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**Senescence of Staphylococci:  
metabolic and environmental factors determining  
bacterial survival and persistence**



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*“To my family, Rupal & Rhea”*

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New insight of a heat shock protein ClpC ATPase as an important physiologic, metabolic and stress regulator in *Staphylococcus aureus*.

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*Staphylococcus aureus* ClpC ATPase, Stress Resistance, & Tricarboxylic Acid Cycle Activity.

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## List of Abbreviations

% v/v	Percent volume/volume
$\Delta A$	Change in absorbance
$\sigma^B$	Sigma B
$^{\circ}C$	degree centigrade
$\mu g$	microgram
$\mu L$	microlitre
2D	2 dimensional
AA	Amino acid
ACN	Aconitase
ADI	Arginine deiminase
ADP	Adenosine diphosphate
ATP	Adenosine 5'-triphosphate
Agr	Accessory gene regulator
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
BHI	Brain-heart infusion (growth medium)
BLAST	“Basic Local Alignment Search Tool”
bp	base-pair
cDNA	complementary DNA
CFU	“colony forming units”
<i>clp</i>	Caseinolytic protease
CO <sub>2</sub>	Carbon dioxide
CP	Capsular polysaccharide
DNA	Deoxyribonucleic acid
DSM	(Also DSMZ) Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, (German Collection of Microorganisms and Cell Cultures)
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid (edetate)
<i>eg.</i>	Example
<i>et al</i>	<i>Et alii</i>

etc.	<i>Et cetera</i>
EtOH	Ethanol
Fig.	Figure
gDNA	genomic DNA
GISA	Glycopeptide-intermediate <i>Staphylococcus aureus</i>
h	Hour(s)
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HSP	Heat Shock Proteins
<i>i.e</i>	<i>Id est</i> (means “that is”)
<i>i.v.</i>	Intra-venous
Kb	Kilo-base pair
LB	Luria and Bertani
M	Molar
MXF	Moxifloxacin
MH	Mueller-Hinton (growth medium)
MIC	minimum inhibitory concentration
min	minute(s)
mL	millilitre(s)
mM	milli Molar
mRNA	messenger RNA
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NAD	Nicotinamide adenine dinucleotide
NCCLS	“National Committee for Clinical Laboratory Standards”
ng	nanogram
OD	Optical density
ORF	Open reading frame
PCD	“Programmed cell death”
pH	a measure of the activity of hydrogen ions (H <sup>+</sup> ) in a solution
PIA	Polysaccharide Intercellular Adhesin
RNA	Ribonucleic acid
ROS	Reactive oxidative species
rpm	Revolutions per minute
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
<i>S. aureus</i>	<i>Staphylococcus aureus</i>

Sar	Staphylococcal accessory regulator
SCV	“Small colony variants”
SD	Standard deviations
secs	second(s)
SEM	Scanning electron microscopy or Standard error of measurement
SXT	(Also TMP-SMX) trimethoprim-sulfamethoxazole
TCA	tricarboxylic acid cycle (or the Krebs Cycle)
TIGR	“The Institute for Genomic Research”
TSA	Tryptic soy broth agar (growth medium)
TSB	Tryptic soy broth (growth medium)
VLEC	Very Low Ethanol Concentration
WT	Wild-type

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# **Chapter 1**

## **INTRODUCTION**

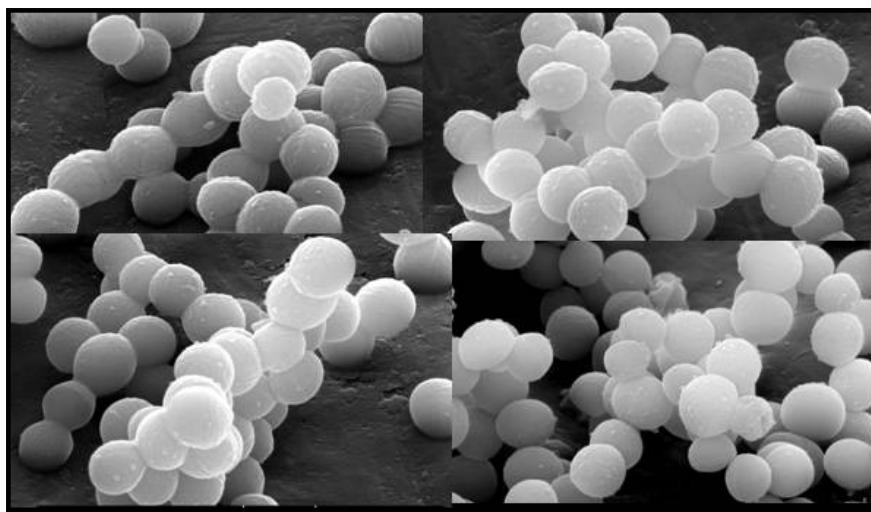


## 1. Introduction

### 1.1 *Staphylococcus aureus*

As early as 1880 and 1882, Ogston described staphylococcal disease and its role in sepsis and abscess formation (Ogston, 1882). More than 100 years later, *Staphylococcus aureus* (*S. aureus*) still remains a versatile and dangerous pathogen in humans. The frequencies of both community-acquired and hospital-acquired staphylococcal infections have increased steadily, with little change in overall mortality. Treatment of these infections has become more difficult because of the emergence of multidrug-resistant strains (Lowy, 1998).

*S. aureus* is a member of the Micrococcaceae family. On microscopically examination, the organisms appear as gram-positive, catalase-positive cocci in grape-like clusters (**Fig.1.1**). *S. aureus* is distinguished from other staphylococcal species on the basis of the gold pigmentation of colonies and positive results of coagulase, mannitol fermentation, and deoxyribonuclease tests. The staphylococcal genome consists of a circular chromosome (of approximately 2800 bp), with prophages, plasmids, and transposons. Genes governing virulence and resistance to antibiotics are found on the chromosome, as well as the extra-chromosomal elements. These genes are transferred between staphylococcal strains, species, or other gram-positive bacterial species through the extra-chromosomal elements (Schaberg and Zervos, 1986).



**Fig.1.1.** Different micrographs of *Staphylococcus aureus* DSM20231 under scanning electron microscope.

### **1.2 Epidemiology of *Staphylococcal* disease**

#### **1.2.1 Colonization and Infection**

Humans are a natural reservoir of *S. aureus*. 30 to 50 % of healthy adults are colonized, with 10 to 20 % persistently colonized (Noble *et al*, 1967; Casewell and Hill, 1986). Both methicillin-sensitive and methicillin-resistant isolates are persistent colonizers (Casewell and Hill, 1986; Sanford *et al*, 1994). Persons colonized with *S. aureus* are at increased risk for subsequent infections (Wenzel and Perl, 1995). Rates of staphylococcal colonization are high among patients with type 1 diabetes (Tuazon *et al*, 1975) intravenous drug users (Tuazon and Sheagren, 1974) patients undergoing hemodialysis (Yu *et al*, 1986), surgical patients (Weinstein, 1959; Kluytmans *et al*, 1995) and patients with the acquired immunodeficiency syndrome (Weinke *et al*, 1992). Patients with qualitative or quantitative defects in leukocyte function are also at increased risk for staphylococcal disease (Waldvogel, 1995).

#### **1.2.2 Transmission**

Persons colonized with *S. aureus* strains are at increased risk of becoming infected with these strains. Most cases of nosocomial infection are acquired through exposure to the hands of health care workers after they have been transiently colonized with staphylococci from their own reservoir or from contact with an infected patient. Outbreaks may also result from exposure to a single long-term carrier or environmental sources, but these modes of transmission are less common (Casewell and Hill, 1986; Sheretz *et al*, 1996).

### **1.3 Diseases caused by *S. aureus***

#### **1.3.1 Bacteremia**

The overall rate of mortality from staphylococcal bacteremia, which has not changed in the past 15 years, ranges from 11 to 43 % (Mylotte *et al*, 1987). Factors associated with increased mortality include an age of more than 50 years, non-removable foci of infection, and serious underlying cardiac, neurologic, or respiratory disease. Bacteremia caused by methicillin-resistant strains is not associated with increased mortality. The frequency of complications from staphylococcal bacteremia is high, ranging from 11 to 53 %. As many as 31 % of patients with bacteremia who do not have evidence of endocarditis do have evidence of metastatic infection (Mylotte *et al*, 1987; Lowy, 1998). An increasing percentage of bacteremic infections are related to catheterization (Steinberg *et al*, 1996).

