manostat Ministaltic circulation pump (Manostat corporation) and the EC 105 power supply (EC Apparatus Corporation, St Petersburg, Fl). The gel was run at 56V for approximately 3 hrs depending on the migration of the tracking dye in phosphate buffer (10 mM Na/Na_2PO_4 [pH 7.0]). When the

electrophoresis was completed, the gel was stained for 15 min in the dark with ethidium bromide at a final concentration of 5 μg/ml. The gel was destained for the same length of time in deionized, DEPC-treated water. The 23S and 16S rRNA bands were visualized using UV light and the bands were documented using the Alpha imager (Alpha Innotech Corporation). The RNA was then transferred onto a MagnaGraph neutral nylon membrane (Osmonics Inc.), as described for southern analysis. The membrane with transferred RNA was then placed in the GS Gene Linker UV chamber (Bio-rad, Hercules, CA), and using the C-3 program designated for damp membrane, the RNA was cross-linked to the membrane. Following that the membrane was immersed in 250 ml of 50 mM NaOH solution made in DEPC-treated water and incubated for 15 sec. This step released the glyoxal from the RNA. The membrane was removed immediately and transferred to the 0.2 M Tris-HCl, pH 7.5-1X SSC neutralization solution for about 30 sec to 1 min until it was transferred to a clean hybridization bottle using that same solution. The membrane was processed as described for southern analysis. The hybridization was carried out for 18-24 hrs at 65°C with constant agitation. Two different Chemiluminescent digoxigenin-labeled probes made with *sap* PCR product and ribosomal RNA fragment 16S were used. The 16S RNA probe was used to re-probe *sap*-probed Northern to verify RNA loads. Following the hybridization, the membrane was processed as described for Southern analysis.

Densitometric analyses of the northern blots bands were performed in order to validate any visual intensity differences between sample bands. The densities of the bands were measured using the imager from Alpha Innotech Corporation. The samples

band density values were normalized by dividing them into their equivalent dilution 16S band density values. The results were displayed in the form of bar graphs generated using the computer application Prism (GraphPad Prism 3.03).

Acid phosphatase activity in spent media isolated from of *S. aureus*.

Qualitative (zymographic) and quantitative (Spectrophotometric) assays were performed to detect and compare Sap activity in the spent media of *S. aureus* RN6390, UAMS-1 and their respective isogenic regulatory mutants.

Isolation of spent media

S. aureus RN6390 and UAMS-1 strains and their respective mutants were cultured for 6 hrs as described in the cells culture section. The optical density at 550 was determined for each culture. Each sample was divided into 2 equal portions in 1.5 microcentrifuge tubes and centrifuged for 5 min at 16,000 x *g* in a mirocentrifuge at 4°C. The supernatants were carefully removed and filter-sterilized using Nalgene 0.20 μm syringe filter (Nalgen Nunc International, Rochester, NY). About 2 ml of each filtrate was loaded onto the YM-3 centricons (Millipore Corporation, Bedford, MA). The centricons were placed into centrifuge adapters for 15 ml corex tubes and centrifuged at 6,500 x *g* overnight. The flow through was removed and discarded and the retentate vials were inverted and the concentrated spent media was collected by centrifugation at 1000 x *g* for 2 min. Typically, 60-80 μl of concentrated spent media were recovered which represented a concentration of 25-33 fold. Samples were stored at -20°C.

Alternatively, 20 ml or 50 ml cultures of *S. aureus* RN6390, UAMS-1 and their respective mutants were grown as described before in the culture condition section of the Materials and Methods. Growth was monitored spectrophotometrically and at various times during growth flaks containing 20 or 50 ml of culture were harvested by centrifugation (10,000 x *g*, 20 min at 4°C). The supernatants were filter-sterilized using 0.22 μm vacuum driven disposable bottle top filters (Millipore Corporation, Bedford, MA). Portions of filtrates were transferred to sterile, 50-ml conical tubes and quick-frozen using a dry ice/ethanol bath. Frozen samples were then stored overnight at -85°C. Frozen samples were lyophilized to dryness using a model FD 3.0 lyophilizer (ATR Inc., Laurel, MD). Upon completion, samples were stored at -85°C until needed. Prior to utilization, the samples were suspended in 0.1 M sodium acetate pH 5.0. The suspension volume was 20 times less than the original volume prior to lyophilization in order to obtain 20X concentration.

SDS-PAGE gel electrophoresis to resolve extracellular proteins present in the spent media.

The proteins were separated under partial denaturing conditions (no addition of reductants) following Laemmeli gel method as described in the Current Protocols in Molecular Bilogy (58 and 35). We routinely used the Mini-protean II cell system or the Mini-protean 3 cell system both from Bio-rad (Bio-rad, Hercules, CA). 12% and 13% separating polyacrylamide gels were routinely prepared. We alternatively used protein samples concentrated by either the centricon or lyophilization methods. The protein samples were diluted 1:1 with 2X SDS sample buffer. The diluted samples were then

incubated at 37°C for 30 min (31). The pre-heated samples were carefully loaded into the wells using a long-tipped pipette. Pre-stained molecular weight markers low or broad range from Bio-Rad (Bio-Rad, Hercules, CA) and purified potato acid phosphatase (Sigma-Aldrich, St. Louis, MO) were also prepared and loaded in a similar manner. Electrophoresis in 1X SDS buffer (25 mM Tris base, 192 mM glycine and 0.1% SDS in water) was carried out at 120V for 75 min. The gels were always run in duplicate so that one could be developed for detection of phosphatase activity and the other stained with Coomassie blue to verify the protein loads. Upon completion the gels were immediately rinsed in sterile, deionized water and processed for either acid phosphatase activity or detection of proteins by Coomassie blue staining (91).

Zymographic detection of phosphatase activity

For these experiments we adopted the method as described by Du Plessis *et al.* (31) with few modifications. The SDS-PAGE gel was incubated for 16-18 hrs in several changes of renaturation buffer. The renaturation buffer consisted of 100 mM Tris-HCl (pH 7.0), containing 2 mM $MgSO_4$, 0.05 mM $ZnCl_2$ and 2% v/v Triton X-100 (44). After renaturation treatment the gel was re-equilibrated for 1 hr at 37°C in 0.1 M sodium acetate buffer pH 5.0. The gel was then developed for acid phosphatase activity by incubation at 37°C for 1-2 hrs in 0.1 M sodium acetate pH 5.0 containing 0.1-0.15% (w/v) α-naphthyl phosphate (Sigma-Aldrich, St. Louis, MO) and 0.1% (w/v) Fast Garnet GBC salt (Sigma-Aldrich, St. Louis, MO). The appearing of brown-black stained bands indicated a positive reaction for acid phosphatase. The developing solution was poured

off and the gel was stored in deionized water at room temperature until documentation and procuration.

Determination of the spent media samples protein concentration using the BCA assay

The BCA reagent kit PIERCE biotechnology (Rockford, IL) was used to determine to total protein concentration in the spent media isolated form *S. aureus* RN6390 and UAMS-1 wild type strains and their isogenic regulatory mutants. The dilution of the standards (Bovine Serum Albumin) was done as directed by the protocol. To prepare the total volume of working reagent (WR) required we used the provided formula: (# standards + # unknowns) x (# replicates) x (volume of WR per sample) = total volume WR required. In our case we assayed for 3 dilutions of each of our samples (NTH207, 208, 209, 210, 13, 332, 333, and 334). The dilutions were 1/25, 1/50 and 1/100 from the spent media prepared to dryness and concentrated by lyophilization. The working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. The 96-wells flat bottom microtiter plates from Costar (Costar, Cambridge, MA) were used. After setting up our dilution as stated above, 25 µl of each standard and unknown were pipetted into the microtiter plate wells. 200 µl of the working reagent was added to each well containing standards or protein samples. The plate contents were mixed thoroughly. The plate was covered with its lid and incubated at 37°C for 30 min. Following incubation the plate was allowed to cool to room temperature and then reading was performed using the Spectra max 340 plate reader (Molecular devices) at 562 nm. The readings of the standards permitted the generation of a standard curve and the

concentration of the samples were determined by linear regression using the computer application Prism (GraphPad Prism 3.03).

Quantitative Sap assay in spent media isolated from *S. aureus* RN6390 and UAMS-1 wild type strains and their isogenic regulatory mutants

The assay was adapted with modifications from the one of Du Plessis *et al.* (31). Three dilutions (1:25, 1:50 and 1:100) were set up for each of the eight *S. aureus* spent media samples (NTH207, 208, 209, 210, 13, 332, 333, and 334). 0.1 M sodium acetate pH 5 was used to make the dilution. Purified potato acid phosphatase (Sigma-Aldrich, St. Louis, MO) used as positive control was also diluted out (1:25, 1:50, 1:100, 1:250, 1:500, and 1:1000 from a stock of 1μg/μl) in 0.1 M sodium acetate pH 5. The substrate used for this assay was *p*-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO). Tablets of substrate were dissolved into 0.1 M sodium acetate pH 5 to a final concentration of 25 mM. Each tablets contained 5 mg of substrate. The reactions were set up in a 96-wells flat bottom microtiter plate (Costar, Cambridge, MA). 25 μl of each sample and control was pipetted into the wells. 75 μl of the substrate solution was added to each well. After mixing the microtiter plate content by brief shacking on a horizontal plan, the plate covered with its lid was incubated at 37°C for 30 min. The reaction was stopped by adding 125 μl of 1 M NaOH to each well. 0.1 M sodium acetate pH 5.0 was loaded in few empty wells to serve as blanks. Acid phosphatase activity was assayed by measuring the liberated *p*-nitrophenol (*p*NP) at 405 nm on the micro plate reader MRX (Dynatech Laboratories). Means of three independent determinations +/- standard deviations were graphed using Prism software (GraphPad Prism 3.03). The statistical analysis of the

results was done using Anova one-way analysis of variance as test and Bonferroni's multiple comparison test run as post-test (GraphPad Prism 3.03).

RESULTS

Polymerase chain reaction with *sap* specific primers yielded the expected fragment

"Touch down" PCR reaction with specific *sap* primers, using the *S. aureus* chromosomal DNA as template, yielded a DNA fragment of size corresponding to 1233 bp as expected (fig. 2). The PCR fragment included the 891 bp *sap* gene, 164 bp of upstream sequence, 158 bp of downstream sequence, and 10 bp at each end due to the insertion of *Kpn*I and *Xba*I restriction sites.

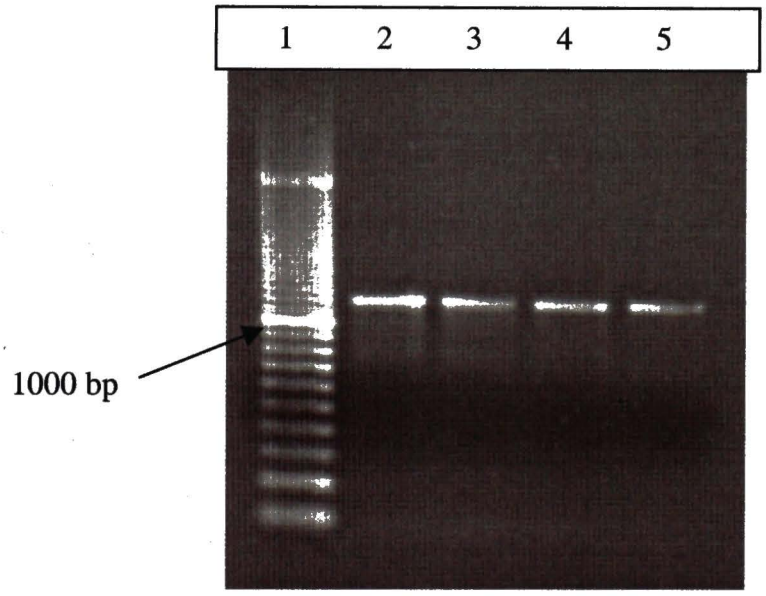


FIGURE 2. *sap* amplicon resolved on a 0.8% agarose gel. Lane 1: 100 bp PCR ladder. Lane 2-5: samples of *sap* PCR reactions.