### 1.3.2 Endocarditis

The incidence of *S. aureus* endocarditis has increased and accounts for 25 to 35 % of cases (Sandre and Shafran, 1996). It occurs in intravenous drug users, elderly patients, patients with prosthetic valves, and hospitalized patients. In all four groups, the initial presentation may be limited to fever and malaise, making diagnosis difficult. Unlike endocarditis caused by less virulent pathogens, *S. aureus* endocarditis is characterized by a rapid onset, high fever, frequent involvement of normal cardiac valves, and the absence of physical stigmata of the disease on initial presentation (Chambers *et al*, 1983). In cases of endocarditis related to intravenous drug use, the disease is frequently right-sided, the patients are young, the mortality rate is low, and the majority of patients do not have antecedent valvular disease. The prognosis is worse for intravenous drug users who have advanced disease associated with human immunodeficiency virus (HIV) infection than it is for those without HIV infection (Pulvirenti *et al*, 1996). In cases of endocarditis that are not related to drug use, the disease is often left-sided, the patients are older, the mortality rate is high (20 to 44 %), and the disease usually involves previously damaged cardiac valves (Chambers *et al*, 1983). The mortality rate for nosocomial endocarditis, regardless of the pathogen, is 40 to 56 %, and the rate is even higher when the pathogen is *S. aureus* (Fernandez-Guerrero *et al*, 1995). In many of these cases, the diagnosis is obscured by other conditions or the administration of antibiotics.

### 1.3.3 Metastatic Infections

*S. aureus* has a tendency to spread to particular sites, including the bones, joints, kidneys, and lungs (Chambers *et al*, 1983; Libman and Arbeit, 1984). Suppurative collections at these sites serve as potential foci for recurrent infections. Patients with persistent fever despite appropriate therapy should be evaluated for the presence of suppurative collections.

### 1.3.4 Toxic Shock Syndrome

Staphylococcal toxic shock syndrome came to prominence in 1980–1981. The disease is characterized by a fulminant onset, often in previously healthy persons. The diagnosis is based on clinical findings that include high fever, erythematous rash with subsequent desquamation, hypotension, and multiorgan damage. Alternative diagnoses, including Rocky Mountain spotted fever, streptococcal scarlet fever, and leptospirosis, must be ruled

out. The toxic shock syndrome often develops from a site of colonization rather than infection (Chesney *et al*, 1981).

### 1.4 Treatment of *S. aureus* infection

Penicillin remains the drug of choice if the isolate is sensitive to it. A semi-synthetic penicillin (nafcillin or oxacillin) is indicated for  $\beta$ -lactamase-producing strains. In patients with histories of delayed-type penicillin allergy, a cephalosporin such as cefazolin or cephalothin is an acceptable alternative. *In vitro* data from experimental and clinical studies suggest that vancomycin is a less effective anti-staphylococcal drug than the  $\beta$ -lactams (Levine *et al*, 1991). Therefore, the selection of vancomycin as an alternative to a  $\beta$ -lactam in a patient with a history of allergy should be carefully considered.

Vancomycin is the drug of choice for methicillin-resistant isolates (MRSA). Patients unable to tolerate vancomycin have been treated with fluoroquinolones, trimethoprim-sulfamethoxazole, clindamycin, or minocycline. Each of these drugs has been effective in cases that require bactericidal therapy (Chambers, 1997). They are not as effective as vancomycin, however, either because they have less anti-staphylococcal activity or because resistance develops during therapy (Chambers, 1997).

Quinolones with enhanced anti-staphylococcal activity have recently become available, but their use may also be limited by the development of resistance during therapy. A number of potentially active drugs are under investigation, including quinupristin-dalfopristin, a new carbapenem, and a new family of antimicrobials drugs, oxazolidinones (Michel and Gutmann, 1997). The glycopeptide-intermediate strains (GISA) reported to date have been variably sensitive to chloramphenicol, gentamicin, rifampin, trimethoprim-sulfamethoxazole, and tetracycline (Hiramatsu *et al*, 1997; Tenover *et al*, 1998). The initial case involving a glycopeptide-intermediate strain was treated with surgical débridement and ampicillin-sulbactam plus an aminoglycoside (Hiramatsu *et al*, 1997).

Rifampin is another potent anti-staphylococcal drug, but resistance invariably develops if it is used alone. Although the efficacy of rifampin as an adjunctive drug in patients with life-threatening infections remains controversial, it is recommended in combination with gentamicin and vancomycin or nafcillin for the treatment of prosthetic-valve endocarditis

(Wilson *et al*, 1995). Rifampin has also been combined with quinolones in an effort to prevent the development of resistance (Dworkin *et al*, 1989). The duration of therapy for invasive, life-threatening infections, including those that cause endocarditis, osteomyelitis, or arthritis, is four weeks or longer. The appropriate duration of treatment for bacteremias originating from a removable focus of infection, such as an intravascular catheter, is controversial.

A very recent publication reported a new class of antibiotics - acyldepsipeptides- with antibacterial activity against Gram-positive bacteria in vitro and in several rodent models of bacterial infection. The acyldepsipeptides are active against isolates that are resistant to antibiotics in clinical application, implying a new target, which we identify as ClpP, the core unit of a major bacterial protease complex (Brötz-Oesterhelt *et al*, 2005). It was further shown that because of their unprecedented target, acyldepsipeptides show no cross-resistance to any antibiotic classes that are currently on the market or in development, which makes them ideally suited as combination partner for the often life-threatening infections caused by (multidrug)-resistant bacterial isolates including *S. aureus*.

### **1.5 Regulation of virulence determinants in *S. aureus***

*Staphylococcus aureus* is a remarkably versatile organism. It is adaptable, flexible and multifaceted in its interactions with its surroundings. It can exist comfortably in inanimate sites as well as in various niches in the animal host. It can exist harmlessly as a commensal, inhabiting the skin or mucous membranes, and it can survive in the blood or in a variety of tissue sites where it is responsible for disease states ranging from minor skin infections to toxinoses and systemic, life-threatening illnesses. This versatility depends on a tremendous range of adaptive or accessory gene systems. A subset of these is involved in pathogenesis, which is best viewed as an adaptation to the hostile environment of the host and its formidable antibacterial defenses. The large number of newly characterized genes reveals the complexity of regulatory circuits, which is consistent with the extraordinary ability of *S. aureus* to adapt to the human host. Most of the accessory genes involved in pathogenesis encode proteins that are either displayed on the bacterial surface (cell surface proteins) or released into the surroundings (toxins) and confers resistance to antimicrobials, which may strengthen its virulence and confer resistance to all families of antibiotics. These enable the organism to evade host defenses, to adhere to cells and the tissue matrix, to spread within

the host and to degrade cells and tissues, for both nutrition and protection. These accessory genes are collectively known as the virulon (Novick, 2003). The extra-bacterial proteins that contribute to the virulence of *S. aureus* can be divided into five major families: adhesion proteins, superantigens, pore-forming toxins, ADP-ribosylating toxins and proteases. Although they all have similar functions, these proteins are not antigenically identical (e.g. leucotoxins and  $\alpha$ -toxin, or superantigens). These virulence factors are also differently distributed among strains and are not always regulated in the same way in different strains. Virulence factor expression appears to be controlled by the concentration of auto-inducing peptides and by bacterial density, pH and CO<sub>2</sub>, and each of these signals controls different regulatory systems. The responses of some virulence factor genes to these regulatory systems have been evaluated, providing a broad view of the different elements influencing the secretion of toxins (Bronner *et al*, 2004). The pathogenicity of *S. aureus* is a complex process involving a diverse array of extracellular and cell wall components that are also coordinately expressed during different stages of infection (*i.e.* colonization → avoidance of host defense → growth and cell division → bacterial spread). The coordinated expression of diverse virulence factors in response to environmental cues during infections (*e.g.* expression of adhesins early during colonization vs. production of toxins late in infection to facilitate tissue spread) hints at the existence of global regulators in which a single regulatory determinant controls the expression of many unlinked target genes (Cheung *et al*, 2004).