Verification of *sap* amplicon by cloning, restriction analysis and DNA sequencing

We were able to successfully clone *sap* PCR fragment using the pGEM-T Easy cloning kit by Promega. Several colonies containing the construct of interest (*sap* ligated

into pGEM-T Easy) were isolated by blue/white screening and resistance to carbenicillin. Restriction digestion of the plasmid constructs isolated from those colonies revealed the presence of an approximately 1200 bp fragment. Six plasmid constructs containing the expected fragment were sequenced. Using the sequence data, we were able to confirm that the insert was indeed the *sap* PCR product by comparing it to the sequence of *S. aureus* strain 154 (31) acid phosphatase gene at GenBank (NCBI) website accession number AY061973. Restriction analysis using computer software (DNAMAN, Lynnon Biosoft, Vaudreuil, Quebec) allowed us to identify all possible restriction sites for the insert including the ones we added when designing the primers. Translational overview of the *sap* sequence revealed a putative open reading frame containing 296 amino acids. When we compared the sequence to the one of Du Plessis *et al.* (31), we were able to identify the signal peptide $_{1}$MNKISKYIAIASLSVAVTVSAPQTTNSTAFA$_{31}$ at the amino terminus KSSAEVQQ. The predicted cleavage site between Ala_{31} and Lys_{32}, which is typical for prokaryotic secreted proteins, was also present (31 and 74). These data suggest that *sap* product is a secreted protein.

The 891 bp *sap* gene is conserved among various *S. aureus* strains

A probe generated using the 1233 bp *sap* PCR fragment, was used Southern in analysis to determine the presence of *sap* in various *S. aureus* and *S. epidermidis* strains. Chromosomal DNA from 7 strains of *S. aureus* and 2 strains of *S. epidermidis* were analyzed. As of result we showed that *sap* was present in the chromosome of the tested *S. aureus* strains but absent in *S. epidermidis* (fig. 3). For *S. aureus* we used one

laboratory strain and six clinical isolates; for *S. epidermidis* we used one ATCC strain and one clinical isolate (table 1). The presence of two bands for each sample (fig. 3) was due to the fact that there is an *EcoR*I restriction site in the open reading frame of *sap* as confirmed by restriction analysis of the sequence data.

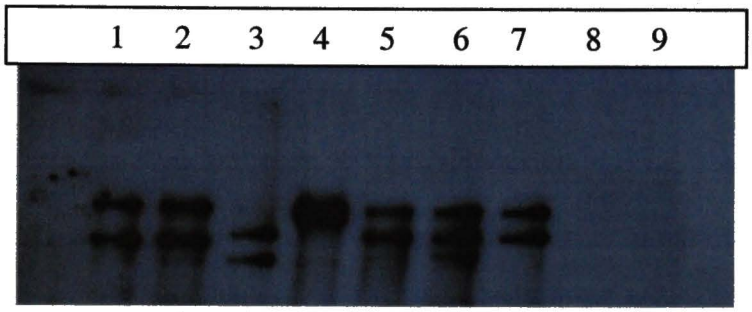


FIGURE 3. Southern analysis with the 1233 bp PCR fragment of *sap* as probe revealed that the gene is conserved among various *S. aureus* strains but absent in *S. epidermidis*. 1: *S. aureus* RN6390; 2: *S. aureus* HAC1; 3: *S. aureus* HAC2; 4: *S. aureus* HAC3; 5: *S. aureus* HAC4; 6: *S. aureus* HAC6; 7: *S. aureus* UAB1; 8: *S. epidermidis* ATCC 12228; 9: *S. epidermidis* HAC33. The chromosomal DNAs were digested with *EcoR*I restriction enzyme.

sap message levels are minimal or absent at twelve hours of growth for *S. aureus* RN6390 but maintained for UAMS-1

Northern analysis of RNA isolated from *S. aureus* RN6390 (NTH207) and UAMS-1 (NTH13) at different times of growth allowed us to examine the expression of *sap* over time. RNAs analyzed were isolated at 3, 6 and 12 hours of growth. Here we

show that *sap* message levels are noticeably decreased at 12 hours of growth for RN6390 (fig. 4) whereas message levels are just slightly decreased for UAMS-1 (fig. 5). The disappearance of the message levels at 12 hours of growth for RN6390 suggests that expression of *sap* is down regulated at this point in growth. Three transcripts were observed for RN6390 (fig. 4) with the major and larger transcript corresponding to the expected size for *sap* (891 bp). The other two transcripts were smaller, and their sizes were estimated to be around 400 bp and 300 bp respectively. UAMS-1 transcript corresponded to the expected size of sap. RNA loads were verified by stripping and probing the same membrane for 16S rRNA (fig. 4 and 5). The significance of the band intensity differences observed at the different time points for both strains was confirmed by the results of densitometric analysis (fig. 6). For *S. aureus* RN6390, a slight decrease of *sap* message level was observed from 3 to 6 hrs and at 12 hrs there was no detectable message (fig. 6a). Whereas, for the UAMS-1 strain, *sap* message was decreased by half from 3 to 6 hrs and maintained the same level from 6 to 12 hours (fig. 6b).

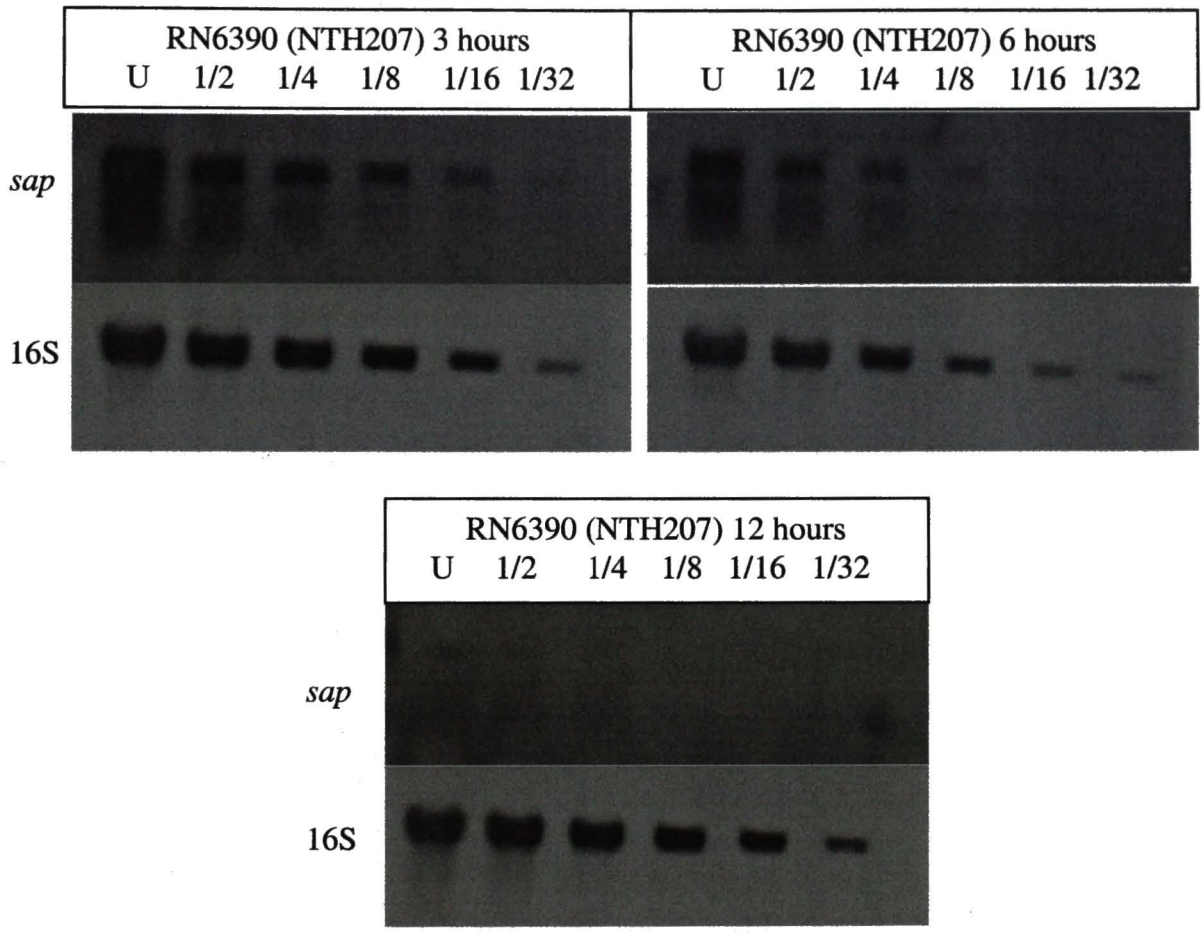


FIGURE 4. Northern analysis of RNA isolated from *S. aureus* RN6390 (NTH207) at 3, 6, and 12 hours of growth and probed for *sap* or 16S rRNA. The *sap* probe used was made from *sap* PCR product that we generated. Membrane was stripped after probing with *sap* and probed for 16S rRNA. The different lanes represent the serial dilutions done for each sample and (U) an undiluted sample of 10 μg of total RNA.

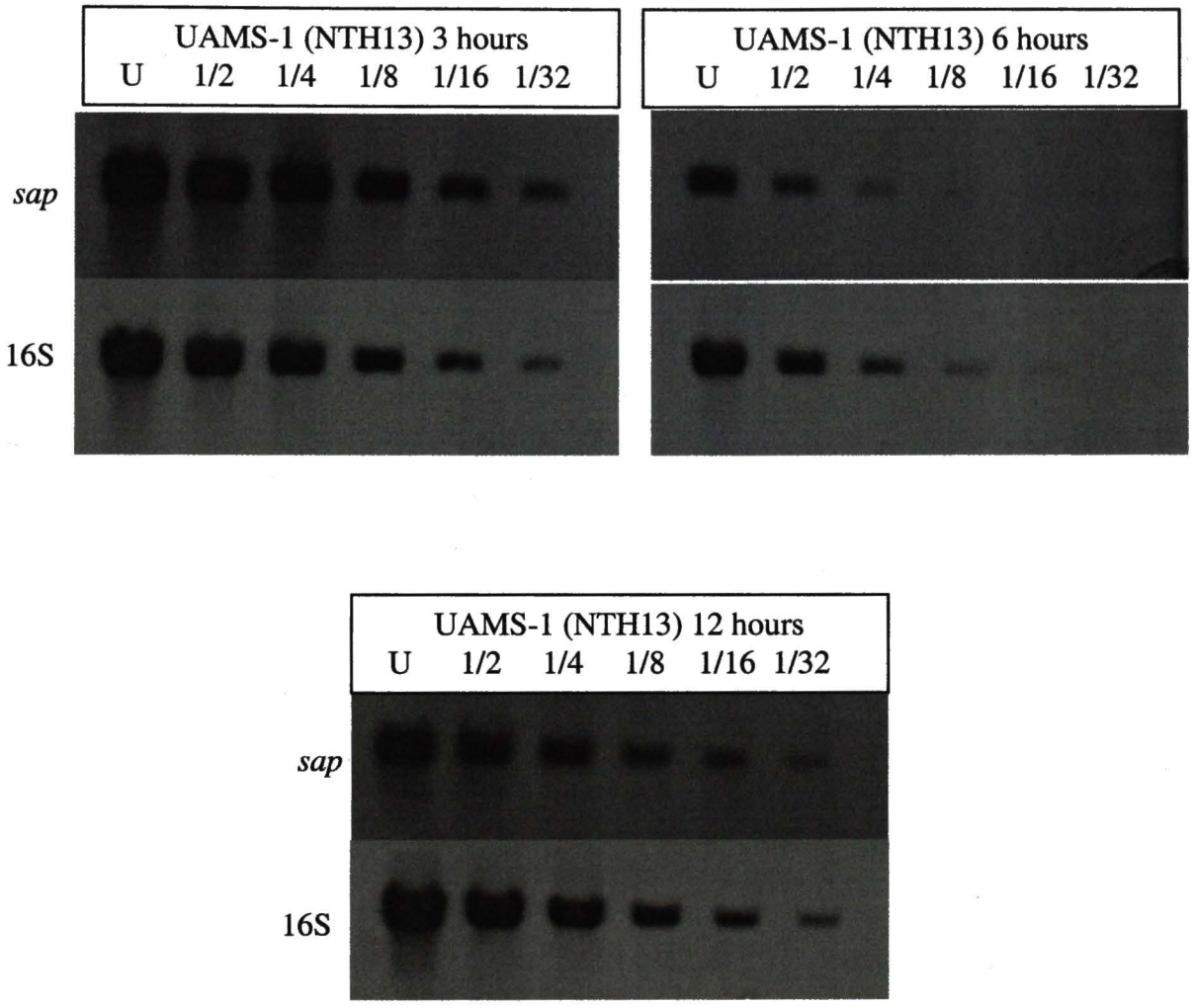
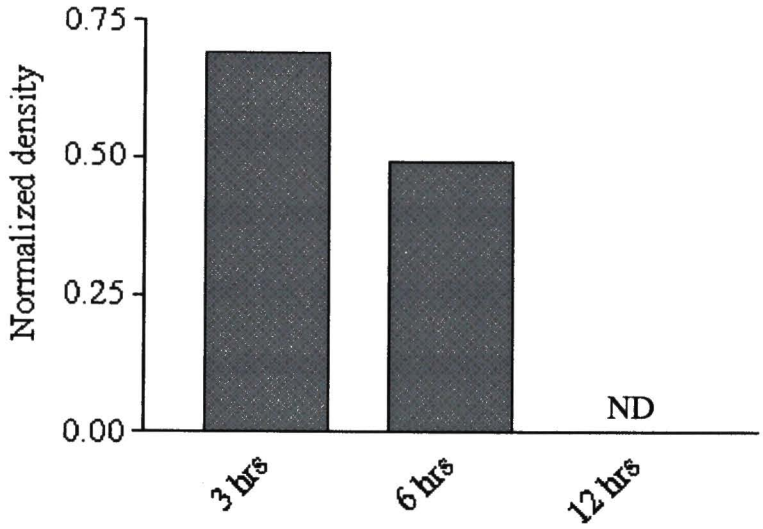


FIGURE 5. Northern analysis of RNA isolated from *S. aureus* UAMS-1 (NTH13) at 3, 6, and 12 hours of growth and probed for *sap* or 16S rRNA. The *sap* probe used was made from *sap* PCR product that we generated. Membrane was stripped after probing with *sap* and probed for 16S rRNA. The different lanes represent the serial dilutions done for each sample and (U) an undiluted sample of 10 μg of total RNA.

a) ***S. aureus* RN6390**



b) ***S. aureus* UAMS-1**

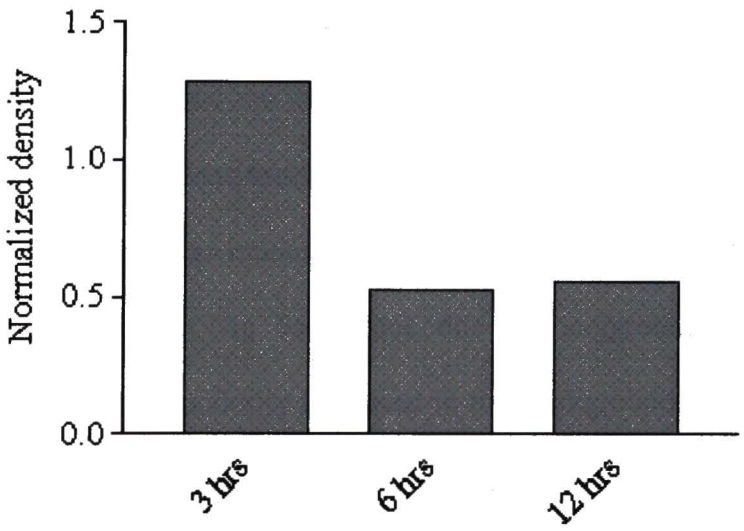


FIGURE 6. Densitometric analysis of northern blots of *sap* expression during growth (fig. 4 and 5). Samples' band densities were normalized with the densities of their corresponding 16S rRNA bands. a) shows the results for *S. aureus* strain RN6390 (ND: not detectable) and b) shows the results for *S. aureus* UAMS-1 strain.

***agr* and *sar* affect the expression of *sap* in a strain-dependent manner**

In an attempt to verify whether the regulatory loci *agr* and *sar* affect the expression of *sap*, we did northern analysis of total RNA isolated from the wild type *S. aureus* RN6390 (NTH207) strain and its isogenic mutants *sar* (NTH208), *agr* (NTH209), and *agr/sar* (NTH210). The RNAs were isolated from cells harvested at six hours of growth. The membrane was probed for *sap* then stripped and re-probed for 16S rRNA (fig. 7). The same banding pattern as seen in the time course study was observed for the *sap* probe. A slight difference in intensity was noticeable between the strains; the wild type and the *sar*-mutant strains appeared a little less intense than the *agr*-mutant and *agr/sar* double mutant strains. Stripping and probing the same membrane for 16S rRNA confirmed intensity differences of the bands among the different strains. To verify the accuracy of our visual observations, we did densitomeric analysis of the bands of each strain on the northern blot films and the densities were normalized with regard to the 16S rRNA probed bands. The densitometry data revealed that there was indeed a modest increase in density for the *agr*-mutant and the *agr/sar* double mutant compared to the wild type and the *sar*-mutant (fig. 9a). Thus, this data suggested that for the RN6390 strains, *sap* transcription is modestly increased in the background of the *agr*-mutant and the *agr/sar* double mutant. The same study was done for the clinical isolate *S. aureus* UAMS-1 (NTH13) strain and its isogenic mutants *sar* (NTH332), *agr* (NTH333), and *agr/sar* (NTH334). As shown on figure 8, visual observations suggested a transcription increase for *sap* in the background of the *sar*-mutant and the *agr/sar* double mutant strains. The message levels of *sap* were comparable for the wild type and the *agr*-

mutant. The RNA loads were also verified by stripping and probing the same membranes for 16S rRNA revealing differences in intensity. As for the RN6390 strains, we also did a densitometric analysis and the densities of the different strain bands were normalized with regard to the density of the 16S rRNA probed bands. This data confirmed a 6-fold increase in the message levels of *sap* for the *sar*-mutant and the *agr/sar* double mutant strains compared to the wild type strain (fig. 9b). The message levels in the *agr*-mutant background were comparable to the wild type. In contrast with what was seen for the RN6390 strains, this data suggest that *sap* transcription is increased for the *sar*-mutant and the *agr/sar* double mutant.

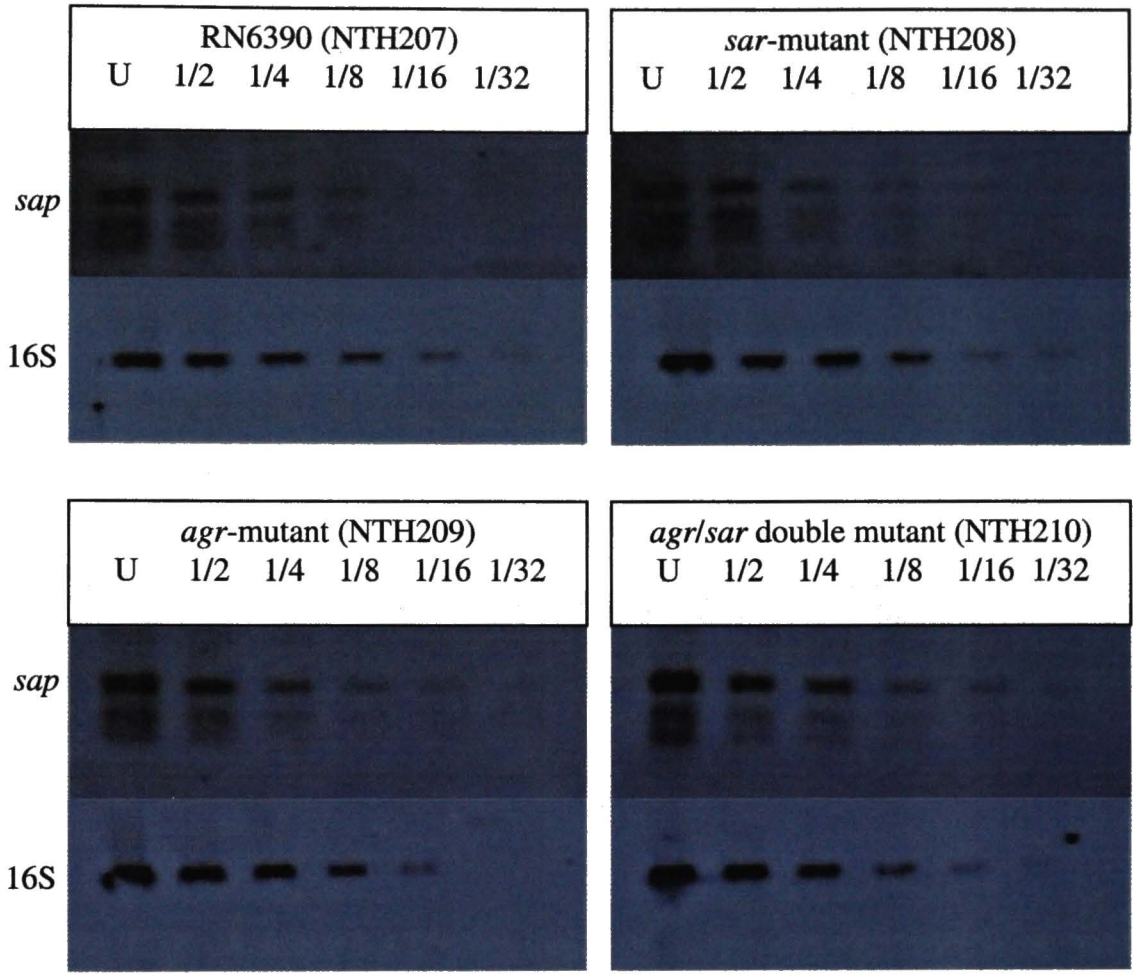


FIGURE 7. Comparative northern analysis of RNA isolated from *S. aureus* RN6390 and its *agr*-, *sar*-, and *agr/sar* double mutants probed for *sap* and 16S rRNA. The RNAs were isolated from cells harvested at six hours of growth. The membrane initially probed for *sap* was stripped and re-probed for 16S rRNA to verify RNA load. Lanes represent a serial, two-fold dilution. Approximately 10 μg of RNA was loaded in lane (U), undiluted of each strain.