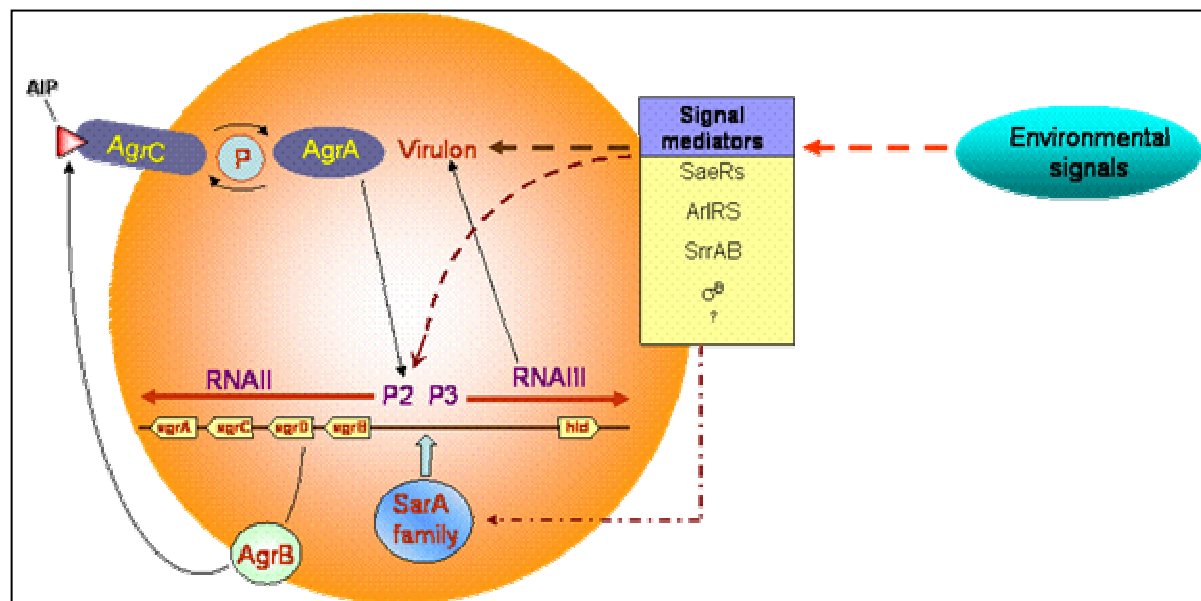
Genomic scans have revealed two major families of global regulators in *S. aureus*.

### 1.5.1 Two-component regulatory systems

Two-component regulatory systems (TCRS), of which there are 16 in the genome (Arvidson and Tegmark, 2001; Cheung and Zhang, 2002; Novick, 2003). Signal receptors are a major source of information on the external environment and, for *S. aureus*, appear to be the primary regulatory modality for expression of the virulon (Novick, 2003). These regulatory systems are sensitive to environmental signals (*e.g.* auto inducing peptide from agr). In *S. aureus*, the best-described two-component system is the Agr system (accessory gene regulator, *agrAC*) (**Fig 1.2**), and consist of two proteins: a sensor histidine kinase and a response regulator. The sensor either directly binds a specific extracellular ligand or is associated with a receptor (Cheung *et al*, 2004). Auto phosphorylation occurs when the extracellular ligand binds to the receptor, leading to the transfer of a phosphate residue from

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ATP to a histidine residue of the cytoplasmic domain of the sensor kinase. This phosphate residue is further transferred to an aspartate residue of the response regulator. The phosphorylation cascade ends with the binding of the response regulator to specific DNA sequences, which activates the transcription-regulating functions. The specific target genes controlled by these DNA-binding proteins encode effectors of the two-component systems (e.g. RNAIII from *agr*) and/or various proteins. The C-terminus domain of the sensor histidine kinase and the N-terminus domain of the response regulator are highly conserved amongst bacterial species and are responsible for general regulatory processes.



**Fig.1.2.** The accessory gene regulator (*agr*) system in *Staphylococcus*. The P2 operon encodes (via RNAII) the signaling mechanism, whereas the transcript of the P3 operon, RNAIII, acts as the effector molecule of the *agr* locus. Additional regulators of the quorum response and virulence genes, described in the text, are listed. Potential regulatory pathways used by environmental signals are indicated by the dashed red lines. system (reproduced from Yarwood and Schlievert, 2003).

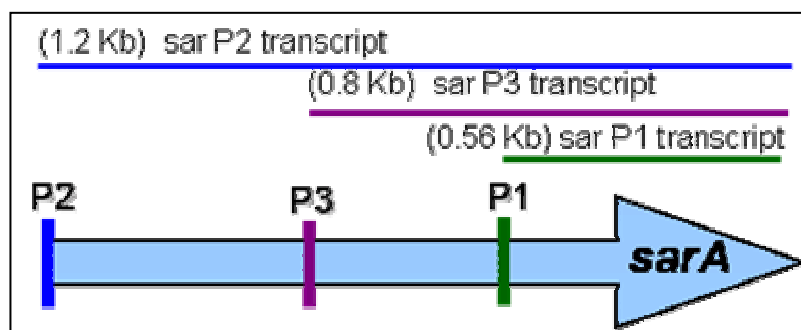
Other two-component systems involved in the regulation of virulence factors have also been described. These are (Bronner *et al*, 2004).

- (a) **sae** (*S. aureus* exoprotein expression),
- (b) **srr** (Staphylococcal respiratory response) or **srhSR**,
- (c) **ArlSR** (Autolysis-related locus), and
- (d) **LytRS**.

### 1.5.2 The SarA homologs.

These are staphylococcal accessory regulator, a family of proteins homologous to SarA, a global regulator of virulence factors (Cheung and Zhang, 2002).

SarA and its homologues (Tegmark *et al*, 2000; Manna and Cheung, 2001) affect a wide variety of genes that may or may not encode virulence or other accessory functions (Dunman *et al*, 2001). The *sarA* operon was cloned and sequenced by Cheung and Projan (Cheung and Projan, 1994), which consists of 3 overlapping transcripts, with common 3' ends. The transcripts, *sarAP1* (0.56 kb), *sarAP3* (0.80 kb) and *sarAP2* (1.2 kb) (**Fig. 1.3**, Novick, 2003), previously termed *sarA*, *sarC*, and *sarB*, respectively, are controlled by the three distinct promoters (P1, P3 and P2, respectively) (Bayer *et al*, 1996; Manna and Cheung, 2001). The three transcripts encode the 14.5-kDa SarA protein (Bayer *et al*, 1996).

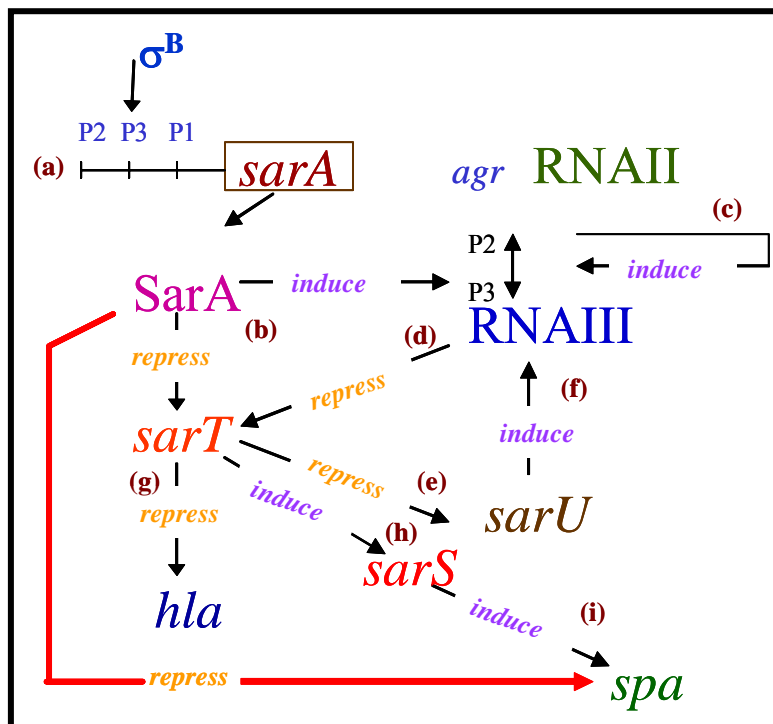


**Fig.1.3.** A cartoon dissipating the predicted SarA transcription from one of three nested promoters (P1, P2, or P3; Schmidt *et al*, 2003).

The expression of *sarA* is growth phase-dependent. The P3 promoter has a similar sequence to stress response sigma B-dependent promoters. The transcription of *sarAP1* and *sarAP2* is initiated by the  $\sigma^A$ -specific promoters P1 and P2, respectively. The multi- $\sigma$ -dependent promoters of the *sarA* operon allow the expression of *sarA* during all growth phases; *sarAP1* and *sarAP2* from mid to exponential growth phase, and *sarAP3* during the late-exponential and stationary phase or during environmental stress (Cheung *et al*, 1992). SarA activates its own expression and is down-regulated by SarR. The influence of the SigB factor on the expression of *sarA* remains unclear and discrepant results have been reported. These differences may be due to the strains or the environmental and growth conditions used in these studies (Manna *et al*, 1998; Cheung *et al*, 1999; Bischoff *et al*, 2001b). The SarA protein is a DNA-binding protein, which binds as a homodimer structure to a



conserved A/T-rich recognition motif in the promoter regions of target genes. SarA was initially described as an activator of the *agr* operon; it binds to the *agr* P2 and P3 promoter regions, thus increasing the levels of both RNAII and RNAIII, and altering the synthesis of virulence factors (*agr*-dependent pathway). SarA can also directly bind to conserved regions, termed Sar boxes, within the promoters of several cell wall-associated proteins (protein A, fibronectin-binding proteins, collagen adhesin) and exoproteins ( $\alpha$ -toxin,  $\delta$ -hemolysin,  $\gamma$ -hemolysin) (*agr*-independent pathway) (Bronner *et al*, 2004). Sequences that share a high level of identity with SarA have been identified in the *S. aureus* genome (The Institute for Genome Research, TIGR) and designated SarA homologues. These includes SarR, SarS (SarH1), SarT and SarU. A simplified overview of the predicted *sarA/agr* regulatory interaction is shown in the **Fig. 1.4**.

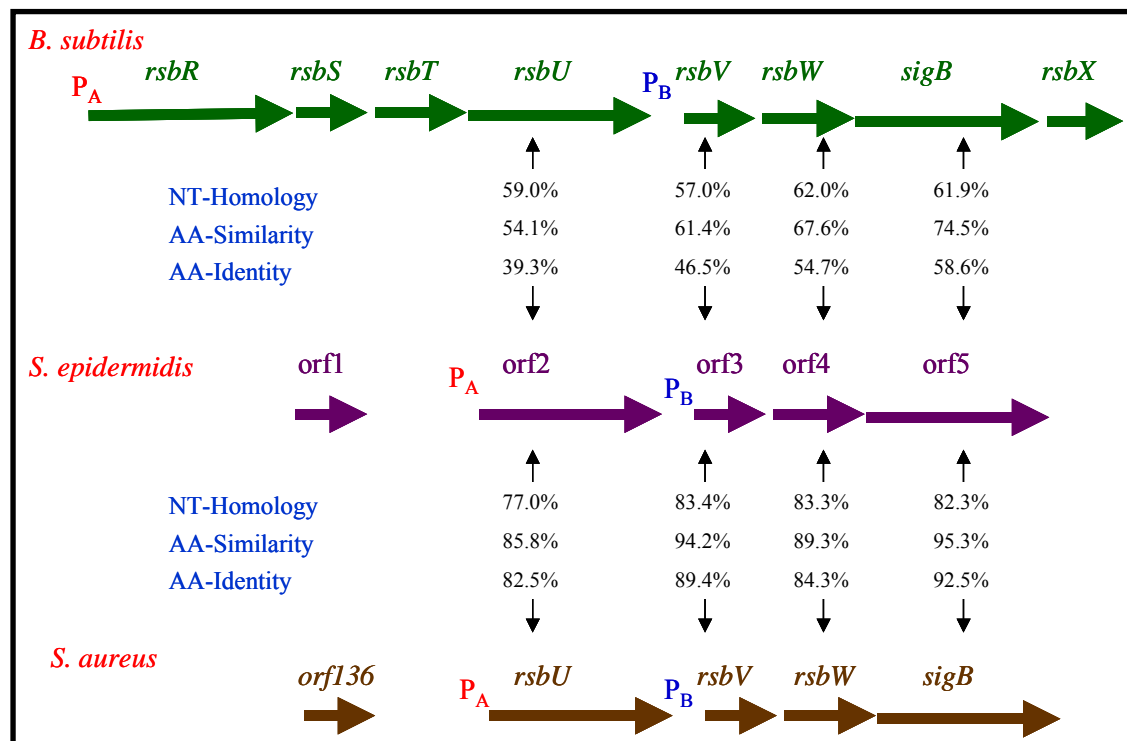


**Fig.1.4.** A simplified overview of the predicted *sarA/agr* regulatory web involving SarT and SarS. (a) SarA is transcribed from one of three nested promoters (P1, P2, or P3). (b) SarA represses expression of *spa* (protein A) and *sarT* (Cheung *et al*, 2001; *sarS*) and induces *agr* RNAII (Chien *et al*, 1998). (c) *agr* RNAII encodes a two-component quorum-sensing system, which activates expression of *agr* RNAIII, a pleiotropic regulator for expression of proteins associated with virulence (Janzon and Arvidson, 1990). (d) *agr* RNAIII represses *sarT*. (e) Increased expression of *sarT* causes repression of *sarU*, particularly during exponential growth (Manna and Cheung, 2003). (f) *sarU* induces expression of *agr* RNAIII (Manna and Cheung, 2003). (g) SarT represses expression of *hla* (Schmidt *et al*, 2001). (h) SarT induces expression of *sarS* (Schmidt *et al*,

2003), and *sarS* induces expression of *spa* (Cheung *et al*, 2001, *sarS*). The conditions and regulators mediating induction of *sarT* have not yet been determined. (reproduced from Schmidt *et al*, 2003).

## 1.6 Alternative sigma factor ( $\sigma^B$ )

In the process of infection and disease, *S. aureus* has to adapt to variable external surroundings. One of the triggers responding to environmental stimuli is alternate transcription factor,  $\sigma^B$ . The *S. aureus sigB* operon comprises the genes *rsbU*, *rsbV*, *rsbW*, and *sigB* (Wu *et al*, 1996; Kullik and Giachino, 1997), which modulate  $\sigma^B$  activity in a sequential fashion (**Fig.1.5**). *RsbW* acts as an anti-sigma factor by sequestering  $\sigma^B$  through protein-protein interactions, and *RsbU* controls, via *RsbV* phosphorylation, the availability of free *RsbW* to interact with  $\sigma^B$  (Bischoff and Berger-Bachi, 2001). The genetic organization of the *S. aureus sigB* operon closely resembles that of the distal part of the well-characterized homologous operon of the soil-borne gram-positive bacterium *Bacillus subtilis* (Kalman *et al*, 1990; Wise and Price, 1995) (**Fig. 1.5**) and contains many components that are perceived to be important for protecting the cell from various environmental stresses.



**Fig.1.5.** Comparison of the organisation of the *sigB* operon of *S. epidermidis* 1457, and that of the *sigB* of *S. aureus* and *B. subtilis* (Knobloch *et al*, 2001)

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The accessory sigma factor  $\sigma^B$  serves as a second major mechanism of response to environmental stimuli, generally activated directly within the cell rather than through signal transduction and has been the subject of much interest in *S. aureus* (Chan and Foster, 1998; Gertz *et al*, 1999; Gertz *et al*, 2000; Giachino *et al*, 2001; Rice *et al*, 2004; Senn *et al*, 2005).  $\sigma^B$  is activated by environmental stress and energy depletion (reduced ATP/ADP ratio), as well as by environmental stimuli such as ethanol (Chan and Foster, 1998) and salicylic acid (Bayer *et al*, 2000), and its activity is regulated by a complex post-translational pathway consisting of *rsbU*, *V* and *W* (Scott *et al*, 1999; Novick, 2003)

It has been shown to be involved in the general stress response (Chan *et al*, 1998; Gertz *et al*, 1999; Gertz *et al*, 2000).  $\sigma^B$  also directly or indirectly influences the expression of a variety of genes (Kullik *et al*, 1998b; Gertz *et al*, 2000), including many associated with virulence, such as  $\alpha$ -hemolysin (Giachino *et al*, 2001; Horsburgh *et al*, 2002), clumping factor (Nair *et al*, 2003), coagulase (Miyazaki, 1999), fibronectin-binding protein A (Nair *et al*, 2003), lipases (Kullik *et al*, 1998), proteases (Karlsson and Arvidson, 2002; Horsburgh *et al*, 2002) and thermonuclease (Kullik *et al*, 1998; Ziebandt *et al*, 2001). In addition,  $\sigma^B$  has been shown to influence the expression of several global virulence factor regulators, including SarA (Bischoff *et al.*, 2001), SarS (also known as SarH1)(Stover *et al*, 2000), and RNAIII (Bischoff *et al*, 2001a; Horsburgh *et al*, 2002). However, no effect of  $\sigma^B$  on *S. aureus* pathogenicity has been demonstrated in any *in vivo* model analyzed to date (Chan *et al*, 1998; Nicholas *et al*, 1999; Horsburgh *et al*, 2002). Besides its function in regulating virulence determinants,  $\sigma^B$  is likely to play a role in mediating antibiotic resistance. Inactivation of the gene coding for  $\sigma^B$ , *sigB*, in the homogeneously methicillin-resistant strain COL increases its susceptibility to methicillin (Wu *et al*, 1996) while mutations within the *rsbU*-defective strain BB255, leading to  $\sigma^B$  hyper production, are associated with an increase in glycopeptide resistance (Bischoff and Berger-Bachi, 2001). Moreover,  $\sigma^B$  was shown to affect pigmentation (Giachino *et al*, 2001), to increase resistance to hydrogen peroxide (Kullik *et al*, 1998; Giachino *et al*, 2001) and UV (Giachino *et al*, 2001), and to promote microcolony formation (Bateman *et al*, 2001) and biofilm production (Rachid *et al*, 2000). DNA microarray technology-based analysis of the general stress response in *B. subtilis* identified 127 genes controlled by  $\sigma^B$  (Price *et al*, 2001), and heat shock studies suggest that the  $\sigma^B$  regulon of this organism comprises up to 200 genes (reviewed in references (Hecker and Engelmann, 2000). Because *S. aureus*  $\sigma^B$  seems to be a pleiotropic