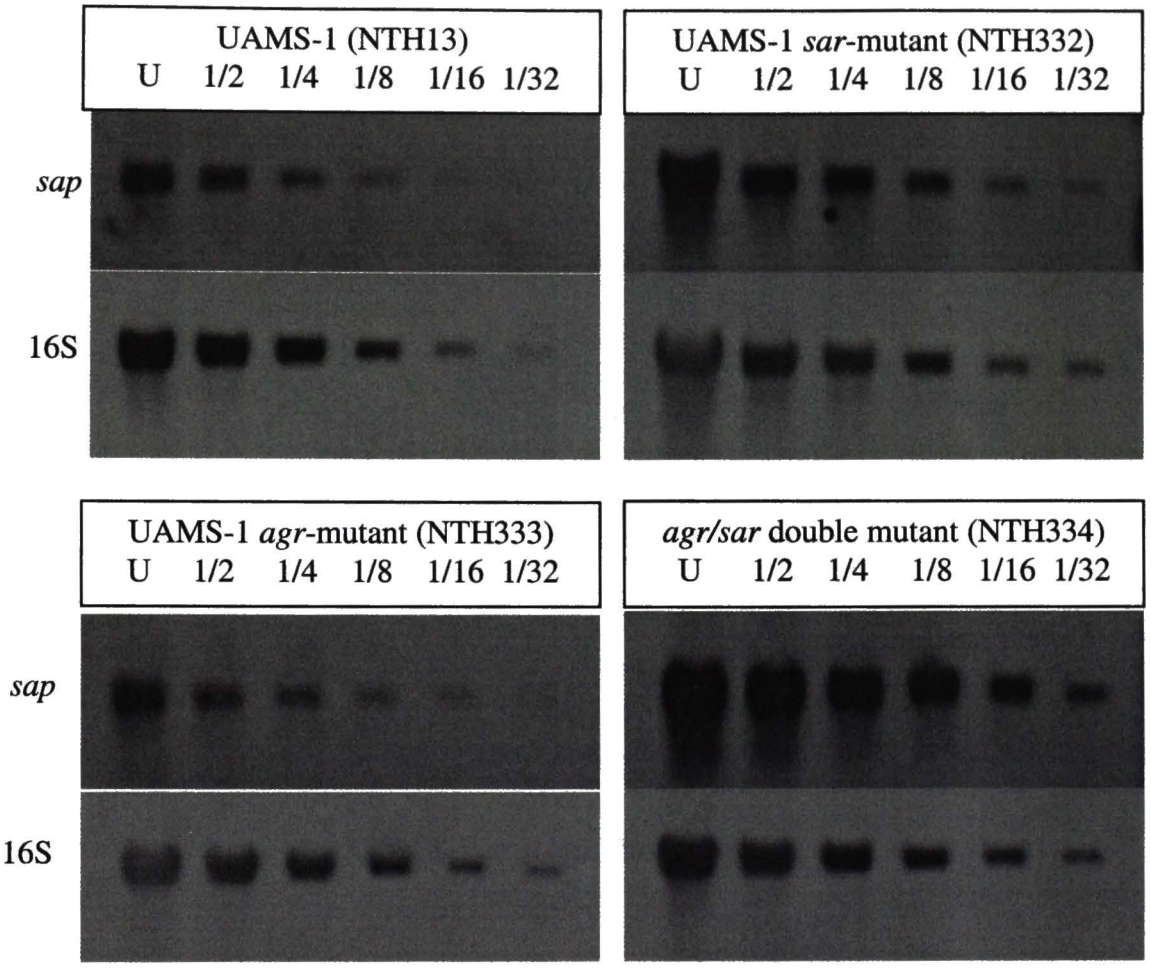
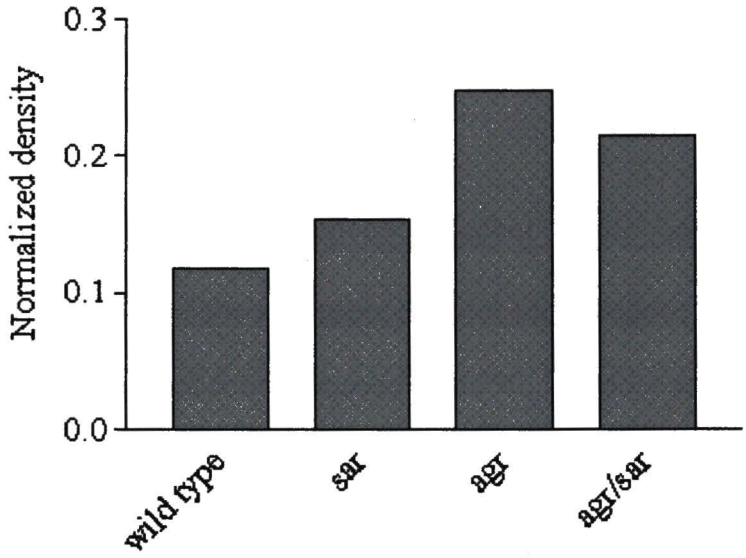


FIGURE 8. Comparative northern analysis of RNA isolated from *S. aureus* UAMS-1 and its *agr*-, *sar*-, and *agr/sar* double mutants probed for *sap* and 16S rRNA. The RNAs were isolated from cells harvested at six hours of growth. The membrane initially probed for *sap* was stripped and re-probed for 16S rRNA to verify RNA load. Lanes represent a serial, two-fold dilution. Approximately 10 μg of RNA was loaded in lane (U), undiluted of each strain.

a) **RN6390 and isogenic mutants**



b) **UAMS-1 and isogenic mutants**

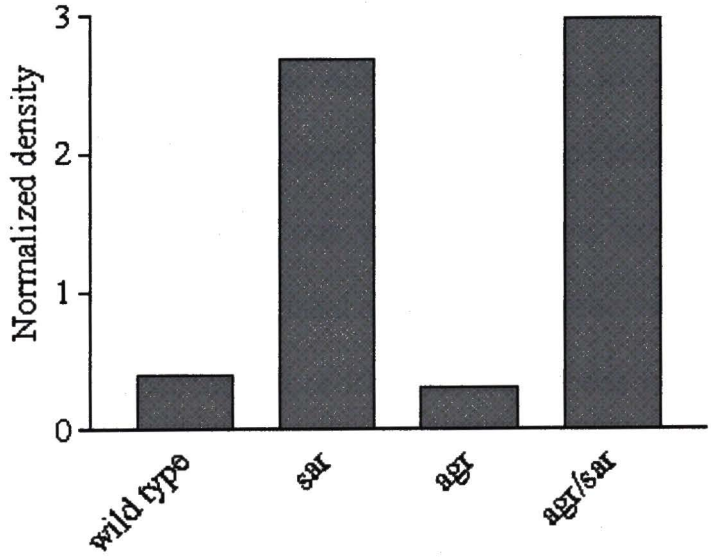


FIGURE 9. Densitometric analysis of *sap* comparative northern blots (fig. 7 and 8). a) shows results for *S. aureus* RN6390 and isogenic *agr*- and *sar*-mutants, and b) shows the results for *S. aureus* UAMS-1 and isogenic *agr*- and *sar*-mutants.

Zymographic detection of Sap activity in spent media isolated from *S. aureus* RN6390 and UAMS-1 and their isogenic *agr* and *sar* regulatory mutants

Spent media isolated from *S. aureus* RN6390, UAMS-1 and their respective *agr*-, *sar*-, and *ag/sar*-mutants were assayed for Sap activity. The bacterial cultures were standardized spectrophotometrically and cell free spent media was prepared to dryness by lyophilization. The substrate used for this assay was α-naphthyl phosphate (Sigma-Aldrich, St. Louis, MO). Acid phosphatase activity appeared between 30 and 60 min after the addition of substrate and as evident by the presence of brown bands. Purified potato acid phosphatase (Pap, Sigma-Aldrich, St. Louis, MO) was used as positive control. A Sap activity band was present for the wild type strain RN6390 and its *agr*-mutant; however, no activity band was visualized for the *sar*-mutant or the *agr/sar* double mutant (fig. 10). Likewise, a band of activity was observed for the UAMS-1 wild type strain but unlike the *sar* mutant of RN6390, a band was present for the UAMS-1 *sar*-mutant. No activity bands were visualized for the UAMS-1 *agr*-mutant and *agr/sar*

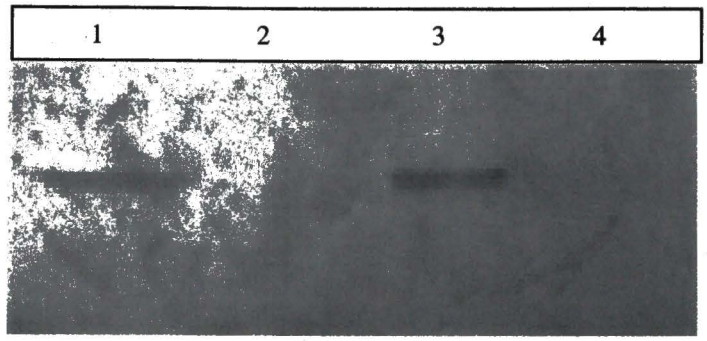


FIGURE 10. SDS-PAGE/zymographic analysis to detect Sap activity using α-naphthyl phosphate as a substrate. Lane 1: RN6390 (NTH207); lane 2: *sar*-mutant (NTH208); lane 3: *agr*-mutant (NTH209); Lane 4: *agr/sar* double mutant (NTH210).

double mutant (fig. 11). The SDS-PAGE polyacrylamide gels were run in duplicate and the second gels were stained with Coomassie blue to verify the protein loads and also to ensure that the proper sample was loaded in the correct well corresponding to our legend. The *agr* and *sar* regulatory mutants are impaired in their ability to produce proteins, and this allows to easily distinguish them from the wild type or from each other on duplicate gels stained with Coomassie blue (fig. 12). Pre-stained SDS-PAGE protein standards (Bio-rad, Hercules, CA) were loaded on each gel and the size of each activity band was estimated to be in the range of 30 kDa.

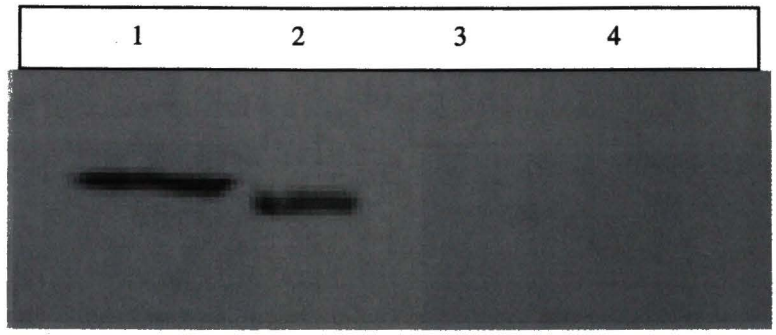


FIGURE 11. SDS-PAGE/zymographic analysis to detect Sap activity using α-naphthyl phosphate as a substrate. Lane 1: UAMS-1 (NTH13); lane 2: *sar*-mutant (NTH332); lane 3: *agr*-mutant (NTH333); Lane 4: *agr*/*sar* double mutant (NTH334).