regulator that plays a role in a number of clinically relevant processes, a number of investigators have begun characterizing the  $\sigma^B$  regulon. Proteomic approaches have identified 27 *S. aureus* cytoplasmic proteins and one extracellular protein to be under the positive control of  $\sigma^B$ , and 11 proteins were found to be repressed by the factor, indicating that the  $\sigma^B$  regulon of this pathogen is likely to comprise a much higher number of genes than known to date (Bischoff *et al*, 2004). Moreover, previous study by Gertz *et al* had also shown the presence of at least two *clpC*-homologous genes in *S. aureus*, out of which one *clpC*-homologous gene seemed to be controlled solely by  $\sigma^B$  while the other was probably controlled the global regulator of class III general stress genes CtsR (Gertz *et al*, 2000) .

### 1.7 Heat Shock Responses

In recent years, it has become clear that, in addition to the regulation of the expression of specific genes, there are global regulatory systems that control the simultaneous expression of a large number of genes in response to a variety of environmental stresses. The first of these global control systems, and of substantial importance, is the heat-shock response. The heat-shock response is characterized by the induction of a large set of proteins (heat-shock proteins-HSPs) upon shifts to higher temperature and upon exposure to conditions in which proteins are denatured. The heat-shock proteins are important for protection against environmental stress, and they produce tolerance against high temperature, high salt, and heavy metals (VanBogelen *et al*, 1987; Hecker and Volker, 1998). Heat-shock proteins also play critical roles in protective systems such as the human immune system (Christians *et al*, 2002; Li *et al*, 2002). The heat-shock response is universal and many of the heat-shock proteins are highly conserved among species. In bacteria, the heat-shock response has been studied extensively in several Gram-positive bacteria (*Bacillus subtilis*) and in the Gram-negative bacteria (*i.e.*, *Escherichia coli*, *Agrobacterium tumefaciens*).

Heat-shock - a rapid up-shift in the environmental temperature - results in various physical and chemical changes in bacterial proteins and membranes. It is presumed that these changes, such as protein unfolding, are detected by cellular systems, which induce the large set of heat-shock proteins to cope with the changes and the potential damage. This heat-shock response is regulated by several control elements, thus dividing the major stimulus of heat-shock proteins into several regulatory groups (regulons). Most stress-induced proteins are molecular chaperones or proteases, consistent with their role in protein

folding and degradation (Gottesman *et al*, 1997). Several heat-shock proteins are found to protect against damage induced by temperature shift-ups (Rosen and Ron, 2002). In the Gram-positive model organism *Bacillus subtilis*, four different classes of heat shock genes can be distinguished:

- a) **class I genes**, encoding classical chaperones (DnaK, GroES, GroEL), are controlled by the HrcA repressor, which recognizes the highly conserved CIRCE operator sequence (TTAGCACTC-N9-GAGTGCTAA);
- b) **class II genes** encode general stress proteins, and their expression requires the  $\sigma^B$  stress sigma factor;
- c) **class III heat shock genes** are repressed by CtsR (Class three stress gene repressor), which recognizes a tandem heptanucleotide direct repeat (A/GGTCAAANANA/GGTCAAA) and,
- d) **class IV genes** are those that are not controlled by HrcA,  $\sigma^B$  or CtsR (Versteeg *et al*, 2003).

The regulatory mechanisms controlling heat shock induction of the first three classes of stress response genes have been studied in some detail in *B. subtilis*. Activity of  $\sigma^B$ , the alternative  $\sigma$ -factor controlling class II genes, is regulated post-translationally by a complex signal transduction network involving multiple protein-protein interactions and serine/threonine phosphorylation (Price *et al*, 2001; Chastanet *et al*, 2003).

### 1.7.1 HSP100 / Clp ATPases (Class III Heat Shock Genes)

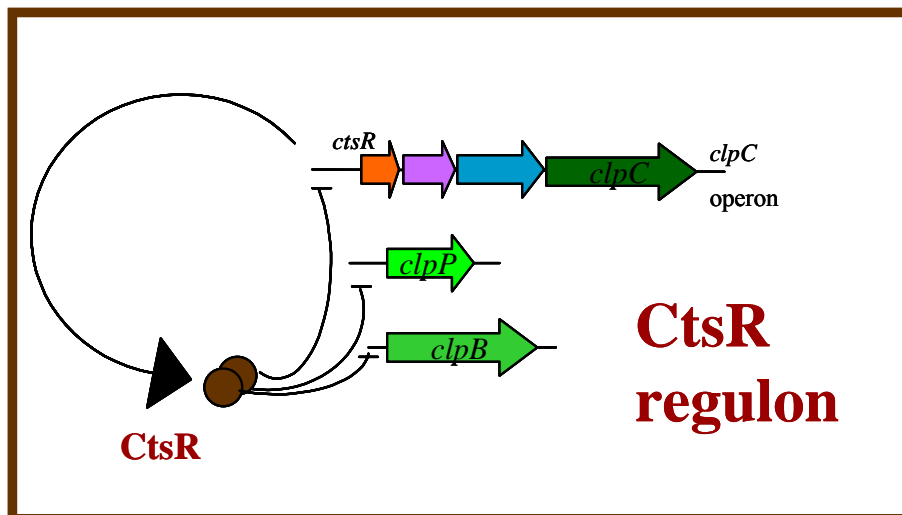
Induction of class III genes is thought to involve targeted degradation of the CtsR repressor by the Clp ATP-dependent protease (Derre *et al*, 2000; Kruger *et al*, 2001). Until recently, surprisingly little was known about stress response regulation in Gram-positive bacteria other than *B. subtilis*, despite the fact that many stress proteins play an important role in virulence, such as ClpX of *S.aureus* (Mei *et al*, 1997), ClpE and ClpC of *Streptococcus pneumoniae* (Polissi *et al*, 1998; Lau *et al*, 2001) or ClpC, ClpE and ClpP of *Listeria monocytogenes* (Rouquette *et al*, 1996; Rouquette *et al*, 1998; Nair *et al*, 1999; Nair *et al*, 2000; Arvidson and Tegmark, 2001; Chastanet *et al*, 2003).

The Hsp100/Clp ATPases constitute a family of closely related proteins that are highly conserved and universal. The Clp ATPase family belongs to the AAA+ superfamily that is characterized by a conserved segment of about 220 amino acids, commonly referred to as the AAA domain, which contains several conserved motifs including those necessary for ATP binding and hydrolysis, the Walker A and Walker B motifs respectively (Neuwald *et al*, 1999). Members of the Clp ATPase family are classified based on the presence of either one or two ATP binding domains (Schirmer *et al*, 1996).

The **class 1 Clp proteins** possess two ATP binding sites, ATP-1 and ATP-2 and are relatively large (ranging from around 70 to 110 kDa). The variable length of the spacer region separating ATP-1 and ATP-2 as well as the occurrence of specific signature sequences is the basis for subdividing into **ClpA**, **ClpB**, **ClpC**, **ClpD**, **ClpE** and **ClpL** families of Clp 1 ATPases. The smaller, **class 2 Clp proteins**, such as **ClpX** and **ClpY**, contain a single ATP binding site with most similarity to ATP-2 (Schirmer *et al*, 1996). A breakthrough in understanding the function of Clp ATPases came when it was shown that ClpA and ClpX have protein reactivating and re-modeling activities typical of molecular chaperones (Wickner *et al*, 1994; Wawrzynow *et al*, 1995). Additionally, ClpA and ClpX can associate with the unrelated ClpP peptidase forming a Clp proteolytic complex, with structural resemblance to the eukaryotic proteasome (Kessel *et al*, 1995). In this proteolytic complex, the ATPase components serve to specifically recognize and subsequently unfold and translocate the substrate into the ClpP proteolytic chamber for degradation (Hoskins *et al*, 1998; Hoskins *et al*, 2000; Arvidson and Tegmark, 2001). Chaperones and ATP-dependent proteases play a major role for bacterial survival under conditions of stress where proteins tend to unfold and aggregate. Hsp104 and HSP70, the yeast ClpB and DnaK

orthologues, respectively, can solubilize protein aggregates during heat shock (Parsell *et al*, 1994), and this ability has been suggested to be a unique property of the Clp/Hsp100 family (Schirmer *et al*, 1996).

The division of heat stress genes into different classes is not mutually exclusive as the example of the Clp proteins shows. They are involved in protein (re)folding and degradation as a result of their nature as HSP100-like ATPases (ClpC, ClpE and ClpX) and as a cognate protease (ClpP) that exerts its degradative function while complexed with an ATPase component (Gottesman *et al*, 1997). ClpC, as part of the tetracistronic *clpC*-operon (*ctsR-mcsA-mcsB-clpC*) (**Fig. 1.6**), ClpE and ClpP exclusively compose class III of the heat stress stimulon, as only their operons are preceded by CtsR recognition motifs. At the same time, *clpP* and the *clpC* operon have a second  $\sigma^B$ -dependent promoter resulting in an overlap between classes III and II. The *clpX* gene seems to be devoid of any known heat stress-specific operator sequences and thus can be assigned to class IV. The Clp proteins are of prime importance for survival during heat stress for major gram-positive bacteria like *Bacillus subtilis*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Streptococcus pyogenes*.



**Fig.1.6.** The CtsR regulon as seen in *S. aureus*. Many of the *clp* genes of *S. aureus* (*clpB*, *clpP*, *clpC*) appear to be controlled by CtrR (reproduced from Chastanet *et al*, 2003)

Irreversibly damaged proteins that cannot be refolded to the native state by the sequential action of chaperones are degraded by stress-inducible ATP-dependent proteases. In several of the pathogenic bacteria, intriguingly, inactivation of *clpP* not only led to stress

sensitivity but also attenuated virulence and reduced expression of major virulence genes of these bacteria. To gain proteolytic activity, ClpP must associate with its Clp ATPase partner that is responsible for substrate recognition. In *B. subtilis*, ClpC and to a lesser extent ClpX seem to target heat-denatured proteins for degradation by ClpP (Kruger *et al.*, 2000). In contrast, ClpC could be deleted in lactococci and streptococci without affecting stress survival (Ingmer *et al.*, 1999; Chastanet *et al.*, 2001). The requirement for Clp proteolytic complexes is paralleled by increased synthesis of the constituents under stress conditions. In several low G+C percentage Gram-positive bacteria, *clp* regulation is mediated by the transcriptional repressor, CtsR, a mechanism that appears to be highly conserved in low G+C percentage Gram-positive bacteria (Derre *et al.*, 1999; Chastanet *et al.*, 2001; Chastanet and Msadek, 2003). However, several additional mechanisms of control for *clp* expression have been characterized in Gram-positive bacteria, such as the alternative sigma factor,  $\sigma^B$  (Gertz *et al.*, 2000), the HrcA repressor in *Streptococcus salivarius* (Chastanet and Msadek, 2003), or the HspR repressor in the high G+C percentage *Streptomyces* bacteria (Grandvalet *et al.*, 1999). Finally, several other *clp* genes have been demonstrated to be heat inducible by unknown mechanisms; an example is *clpX* of *B. subtilis* and *S. aureus* (Gerth *et al.*, 1996; Frees *et al.*, 2004).

In *S. aureus*, several genes encoding Clp ATPase homologues have been identified: *clpC* (SA 0483 according to the *S. aureus* N315 genome designation [(Kuroda *et al.*, 2001)]), *clpB* (SA 0835), *clpY* (SA 1097), *clpX* (SA 1498), and *clpL* (SA 2336). In addition, two genes encoding Clp proteases are present: *clpP* (SA 0723) and *clpQ* (SA 1096). While little is known about the role of ClpCP in *S. aureus*, this complex was shown to play crucial roles in targeting MecA and ComK in *Bacillus subtilis* and thus controlling growth at high temperature, cell division, sporulation, competence for genetic transformation, and degradative-enzyme synthesis (Msadek *et al.*, 1994; Kruger *et al.*, 1997; Turgay *et al.*, 1998). Furthermore, it is a general stress protein required for in vivo survival of *Listeria monocytogenes*, being involved in early bacterial escape from the phagosomes of macrophages (Rouquette *et al.*, 1996; Rouquette *et al.*, 1998). These different roles of ClpCP-mediated proteolysis hint on important regulatory functions in gram-positive bacteria, even under non-stress conditions (eg. post-stationary phase growth). To investigate about the different roles of ClpC ATPase, it was further characterized in a biofilm-forming *Staphylococcus aureus* strain DSM20231 (Becker *et al.*, 2001) and is described in Chapter 3.1, Chapter 3.2 and Chapter 4.1.



### 1.8 *Staphylococcus aureus* & Very Low Ethanol Concentrations (VLEC)

Ethanol is abundant, not only for human consumption, but also in large areas in modern medicine. Alcoholic compounds have numerous applications as stabilizers, solvents and disinfectants. A large variety of therapeutics (typically, liquids for oral application, e.g. cough suppressants, expectorants, oral tranquilizer suspensions) contain ethanol at various concentrations. Furthermore, a number of pharmaceuticals for intravenous (*i.v.*) treatment also contain ethanol at concentrations varying between 1% v/v and 96% v/v.

A variety of invasive devices are required for diagnosis and life maintaining therapy and these include intravascular catheters ('drips'), urinary catheters, cerebrospinal fluid shunt catheters to prevent intracranial pressures increasing to dangerous levels, vascular grafts, etc. Such devices represent a possible entry point for micro-organisms that can cause infection. As an example, antibiotic lock therapy of implanted *i.v.* catheters uses alcohol as an antimicrobial disinfectant (Dannenberg *et al*, 2003) and are widely applied, particularly in paediatrics (Seifert, 2005). Most alcohol-based disinfectants contain ethanol, typically at a concentration at ~70-85% v/v.

Alcoholic disinfectants are recommended for surgical hand disinfection, cutaneous antisepsis before the insertion of catheters. Though its usage results in a high extent of bacterial elimination, small numbers of bacteria still survive on skin. In recent years *Staphylococcus aureus* has been isolated with increasing frequency as a causative pathogen of nosocomial infections, which are often foreign body related. The major pathogenic factor is the ability to form biofilms on polymeric surfaces.

Ethanol may also be used for food preservation (Seiler and Russel, 1991). Given the large range of ethanol concentrations in the different preparations, and considering wash-out, dilution, and evaporation, the actual concentrations *in situ* are anticipated to be more diverse and would include very low concentrations of ethanol (VLEC); hence, a fundamental understanding of the effects of VLEC on bacterial physiology is important.

Most important for the field of microbiology in research and diagnostics is the fact that ethanol or related alcohols are routinely used in medical microbiology for *in vitro* testing as a solvent: According to NCCLS guidelines (NCCLS, 2002), 95% ethanol or

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methanol is recommended to dissolve various macrolides, chloramphenicol, and rifampin. The final concentration of ethanol in the medium depends of the concentration of antimicrobial selected; for instance, a solution containing 10 µg/mL of the respective antimicrobial also contains 0.1% v/v ethanol. Furthermore, bacterial genetic research employing erythromycin resistance as a marker for selection typically employs final concentrations of 10 µg/mL erythromycin, *i.e.* a solution containing 0.1% v/v ethanol.