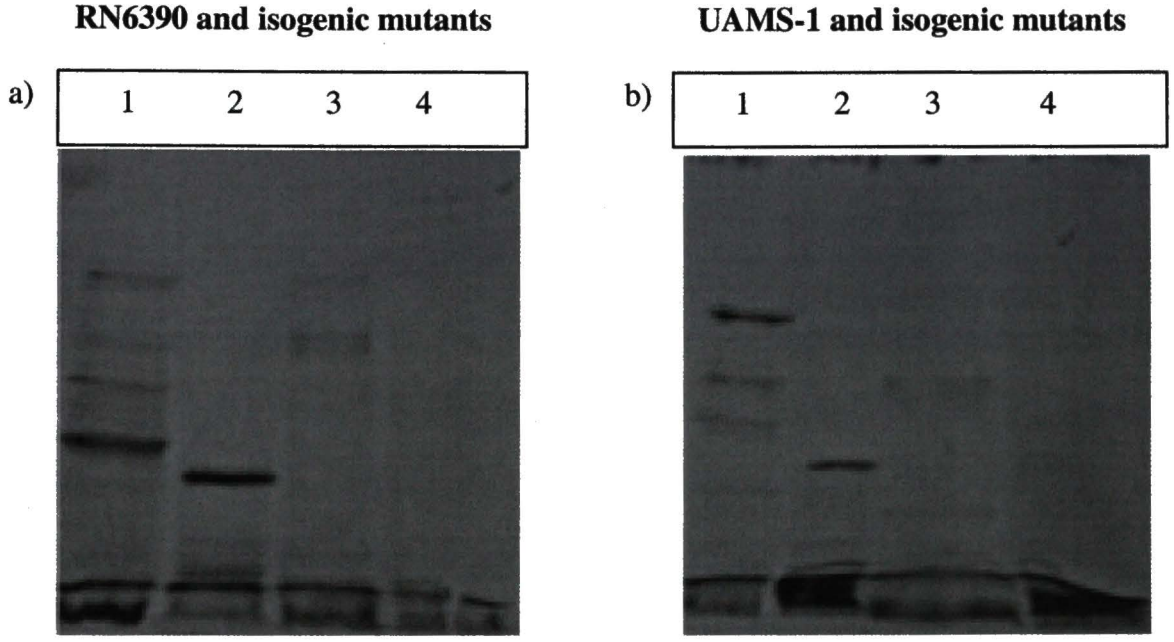
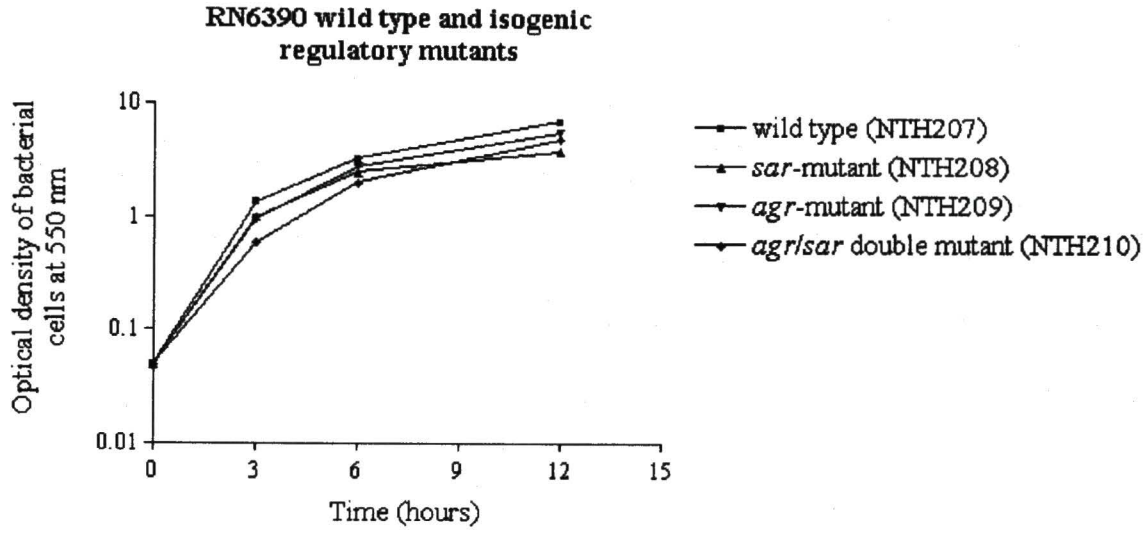


FIGURE 12. Duplicate SDS-PAGE gels of zymographic analysis stained with Coomassie blue. This staining was done to verify and compare the protein profile of the spent media from each strain. a) shows the duplicate gel of *S. aureus* RN6390 and its isogenic regulatory mutants, and b) shows the same thing for *S. aureus* UAMS-1 and its isogenic regulatory mutants. On both gels the wild type spent media was loaded in lane 1, the *sar*-mutant spent media was loaded in lane 2, the *agr*-mutant spent media was loaded in lane 3, and the *agr/sar* double mutant was loaded in lane 4.

a)



b)

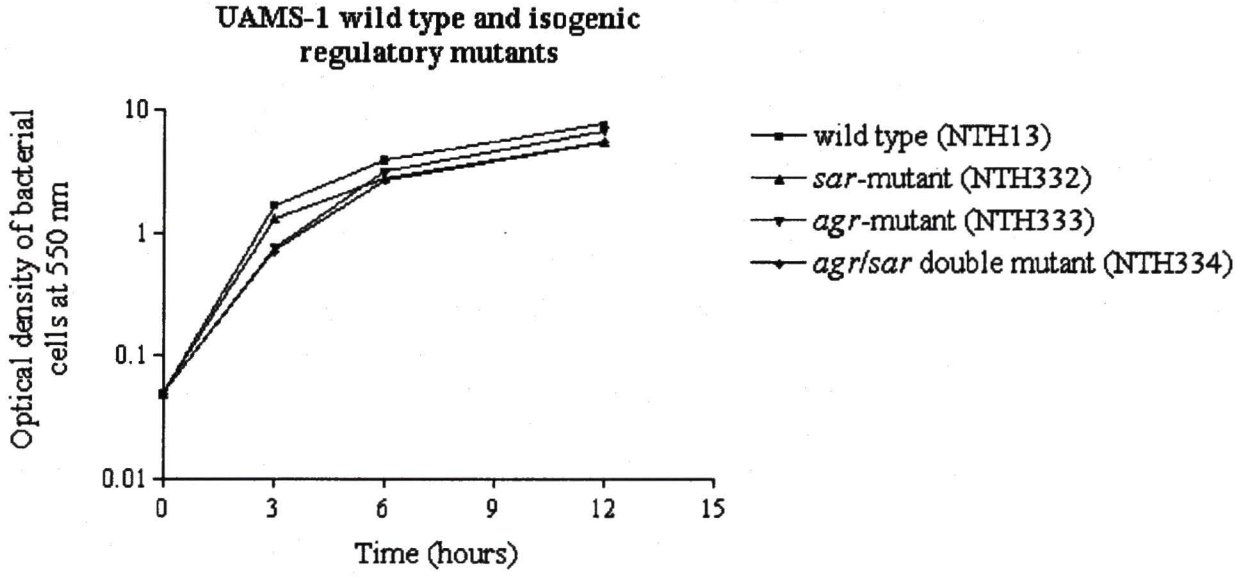


FIGURE 13. Growth curves of *S. aureus* strains RN6390 and UAMS-1 and their isogenic regulatory mutants. Optical densities (OD) of cells were measured spectrophotometrically at 550 nm. The OD axis was converted to a logarithmic scale. a)

shows the RN6390 strains group and b) shows the UAMS-1 strains group. Cells were harvested at 3, 6, and 12 hrs of growth.

Quantitative Sap activity assay with *p*-nitrophenyl phosphate (*p*NPP) as substrate

In order to quantify the activity of Sap a spectrophotometric assay using *p*NPP (Sigma-Aldrich, St. Louis, MO) as substrate was used (31). In this assay, Sap activity was determined indirectly by measuring the absorbance of liberated *p*-nitrophenol at 405 nm. Spent media isolated from RN6390 and UAMS-1 and their isogenic *agr* and *sar* regulatory mutants were standardized spectrophotometrically, and cell free spent media were prepared to dryness by lyophilization. Total proteins concentrations in the spent media were determined using the BCA protein assay (PIERCE Biotechnology, Rockford, IL). The activity of Sap was determined in arbitrary units (AU). Figure 14a shows the activity determined directly from each spent media samples (standardized spectrophotometrically). For the RN6390 strains, the most activity was observed for the *agr* mutant and the wild type. For the UAMS-1 strains, Sap activity was greater for the wild type and the *sar* mutant. Activity of Sap per μg of total protein in the spent media was also determined as shown on figure 14b. The *sar* mutant revealed more Sap activity per total protein than the other strains in both group (fig. 14b). Purified Pap used as positive control was extremely reactive (not shown on graphs). The result of Sap activity assay shown in figure 14a corresponded to the results of Sap activity detected by zymographic analysis, and in both experiments the spent media were isolated from spectrophotometrically standardized bacterial cultures. Figure 13 and Table 3 show that

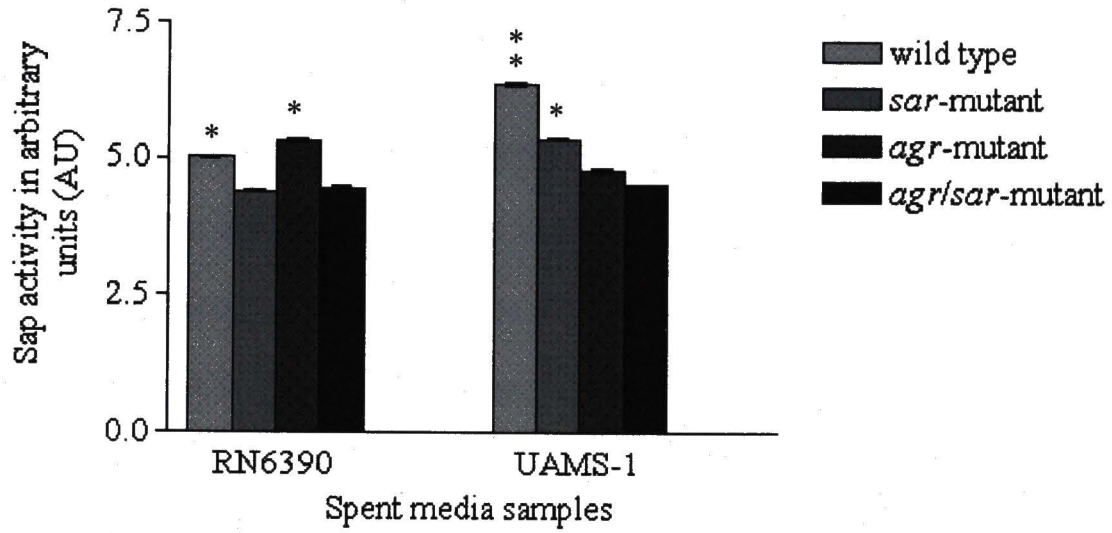
overall *S. aureus* UAMS-1 strains grew better than RN6390 strains and in addition to that their ability to release proteins in the spent media was also increased (Table 3). Statistical aanalysis of the results, using Anova one-way analysis of variance as test and Bonferroni's multiple comparison test run as post-test (GraphPad Prism 3.03), confirmed the significance of the activity level differences observed ($p< 0.0001$).

TABLE 3. Differences in growth and total proteins in the spent media of *S. aureus* strains.