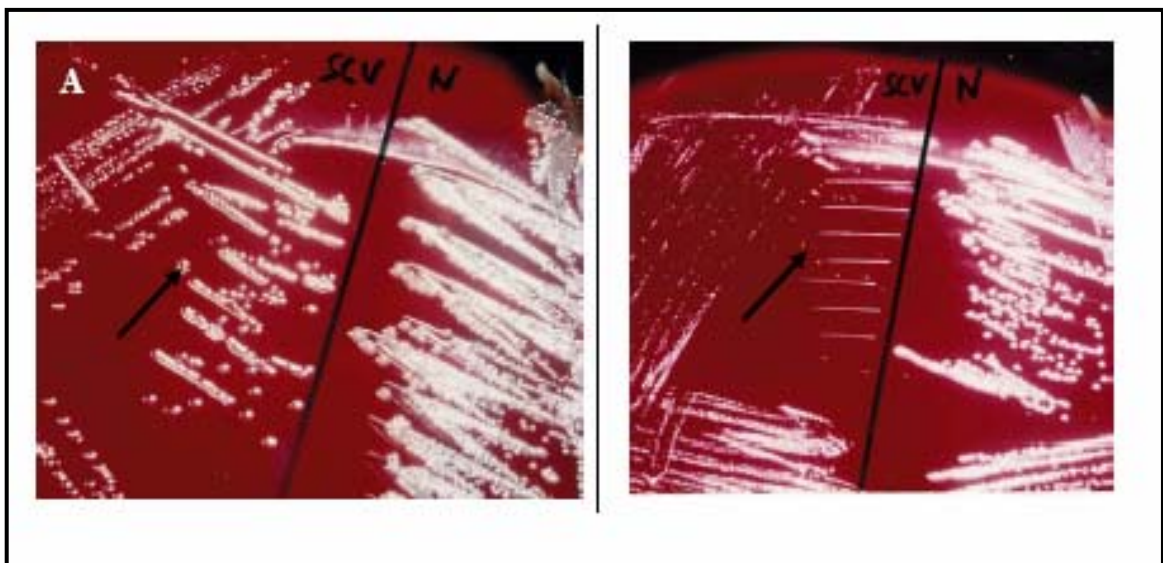
The bactericidal activity of ethanol is due to several factors: disruption of membrane structure or function (Fried and Novick, 1973; Haleboua and Inouye, 1979; Barker and Park, 2001; Silveira *et al*, 2004); interference with cell division, affecting steady-state growth (Fried and Novick, 1973); variations in fatty acid composition and protein synthesis (Chiou *et al*, 2004); inhibition of nutrient transport via membrane-bound ATPases (Bowles and Ellefson, 1985); alteration of membrane  $\Delta\text{pH}$  (Bowles and Ellefson, 1985; Terracciano and Kashket, 1986) and  $\Delta\psi$  (Terracciano and Kashket, 1986); and decrease of intracellular pH (Bowles and Ellefson, 1985; Huang *et al*, 1986; Terracciano and Kashket, 1986). In a recent study with a gram-positive *Bacillus subtilis*, it was demonstrated that treatment with subinhibitory concentrations of ethanol (not affecting vegetative growth) inhibited the initiation of spore development through a selective blockage of key developmental genes under the control of the master transcription factor Spo0A~P (Gottig *et al*, 2005). These toxic effects have been described for a wide variety of microbial species, and for use of different concentrations of ethanol, ranging from 2.5% to 70% (Lacy, 1968; Dombek and Ingram, 1984; Ingram and Buttke, 1984; Basu and Poddar, 1994; Barker and Park, 2001; Chiou *et al*, 2004; Silveira *et al*, 2004). Surprisingly, very little is known about the physiological effects of VLEC.

Therefore, the purpose of our part of this thesis work was to determine the effects VLEC on medically important staphylococci at a concentration frequently encountered in the hospital and laboratory. In *Chapter 3.3* and *Chapter 4.2*, we report major effects of VLEC on *S. aureus* cell integrity, survival and growth recovery, and describe the effects of VLEC on metabolism and transcription of select staphylococcal genes.

## 1.9 *Staphylococcus aureus* & thymidine-dependent small colony variants (Thy-SCV)

The persistence of *S. aureus* in CF and other persistent infections such as osteomyelitis and device-related infections has been associated with the isolation of *S. aureus* small-colony variants (SCVs) (Gilligan *et al*, 1987; von Eiff *et al*, 1997). In contrast to the normal *S. aureus* phenotype, SCVs grow as tiny, non-pigmented, and non-hemolytic colonies. SCVs

- produce greatly reduced amounts of  $\alpha$ -hemolysin;  
persist within host cells of *in vitro* assays (von Eiff *et al*, 2001);
- are auxotrophic for substrates such as menadione, hemin, thiamine, or thymidine (**Fig. 1.8**) and,
- can revert to their normal phenotype (Balwit *et al*, 1994).



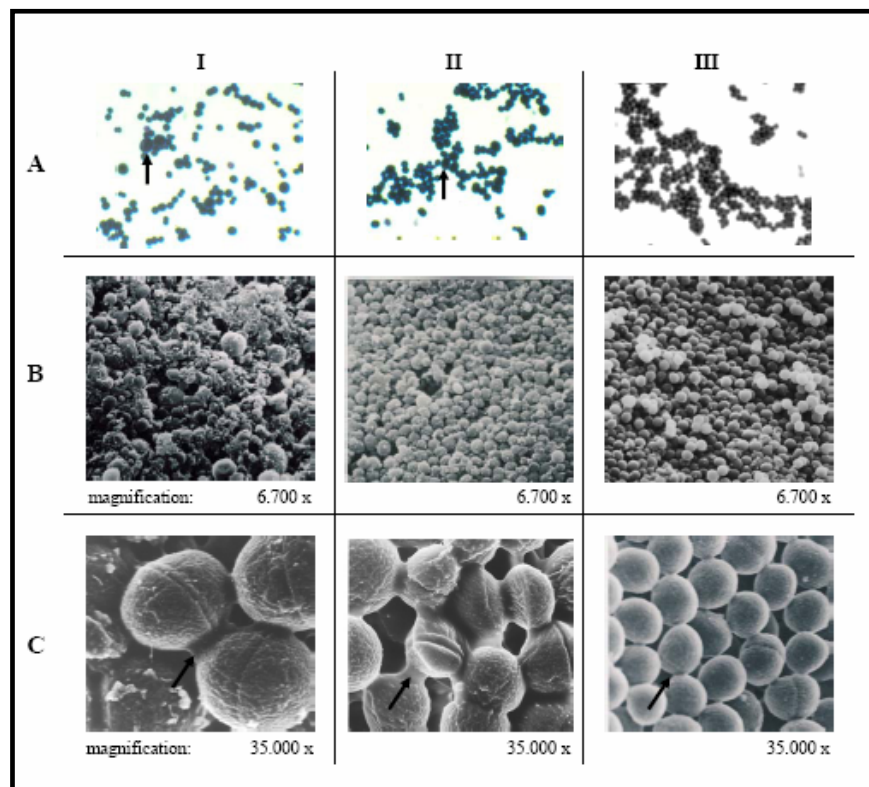
**Fig.1.8** Growth of thymidine-dependent SCVs with fried-egg appearance and with pinpoint colonies on Columbia blood agar. (A) Fried-egg thymidine-dependent SCVs (left, arrow) in comparison to the isogenic normal *S. aureus* (right, N). (B) Pinpoint SCVs (left, arrow) in comparison to the isogenic normal *S. aureus* (right, N). All strains were grown overnight at 37°C. (reproduced from Kahl *et al*, 2003a).

In a 6-year prospective study, persistence of clonal SCVs and/or normal *S. aureus* has been shown in the airways of CF patients over extended periods, as disclosed by pulsed-field gel electrophoresis. From this study, 212 SCVs were available for auxotrophism testing. A total of 122 SCVs were thymidine dependent, and 26 SCVs were both thymidine and hemin dependent. As a result of long-term trimethoprim-sulfamethoxazole (SXT)

treatment, SXT-resistant and thymidine-dependent SCVs emerged, while the corresponding normal strains remained SXT susceptible (Kahl *et al*, 1998). While SXT interferes with the tetrahydrofolic acid pathway, tetrahydrofolic acid acts as a coenzyme for thymidylate synthetase, which catalyzes the synthesis of dTMP from dUMP (Stryer, 1995). Since dTMP is essential for DNA synthesis, susceptible *S. aureus* strains are affected by SXT therapy. However, thymidine-dependent SCVs are resistant to SXT and survive if extracellular thymidine is provided.

Phenotypically, thymidine-dependent SCVs display two different colony types,

- (a) fried-egg SCVs with translucent edges surrounding a smaller, elevated pigmented center and,
- (b) pin-point colonies, which are nearly 10 times smaller than the normal *S. aureus* colony (**Fig. 1.9**; Kahl *et al*, 2005).



**Fig.1.9.** Gram staining and SEM of fried-egg SCVs (panels I), pinpoint SCVs (panels II), and normal *S. aureus* (panels III). (A) Gram staining. Arrows indicate large cocci. (B and C) Low- and high-magnification SEM, respectively. Arrows indicate the enhanced intercellular substance present in SCVs compared to normal *S. aureus* (reproduced from Kahl *et al*, 2003a).

## 1. Introduction

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To investigate if differential gene expression is one of the many factors leading to the thymidine-dependent SCV phenotype, a study was performed presented in *Chapter 3.4* and *Chapter 4.3*.