Strains	OD_{550}	Total protein (μg/μl)
RN6390	3.255	65.68
RN6390 *sar*-mutant	2.480	46.03
RN6390 *agr*-mutant	2.821	39.14
RN6390 double mutant	2.015	32.66
UAMS-1	3.906	75.32
UAMS-1 *sar*-mutant	2.759	48.66
UAMS-1 *agr*-mutant	3.162	39.47
UAMS-1 double mutant	2.697	38.18

The values were determined from cultures at 6 hours of growth. The cells optical densities (OD) were measured using a spectrophotometer at a wavelength of 550nm. The concentration of total protein in the spent media was determined by BCA protein assay.

a)



b)

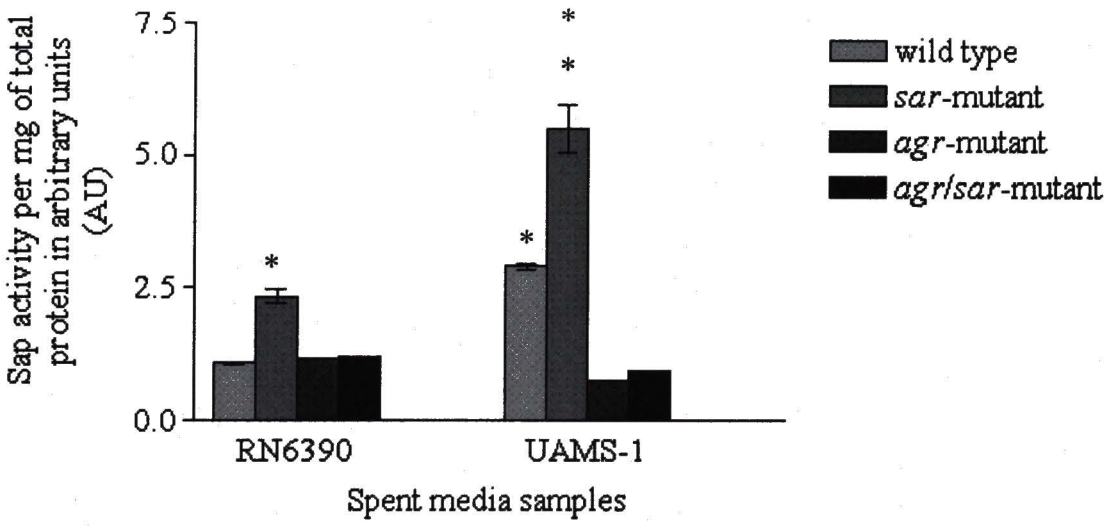


FIGURE 14. Sap activity quantified by measuring the liberation of *p*-nitrophenol. Liberated *p*-nitrophenol absorbance was measured at 405 nm. Panel a) represents Sap activity in spent media isolated from *S. aureus* cultures standardized

spectrphotometrically. Sap activity was determined using the following formula: Absorbance at 405 x extinction coefficient of *p*-nitrophenol x dilution factor. Panel b) shows Sap activity per μg of total protein in the spent media. The formula used to determine activity was: (Absorbance at 405 x extinction coefficient of *p*-nitrophenol x dilution factor)/(sample total protein concentration x volume of protein loaded). The extinction coefficient of *p*-nitrophenol is 18.5 $cm^2/\mu mol$ (105). Sap activity is in arbitrary units (AU). The values represent the mean of 3 independent determinations +/- standard deviation. Statistical analyses were done by Anova one-way with Bonferroni's multiple comparison post-test using Prism.

* Significantly higher and * * twice significantly higher.

DISCUSSION

Our attempt to characterize the Staphylococcal acid phosphatase gene (*sap*), with regard to the well-known *agr* and *sar* global regulators of virulence genes in *S. aureus*, was based on the fact that we think Staphylococcal acid phosphatase might be involved in the virulence process. Many of the cell wall-associated and secreted proteins of *S. aureus* contribute to its virulence (50 and 61). The expression of most known staphylococcal virulence factors is regulated by *agr* and *sar* in conjunction or any of them independently, or in conjunction with other recently discovered regulatory loci (85, 21, 68, 98, 64, and 2). We were able to isolate and subsequently clone the gene for *sap* by PCR. The resulted 1,233 bp amplicon was shown to be identical to the gene reported by Du Plessis *et al.* (31) by restriction digestion, sequencing and comparing the sequences. Analysis of the amino acid sequence of a putative open reading frame also showed that the predicted product of *sap* was identical to the one described by Du Plessis *et al.* (31). The predicted cleavage site between Ala_{31} and Lys_{32} was also present (31 and 74). With these data we demonstrated that *S. aureus* RN6390 Sap was most likely targeted to the cell wall and subsequently secreted as reported by Du Plessis *et al.* (31) for the acid phosphatase of *S. aureus* strain 154.

Southern analysis of chromosomal DNA isolated from various staphylococcal species and probed for *sap*, demonstrated that *sap* is conserved in all the *S. aureus* laboratory and clinical strains that we examined. However, *sap* was not present in the two strains of *S. epidermidis* that we included in our study. This is not to say that *S.*

epidermidis does not produce acid phosphatase, however these data suggest that this particular acid phosphatase is specific to *S. aureus*. Other staphylococcal species may also produce specific acid phosphatases as well. In addition, *S. epidermidis* is a member of the coagulase negative Staphylococci, which are known to be less virulent than *S. aureus*.

Our data indicate that *sap* message was maximally expressed at three and six hours of growth and almost nonexistent by 12 hours of growth in the RN6390 laboratory strain. However, the message levels were only reduced by half at 6 and 12 hours of growth in the UAMS-1 laboratory strain. These results suggested that factors affecting the expression of *sap* are strain-dependent. In general, the 12 hours time point corresponds to the post-exponential phase of growth when staphylococcal exoprotein genes are activated (21, 56, and 75). When we compared the expression of *sap* in the background of RN6390 and its isogenic *agr* and *sar* regulatory mutants, message levels were modestly increased for the *agr*-mutant and the *agr/sar* double mutant strains compared to the wild type and the *sar*-mutant strains. In contrast, for the UAMS-1 strains, significant message levels increase were observed for the *sar*-mutant and the *agr/sar* double mutant strains compared to the wild type and the *agr*-mutant. At this point, these data suggest that the effects of *agr* and *sar* on *sap* expression are strain-dependent. We also consistently observed that *sap* message levels were higher for the UAMS-1 strains in general compared to the RN6390 strains. There were consistently three transcripts on the northern blots when we probed total RNA isolated from the RN6390 strains for *sap*. The larger transcript size corresponded to *sap* (891 bp), whereas

the other two smaller transcripts sizes ranged between 300 and 400 bp. We think that this could be the result of some sort of consistent systematic degradation of the *sap* transcript. The possibility of non-specific hybridization of *sap* probe occurring can be ruled out by the results the southern analysis and the northern analysis of the UAMS-1 strains.

In order to further investigate the effects of *agr* and *sar* on the production of Sap, we compared its activity in the background of those regulatory mutants. Zymographic assays revealed a single Sap activity band for the RN6390 wild type and its *agr*-mutant. The Zymographic analysis result did not completely correspond to results of studies at the transcriptional level. For example, the message levels, for the *agr/sar* double mutant, were as elevated as for the *agr*-mutant but no activity band was detected. In light of that data, we anticipated that certainly other factors contribute to control the production of Sap at the post-transcriptional, post-translational or secretion stage. The same zymographic assay was performed for the UAMS-1 strain and its isogenic regulatory mutants. A single activity band was detected for both the wild type and the *sar*-mutant. Likewise what was observed for the RN6390 strains, no activity band was detected for the *agr/sar* double mutant in spite of the increased message levels for *sap* shown by the northern analysis. It also appears clearly that even though *agr* and *sar* may partially affect Sap production, other factors are also involved in the overall process. The regulatory differences observed for Sap activity between RN6390 and UAMS-1 strains support the suggestion that the control of Sap production may be strain-dependent. The characteristic bands pattern of extracellular proteins for each strain (wild types and mutants) on the duplicate gels stained with Coomassie blue support the accuracy of our

results. The data from the time course study of *sap* message levels also support the hypothesis of strain-dependent overall regulation of Sap production. The activity bands of Sap were more intense for the UAMS-1 strains compared to the RN6390 strains, which was also consistent with similar observations made at the transcriptional level. The acceptability of this divergent data is supported by the recent report that *agr* and *sarA* showed strain-dependent differences in their regulatory roles in *S. aureus* (10). Blevins *et al.* (10) showed that mutation of *sarA* in RN6390 caused a reduction of the hemolytic activity while the opposite effect was observed in all other strains in their study including UAMS-1. Other investigators have also demonstrated that mutation of *sarA* in *S. aureus* DB (blood isolate) caused an increase in its hemolytic activity, whereas the opposite effect was observed in RN6390 (21 and 24). These contrasting results can be explained by the fact that it was recently shown that RN6390 and its related strains (8325-4, RN6390 and BB255), derived from 8325, have a deletion in *rsbU* gene which encodes for an activator of the stress response mediated through a sigma factor (SigB; 36, 39, and 57). Mana *et al.* (65) analysis of *sarA* promoters showed that one of them was *sigB* dependent, and recently Gertz *et al.* (37) showed that mutation of *sigB* in *S. aureus* strain COL resulted in reduced SarA levels. Based on these studies, it appears more than likely that results of regulatory studies done with RN6390 may not be generalized to all other strains.

Quantitative assays for Sap activity done with spent media isolated from *S. aureus* laboratory strains (RN6390 and UAMS-1) and their isogenic regulatory mutants standardized spectrophotometrically gave a result corresponding to the zymographic

analysis. Yet, we think that the assay sensitivity is different based on the substrate used because significant Sap activity of various levels was detected for all strains when *p*NPP was used. Therefore, it appears that Sap activity towards α-naphthyl phosphate may need to reach a certain level in order to be detectable by zymographic analysis. Sap activity levels of *S. aureus* UAMS-1 strains were also elevated compared to RN6390 strains, thus maintaining the consistency with northern and zymographic analyses results. Overall, the UAMS-1 showed increased growth abilities and total proteins production (Table 3), yet the cell cultures were standardized with regard to optical density. Therefore, these data strongly suggest the occurrence of strain-dependent events controlling the production of Sap. The determination of Sap activity per μg of total proteins suggests that Sap constitutes a major protein produced by the *sar*-mutant of both strain groups (RN6390 and UAMS-1).

In this study, we have shown that Sap gene is conserved among the strains of *S. aureus* that we included in our study regardless of their laboratory or clinical origins. However, Sap gene was not present in the *S. epidermidis* strains analyzed. We confirmed that Sap is a secreted protein and that events controlling its production are strain-dependent. We also demonstrated that *agr* and *sar* partially affect the production of Sap on a strain-dependent basis at both the transcriptional and activity levels. These findings are unique in the sense that *agr* and *sar* have consistently been shown to drastically affect the expression of most known staphylococcal exoproteins genes. Therefore, it adds to the complexity of genetic and molecular mechanisms occurring in *S. aureus*. Based on these results we speculate that other regulatory factors are certainly involved and that *agr* and

sar effects on Sap production are most likely indirect. Our data reveal that the production of Sap is regulated at both transcriptional and post-transcritional stages. The post-transcriptional events may be at the translational, post-translational or even at the secretion level. At this point, more in-depth studies are required in order to definitively characterize the regulation of Sap within *S. aureus*. The actual role of Sap in *S. aureus* is yet to be elucidated; however, the fact that the global regulators of virulence may partially or indirectly affect its production is supportive of the hypothesis that Sap may also contribute to virulence in *S. aureus*. The generation of a *sap*-mutant strain of *S. aureus* is necessary in order to do comparative virulence studies. We believe that Sap could be playing a role in the survival of the pathogen within phagocytic cells.

BIBLIOGRAPHY

1. **Almeida, R. A., K. R. Matthews, E. Cifrian, A. J. Guidry, and S. P. Oliver.** 1996. *Staphylococcus aureus* invasion of bovine mammary epithelial cells. J. Dairy Sci. *79*:1021-1026.
2. **Arvidson, S., and K. Tegmark.** 2001. Regulation of virulence determinants in *Staphylococcus aureus*. Int. J. Med. Microbiol. *291*:159-170.
3. **Ausubel. *Et al.* 1994.** Current Protocols in Molecular Biology. Vol. 1. John Wiley and Sons, Inc.
4. **Baca, O. G., M. J. Roman, R. H. Glew, R. F. Christner, J. E. Buhler, and A. S. Aragon.** 1993. Acid phosphatase activity in *Coxiella burnetii*: a possible virulence factor. Infect. Immun. *61*:4232-4239.
5. **Barrett, F. F., R. F. Jr. McGehee, and M. Findland.** 1968. Methicillin-resistant *Staphylococcus aureus* at Boston City Hospital: Bacteriologic and epidemiologic observations. New Engl. J. Med. *279*:441-8.
6. **Bayer, M. G., J. H. Heinrichs, and A. L. Cheung.** 1996. The molecular Architecture of the *sar* locus in *Staphylococcus aureus*. J. Bacteriol. *178*:4563-4570.
7. **Bayles, K., C. Wesson, L. Liou, L. Fox, G. Bohach, and W Trumble.** 1998. Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells. Infect. Immun. *66*:336.

8. **Beaujean, D. J., A. J. Weersink, H. E. Blok, H. M. Frenay, and J. Verhoef.** 1999. Determining risk factors for methicillin-resistant *Staphylococcus aureus* carriage after discharge from hospital. J. Hosp. Infect. *42*(3):213-8.

9. **Blevins, J., A. Gillaspy, T. Rechtin, B. Hurlburt, and M. Smeltzer.** 1999. The staphylococcal accessory regulator (*sar*) represses transcription of the *Staphylococcus aureus* collagen adhesin gene (*cna*) in an *agr*-independent manner. Mol. Microbiol. *33*:317-326.

10. **Blevins, J. S., K. E. Beenken, M. O. Elasri, B. K. Hurburt, and M. S. Smeltzer.** 2002. Strain-dependent differences in the regulatory roles of *sarA* and *agr* in *Staphylococcus aureus*. Infect. Immun. *70*(2):470-480.

11. **Bone, RC.** 1994. Sepsis and its complications: the clinical problem. Crit. Care Med. 22:S8.

12. **Booth, M. C., A. L. Cheung, K. L. Hatter, B. D. Jett, M. C. Callegan, and M. S. Gilmore.** 1997. Staphylococcal accessory regulator (*sar*) in conjunction with *agr* contributes to *Staphylococcus aureus* virulence in endophthalmitis. Infect. Immun. *65*:1550-1556.

13. **Boyce, J. M.** 1998. Are the epidemiology and microbiology of methicillin-resistant *Staphylococcus aureus* changing? JAMA *279*:623-4.

14. **Brown, E. J.** 1986. The role of extracellular matrix proteins in the control of phagocytosis. J. Leuk. Biol. *59*:579.

15. **Centers for Disease Control and Prevention.** 1996. National Nosocomial Infection Surveillance System report: data summary from October 1986-April 1996. U.S. Department of Health and Human Services, Atlanta, Ga.

16. **Centers For Disease Control and Prevention.** 1999. Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus*: Minnesota and Dakota, 1997-1999. Morb. Mortal. Wkly. Rep. *48*:707-10.

17. **Centers for Disease Control and Prevention.** 2000. *Staphylococcus aureus* with reduced susceptibility to vancomycin-Illinois, 1999. Morb. Mortal. Wkly. Rep. *48*:1165-1167.

18. **Centers for Disease Control and Prevention.** 2002. *Staphylococcus aureus* resistant to vancomycin: United States, 2002. Morb. Mortal. Wkly. Rep. *51*:565-7.

19. **Chhatwal, G. S., M. J. Walker, H. Yan, K. N. Timmis, and C. A. Guzman.** 1997. Temperature dependent expression of an acid phosphatase by *Bordetella bronchiseptica*: role in intracellular survival. Microb. Pathogen. *22*:257-264.

20. **Cheung, A. L., and G. Zhang.** 2002. Global regulation of virulence determinants in *Staphylococcus aureus* by the SarA protein family. Front. Biosci. *7*:d1825-42.

21. **Cheung, A. L., J. M. Koomey, C.A. Butler, S. J. Projan, and V. A. Fischetti.** 1992. Regulation of exoprotein expression in *Staphylococcus*

aureus by a locus (*sar*) distinct from *agr*. Proc. Natl. Acad. Sci. USA *89*:6462-6466.

22. **Cheung, A. L., M. G. Bayer, and J. H. Heinrichs.** 1997. *sar* genetic determinants necessary for transcription of RNAII and RNAIII in the *agr* locus of *Staphylococcus aureus*. J. Bacteriol. *179*:3963-3971.
23. **Cheung, A. L., K. Eberhardt, and J. H. Heinrichs.** 1997. Regulation of protein A synthesis by the *sar* and *agr* loci of *Staphylococcus aureus*. Infect. Immun. *65*:2243-2249.
24. **Cheung, A. L., and P. Ying.** 1994. Regulation of α- and β-hemolysins by the *sar* locus of *Staphylococcus aureus*. J. Bacteriol. *176*:580-585.
25. **Cheung, A. L., and S. Projan.** 1994. Cloning and sequencing of *sarA* of *Staphylococcus aureus*, a gene required for the expression of *agr*. J. Bacteriol. *176*:4168-4172.
26. **Chien, Y. -T., A. C. Manna, S. J. Projan, and A. L. Cheung.** 1998. sarA level is a determinant of *agr* activation in *Staphylococcus aureus*. Mol. Microbiol. *30*:991-1001.
27. **Chien, Y.-T., A. C. Manna, S. J. Projan, and A. L. Cheung.** 1999. SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar*-dependent gene regulation.
28. **Cicalini, S., G. Forcina, and F. G. Rosa.** 2001. Infective endocarditis in patients with immunodeficiency virus infection. J. Infect. *42*(4):267-71.

29. **Coleman, G.** 1967. Studies on the regulation of extracellular enzymes formation by *Bacillus subtilis*. J. Gen. Microbiol. *49*:421-431.

30. **Coleman, G., C. Jakeman, and N. Martin.** 1978. Patterns of extracellular protein secretion by a number of clinical isolated strains of *Staphylococcus aureus*. J. Gen. Microbiol. *107*:189-192.

31. **Du Plessis, E. M., J. Theron, L. Joubert, T. Lotter, and T. G. Watson.** 2002. Characterization of a phosphatase secreted by *Staphylococcus aureus* strain 154, a new member of the bacterial class C family of nonspecific acid phosphatases. System. Appl. Microbiol. *25*:21-30.

32. **Easmon, C. S. F., and C. Adlam**. 1983. Staphylococci and staphylococcal infections, volume 1: Clinical and epidemiological aspects. Academic Press Inc. New York, N. Y.

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

34. **Fridkin, S. K.** 2001. Vancomycin-intermediate and –resistant *Staphylococcus aureus*: what the infectious disease specialist needs to know. Clin. Infect. Dis. *32*:108-15.

35. **Gallagher, S. R.** 1999. Current Protocol in Molecular Biology. 10.2A.1-10.2A.34. John Wiley and Sons, Inc.

36. **Gertz, S., S. Engelmann, R. Schmid, K. Ohlsen, J. Hacker, and M. Hecker.** 1999. Regulation of σ^B-dependent transcription of *sigB* and *asp23*

in two different *Staphylococcus aureus* strains. Mol Gen. Genet. *261*:558-566.

37. **Gertz, S., S. Engelmann, R. Schmid, A. K. Ziemandt, K. Tscher *et al.*** 2000. Characterization of the σ^B regulon in *Staphylococcus aureus*. J. Bacteriol. *182*:6983-6991.

38. **Gillaspy, A. F., C. Y. Lee, S. Sau, A. L. Cheung, and M. S. Smeltzer.** 1998. Factors affecting the collagen binding capacity of *Staphylococcus aureus*. Infect. Immun. *66*:3170-3178.

39. **Giarchino, P., S. Engelmann, and M. Bischoff.** 2001. σ^B activity depends on RsbU in *Staphylococcus aureus*. J. Bacteriol. *183*:1843-1852.

40. **Giraudo, A. T., A. Calzolari, A. A. Cataldi, C. Bogni, and R. Nagel.** 1999. The *sae* locus of *Staphylococcus aureus* encodes a two-component regulatory system. FEMS Microbiol. Lett. *177*(1):15-22.

41. **Giraudo, A. T., A. L. Cheung, and R. Nagel.** 1997. The *sae* locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level. Arch. Microbiol. *168*(1):53-8.

42. **Giraudo, A. T., C. G. Raspanti, A. Calzolari, and R. Nagel.** 1994. Characterization of a Tn*551*-mutant of *Staphylococcus aureus* defective in the production of several exoproteins. Can. J. Microbiol. *40*(8):677-81.

43. **Gresham, H. D., J. H. Lowrance, T. E. Caver, B. S. Wilson, A. L. Cheung, and F. P. Lindberg.** 2000. Survival of *Staphylococcus aureus* inside Neutrophils contributes to infection. J. Immun. *164*:3713-3722.

44. **Hamilton, A., D. Harrington, and I. C. Sutcliffe.** 2000. Characterization of acid phosphatase activities in the equine pathogen *Streptococcus equi.* System. Appl. Microbiol. *23*:325-329.

45. **Hart, M. E., M. S. Smeltzer, and J. J. Iandolo.** 1993. The extracellular protein regulator (*xpr*) affects exoprotein and *agr* mRNA levels in *Staphylococcus aureus*. J. Bacteriol. *175*(24):7875-7879.

46. **Heinrichs, J. H., M. G. Bayer, and A. L. Cheung.** 1996. Characterization of the *sar* locus and its interactions with *agr* in *Staphylococcus aureus*. J. Bacteriol. *178*:418-423.

47. **Herold, B. C., L. C. Immergluck, M. C. Maranan, D. S. Lauderdale, *et al.*** 1998. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. JAMA *279*:593-8.

48. **Hiramatsu, K., H. Hanaki, T. Ino, K. Yabuta, T. Oguri, and F. C. Tenover.** 1997. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J. Antimicrob. Chemother. *40*:135-6.

49. **Hudson, M., W. Ramp, N. Nicholson, A. Williams, and M. Nousiainen.** 1995. Internalization of *Staphylococcus aureus* by cultured osteoblasts. Microb. Pathog. *19*:409.

50. **Iandolo, J. J.** 1989. Genetic analysis of extracellular toxins of *Staphylococcus aureus*. Annu. Rev. Microbiol. *43*:375-402.

51. **Janzon, L., S. Arvidson.** 1990. The role of the delta-lysin gene (hld) in the regulation of virulence genes by the accessory gene regulator (*agr*) is *Staphylococcus aureus*. EMBO J. *9*(5):1391-1399.

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

53. **Ji, G., R. C. Beavis, and R. P. Novick.** 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc. Natl. Acad. Sci. USA *92*:12055-12059.

54. **Ji, G., R. C. Beavis, and R. P. Novick.** 1997. Bacterial interference caused by autoinducing peptide variants. Science *276*:2027-2030.

55. **Kapral, F., and f. Shayegani.** 1959. Intracellular survival of staphylococci. J. Exp. Med. *110*:123.

56. **Kornblum J., Kreiswirth, S.J. Projan, H. Ross, and R.P. Novick.** 1990. *agr*, a polycistronic locus regulating exoprotein synthesis in *Staphylococcus aureus*. p. 373-402. In R. P. Novick (ed.), Molecular biology of the staphylococci. VCH Publishers, New York, N.Y.

57. **Kullik, I., P. Giachino, and T. Fuchs.** 1998. Deletion of the alternative sigma factor σ^B in *Staphylococcus aureus* reveals its function as a global regulator of virulence genes. J. Bacteriol. *180*:4814-4820.

58. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bateriophage T4. Nature *227*:680-685.

59. **Lina, G., S. Jarraud, G. Ji, T. Greenland, A. Pedraza *et al.*** 1998. Transmembrane topology and histidine protein kinase activity of AgrC, the *agr* signal receptor in *Staphylococcus aureus*. Mol. Microbiol. *28*:655-662.

60. **Longuet, P., M. C. Douard, G. arlet, J. M. Molina, C. Benoit, and C. Leport.** 2001. Venous access port-related bacteremia in patients with immunodeficiency syndrome or cancer: the reservoir as a diagnostic and therapeutic tool. Clin. Infect. Dis. *32*(12):1776-83.

61. **Lowy, F.** 1998. *Staphylococcus aureus* infections. New Engl. J. Med. *339*:520-532.

62. **Lublin, M., D. L. Bartlett, D. N. Danforth, H. Kauffman *et al.*** 2002. Hepatic abscess in patients with chronic granulomatous disease. Ann. Surg. *235*(3):383-91.

63. **Manfredi, R., L. Calza, and F. Chiodo.** 2002. Epidemiology and microbiology of cellulites and bacterial soft tissue infection during HIV disease: a 10-year survey. Cutan. Pathol. *29*(3):168-72.

64. **Manna, A., and A. L. Cheung.** 2001. Characterization of *sarR*, a modulator of *sar* expression in *Staphylococcus aureus*. Infect. Immun. *69*:885-896.

65. **Manna, A. C., M. G. Bayer, and A. L. Cheung.** 1998. Transcriptional analysis of different promoters in *sar* locus in *Staphylococcus aureus*. J. Bacteriol. *180*:3828-3836.

66. **Mansour, A. J., M. Grinberg, P. L. da Luz, G. Bellotti.** 1992. The complication of infective endocarditis; a reappraisal in the 1980s. Arch. Intern. Med. 152:2428.

67. **Mayville, P., G. Ji, R. Beavis, H. Yang, M. Goger, R. P. Novick, and T. W. Muir.** 1999. Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. Proc. Natl. Acad. Sci. USA *96*:1218-1223.

68. **McNamara, P. J., K. C. Milligan-Monroe, S. Khalili, and R. A. Proctor.** 2000. Identification, cloning, and initial characterization of *rot*, a locus encoding a regulator of virulence factor expression in *Staphylococcus aureus*. J. Bacteriol. *182*:3197-3203.

69. **Melly, M., J. Thomison, and D. Rogers.** 1960. Fate of staphylococci within human leukocytes. J. Exp. Med. *112*:1121.

70. **Mills, E. L., and F. J. D. Noya.** 1993. Congenital neutrophil deficiencies. P. 183. In J. S. Abramson, J. G. Wheeler (ed.): The Neutrophil. IRL Press, Oxford.

71. **Morfeldt, E., D. Taylor, A. von Gabin, and S. Arvidson.** 1995. Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. EMBO J. *14*:4569-4577.

72. **Mortfeldt, E., K. Tegmark, and S. Arvidson.** 1996. Transcriptional control of the *agr*-dependent virulence gene regulator, RNAIII, in *Staphylococcus aureus*. Mol. Microbiol. *21*:1227-1237.

73. **Morfeldt E., L. Janzon, S. Arvidson, and S. Lofdahl.** 1988. Cloning of a chromosomal locus (exp) which regulates the expression of several exoprotein genes in *Staphylococcus aureus*. Mol. Gen. Genet. *211*:435.

74. **Nielsen, H., J. Engelbrecht, S. Brunak, and G. Von Heijne.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Prot. Eng. *10*:1-6.

75. **Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh.** 1993. Synthesis of Staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. *12*:3967-3975.

76. **Novick, R. P., H. Ross, A. Figueiredo, G. Abramochkin *et al.*** 2000. Activation and inhibition of the staphylococcal *agr* system. Science *393*:391.

77. **Novick, R. P., S. J. Projan, J. Kornblum *et al.*** 1995. The *agr* P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol. Gen. Genet. *248*:446.

78. **O'Toole, R. D., W. L. Drew, B. J. Dahlgren, and H. N. Beaty.** 1970. an outbreak of methicillin-resistant *Staphylococcus aureus* infection: Observations in hospital and nursing home. J. Am. Med. Assoc. *213*:251-3.

79. **Otto, M., R. Sussmuth, G. Jung, and F. Gotz.** 1998. Structure of the pheromone peptide of the *Staphylococcus epidermidis agr* system. FEBS Lett. *424*:89-94.

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

81. **Peterson, P. K., B. J. Wilkinson, Y. Kim *et al.*** 1978. Influence of encapsulation on staphylococcal opsonization and phagocytosis by human polymorphonuclear leukocytes. Infect. Immun. *19*:943.

82. **Peterson, P. K., J. Verhoef, L. D. Sabath, P. G. Quie.** 1977. Effect of protein A on staphylococcal opsonization. Infect. Immun. *15*:760.

83. **Peterson, P. K., Y. Kim, B. J. Wilkinson *et al.*** 1978. Dichotomy between opsonization and serum complement activation by encapsulated staphylococci. Infect. Immun. *20*:770.

84. **Projan, S. J., and R. P. Novick.** 1997. The molecular basis of pathogenicity. p. 55-81. In K. B. Crossley and G. L. Archer (ed.): The staphylococci in human disease. Churchill Livingston. New York, N. Y.

85. **Recsei, P., B. Kreiswirth, M. O'Reilly, M. Schlievert *et al.*** 1986. Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agr*. Mol. Gen. Genet. *202*:58-61.

86. **Remaley, A. T., S. Das, P. I. Campbell, G. M. LaRocca, M. T. Pope, and R. H. Glew.** 1985. Characterization of *Leishmania donovani* acid phosphatases. J. Biol. Chem. *260*(2):880-6.

87. **Reilly, T. J., G. S. Baron, F. E. Nano, and M. S. Kuhlenschmidt.** 1996. Characterization and sequencing of respiratory burst-inhibiting acid

phosphatase from *Francisella tularensis*. J. Biol. Chem. *271*(18):10973-10983.

88. **Rogers, D., and R. Tompsett.** 1952. The survival of staphylococci within human leukocytes. J. Exp. Med. *95*:209.

89. **Saha, A. K., J. N. Dowling, K. L. LaMarco, S. Das, A. T. Remaley *et al.*** 1985. Properties of an acid phosphatase from *Legionella micdadei* which blocks superoxide anion production by human neutrophils. Arch. Biochem. Biophys. *243*(1):150-60.

90. **Said-Salim, B., P. M. Dunman, F. M. McAleese, D. Macapagal *et al.*** 2003. Global regulation of *Staphylococcus aureus* genes by Rot. J. Bacteriol. *185*:610-619.

91. **Sasse, J., S. R. Gallagher.** 2000. Current Protocols in Molecular Biology. 10.6.1-10.6.13. John Willey and Sons, Inc.

92. **Sau, S., J. Sun, and C. Y. Lee.** 1997. Molecular characterization and transcriptional analysis of type 8 capsule genes in *Staphylococcus aureus*. J. Bacteriol. *179*(5):1614-21.

93. **Schmidt, K. A., A. C. Manna, S. Gill, and A. L. Cheung.** 2001. SarT, a repressor of α-hemolysin in *Staphylococcus aureus*. Infect. Immun. *69*:4749-4758.

94. **Scriven, J. M., P. Silva, R. A. Swann, M. M. Thompson, *et al.*** 2003. The acquisition of methicillin-resistant *Staphylococcus aureus* (MRSA) in vascular patients. Eur. J. Endovasc. Surg. *25*(2):147-51.

95. **Senthilkumar, A., S. Kumar, and J. N. Sheagren.** 2001. Increased incidence of *Staphylococcus aureus* bacteremia in hospitalized patients with acquired immunodeficiency syndrome. Clin. Infect. Dis. *33*(8):1412-6.

96. **Smith, T. L., M. L. Pearson, K. R. Wilcox, *et al.*** 1999. N. Engl. J. Med. *340*:493-501.

97. **Spika, J. S., H. A. Verbrugh, and J. Verhoef.** 1981. Protein A effect on alternative pathway complement activation and opsonization of *S. aureus*. Infect. Immun. *34*:455.

98. **Tegmark, K., A. Karlson, and S. Arvidson.** 2000. Identification and characterization of sarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus*. Mol. Microbiol. *37*:398-409.

99. **Vandenesch, F., J. Kornblum, and R. P. Novick.** 1991. A temporal signal, independent of *agr*, is required for *hla* but not *spa* transcription in *Staphylococcus aureus*. J. Bacteriol. *173*:6313-6320.

100. **Vann, J. M., and R. A. Proctor.** 1987. Ingestion of *Staphylococcus aureus* by bovine endothelial cells results in time- and dose-dependent damage to endothelial cell monolayers. Infect. Immun. *55*:2155-2163.

101. **Verbrugh, H. A., W. C. Van Dijk, R. Peters *et al.*** 1979. Role of *S. aureus* cell wall peptidoglycan, teichoic acid and protein A in the processes of complement activation and opsonization. Immunology *37*:615.

102. **Verhoef, J., and M. R. Visser.** 1993. Neutophil phagocytosis and killing: normal function and microbial evasion. P. 109. In J. S. Abramson, J. G. wheeler (ed.): The Neutrophil. IRL Press, Oxford.

103. **Wadstrom, T.** 1987. Molecular aspects on pathogenesis of wound and foreign body infections due to staphylococci. Zentralbl. Bakteriol. Mikrobiol. Hyg. *266*(1-2):191-211.

104. **Waldvogel, F. A.** 1990. *Staphylococcus aureus* (including Toxic shock syndrome). p. 1754-1777. In Mandell, Douglas and Bennett's Principles and practice of infectious diseases, volume 2. Churchill Livingston. New York, N. Y.

105. **Walter, K., and C. Schutt.** 1974. Acid and Alkaline phosphatase in serum, pp. 856-860. In Bergmeyer, H. U. (ed.): Methods of enzymatic analysis. New York, Academic Press.

106. **Watanakunakorn, C.** 1987. Bacteremic *Staphylococcus aureus* pneumonia. Scand. J. Infect. Dis. *19*:623.

107. **Wolz, C., P. Pohlmann-Dietze, A. Steinhuber, Y.-T. Chien *et al.*** 2000. *agr*-independent regulation of fibronectin-binding protein(s) by the regulatory locus *sar* in *Staphylococcus aureus*. Mol. Microbiol. *36*:230-243.

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