## **Chapter 2**

# **EXPERIMENTAL PROCEDURES**

## 2. Experimental procedures

### 2.1 Bacterial strains and growth conditions

#### 2.1.1 Aerobic growth conditions

The parental strain used in the study in *Chapter 3.1* and *Chapter 3.2* was *Staphylococcus aureus* DSM20231 (DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; = ATCC 12600), which is a standard laboratory type strain (Silvestri and Hill, 1965). For starvation survival studies, *S. aureus* strains were grown in Brain-Heart Infusion (BHI; Oxoid) medium or on Mueller-Hinton containing 1.5 % agar (MHA). All bacterial cultures were inoculated from an overnight culture and diluted to an OD<sub>600</sub> = 0.1 into BHI incubated at 37°C. For generation of aerobic growth condition, Erlenmeyer flasks (1 L) were incubated with a flask-to-medium ratio of 10:1 and shaken at 230 rpm. Aliquots were removed at indicated time points, and bacterial growth or CFU were determined. Bacterial growth was assessed by measuring the optical density at 600 nm. For stress tolerance studies, *S. aureus* strains were grown in tryptic soy broth (TSB; Oxoid) under aerobic growth conditions or on tryptic soy broth containing 1.5 % agar (TSA) incubated at 37°C. All strains used in *Chapter 3.1*, *Chapter 3.2* and *Chapter 3.3* are summarized in **Table 1A**.

**Table 1A.** Table showing list of bacterial strains used.

Strains	Genotype or relevant characteristics	Reference(s) or source
<i>S. aureus</i> strains		
DSM20231	Wild-type standard laboratory strain (DSMZ )	(Silvestri and Hill, 1965)
SH1000	8325-4, <i>rsbU</i> <sup>+</sup>	(Horsburgh <i>et al.</i> , 2002)
PBM001	DSM20231, <i>clpC::ermB</i>	This study
HCH001	SH1000, <i>clpC::ermB</i>	This study
MGM001	PBM001, (pCX19clpC)	This study
GP266	RN4220, <i>rsbU</i> <sup>+</sup> <i>sigBI</i> (Am) Tc <sup>r</sup>	(Bischoff <i>et al.</i> , 2001)
MB290	DSM20231, <i>sigBI</i> (Am) Tc <sup>r</sup>	This study
MB288	PBM001, <i>sigBI</i> (Am) Erm <sup>r</sup> Tc <sup>r</sup>	This study
SA133	Restriction-negative mutant of <i>S.aureus</i> 8325-4	(Iordanescu and Surdeanu,

## 2. Experimental Procedures

		1976)
<i>S. carnosus</i> TM300	Invasion-negative control strain	((Schleifer and Fischer, 1982)

### 2.1.2 Microaerobic growth conditions

*Staphylococcus aureus* DSM20231 (Silvestri and Hill, 1965) (from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [DSMZ], Braunschweig, Germany) (ATCC12600; Cowan serotype 3) was used throughout the study in *Chapter 3.3*. In select experiments, *S. aureus* strain SH1000 was used (Horsburgh *et al*, 2002). Strains were grown in brain-heart infusion (BHI; Oxoid) medium or on Mueller-Hinton containing 1.5 % agar. All bacterial cultures were inoculated from an overnight culture and diluted to an OD<sub>600</sub> = 0.1 into BHI incubated at 37°C. For generation of microaerobic growth condition, Erlenmeyer flasks (100 ml) were incubated with a flask-to-medium ratio of 2:1 and shaken at 150 rpm. For generation of aerobic growth conditions, Erlenmeyer flasks (1L) were incubated with a flask-to-medium ratio of 10:1 and shaken at 230 rpm. In a part of experiments, sterile-filtered ethanol was added to the BHI media to a final concentration of 0.1% (vol/vol). For these experiments employing ethanol, this concentration of was selected because it represents a concentration frequently employed *in vitro* bacterial genetic research (see *Chapter 1.8*). These media conditions were designated as “very low ethanol concentration-positive” (VLEC+) and compared with unsupplemented media (VLEC-). In the respective sections of this work describing effect of ethanol (*Chapter 3.3*), these descriptions are used according to the medium supplementation conditions established upon starting the cultures; as ethanol is rapidly metabolized or dissipates (see results), the designation VLEC+ also applies to conditions encountered during later stages of growth without detectable ethanol concentrations in the media. Aliquots (100 µl) were removed at indicated time points and cell densities, and the pH of the culture medium and CFU were determined. For L-amino acid supplementation experiments, L-amino acid (asparagine, citrulline, glutamine, glutamic acid, glycine, methionine, ornithine, proline, serine and valine) stock solutions were added to BHI media at a final concentration of 2 mM; L-arginine was supplemented at either 2 mM or 5 mM. Bacterial growth was assessed by measuring the optical density at 600 nm.



### 2.1.3 Growth conditions of small colony variants

Three normal and *S. aureus* SCV strain pairs previously described were used in *Chapter 3.4* (Kahl *et al.*, 2003b). These strain pairs were cultured from individual CF patients persistently infected by *S. aureus*. All SCVs were thymidine dependent and clonal to their normal counterpart, as determined by pulsed-field gel electrophoresis as described previously (Kahl *et al.*, 1998). To ensure stability of the SCV phenotype, the strain pairs were sub-cultured at least 10 times in the laboratory.

Normal *S. aureus* isolates and SCVs were grown in brain heart infusion (BHI; Merck, Darmstadt, Germany) broth, which supports the growth of SCVs. For the growth curve analysis, cultures were grown at 37°C on a rotary shaker at 160 rpm. For the determination of the optical density at 600 nm beyond a value of 1, the value of the ODs was calculated from readings of diluted cultures. For supplementation studies, thymidine (Fluka Chemie, Buchs, Switzerland) was added to BHI broth at a concentration of 100µg/ml.

## 2.2 DNA manipulations.

DNA manipulations, DNA sequencing, PCR reactions and plasmid isolation were performed using standard procedures (Sambrook and Russel, 2001) or according to manufacturers instruction.

## 2.3 Construction of *S. aureus clpC* mutant

### 2.3.1 Construction of *S. aureus clpC* mutant (PBM001) (using molecular cloning)

This work was done by P. Becker, Münster, Germany. A 988-bp fragment (nucleotide -215 to +773 relative to the *clpC* start codon) was amplified from chromosomal DNA of *S. aureus* DSM20231 by PCR with primers (*MclpCForw* and *MclpCRev*; see **Table. 1B**) that contained restriction sites for *BamHI*, and was cloned into pCRII (Invitrogen, USA) to generate the plasmid pClpC. The 1467-bp *ermB* cassette of pEC4 (Bruckner, 1997) was amplified using primers *ermBFor* and *ermBRev* (**Table. 1B**) and was inserted into the blunt ended *ClaI* fragment contained within the *clpC* fragment of pClpC to yield pClpCII. The *clpC-ermB-clpC* fragment was isolated from pClpCII as a 2455-bp *BamHI-BamHI* fragment, and cloned into the temperature-sensitive shuttle vector pBT2 (Bruckner, 1997) to generate the plasmid pBclpC. The plasmid pBclpC was propagated in strain SA113 (Iordanescu and

## 2. Experimental Procedures

and Surdeanu, 1976) then transferred to *S.aureus* DSM20231 by protoplast transformation (Götz and Schumacher, 1987). This was followed by integration of the *ermB* gene into the *S. aureus* chromosome and was selected for by a temperature shift (Palma *et al*, 1996). For verification, the PCR product of the *clpC* mutant was analyzed with primers *MclpC*Forw and *clpC2* (Table. 1B) and was 1.47-kb larger than the product of the wild type strain *S. aureus* DSM20231. Pulsed field gel electrophoresis showed similar bands for both WT and PBM001 and no deviation in the band pattern could be observed due to insertion of the *ermB* cassette. Finally, all ID 32 Staph-Systems obtained reaction results identical for both the strains.

**Table. 1B.** Nucleotide sequences of various primers used in this thesis work.

Primers	Nucleotide sequence	Function/Reference
<i>acsA</i> fl	5'-AAGATATGCAACGGTTATCTAATAAAGCA-3'	Real time RT-PCR, Chapter 3.1
<i>acsA</i> r1	5'-AGGTGTACGCGACATAAAATAAAACTCT-3'	Real time RT-PCR, Chapter 3.1
<i>adhE</i> fl	5'-CACAAAGGTATTGCATTAGTTCTAGCA-3'	Real time RT-PCR, Chapter 3.3
<i>adhE</i> r1	5'-CGTTACCTGGTCCCACACCTA-3'	Real time RT-PCR, Chapter 3.3
<i>ahpC</i> fl	5'-CGTAGTATGCTTCTATCCTGCTGACTT-3'	Real time RT-PCR, Chapter 3.2
<i>ahpC</i> r1	5'-CATTTACGCCTAATTTTTGTAATTCCT-3'	Real time RT-PCR, Chapter 3.2
<i>ahpF</i> fl	5'-GTACTGGTATAGTTGCTGATCGTATCG-3'	Real time RT-PCR, Chapter 3.2
<i>ahpF</i> r1	5'-AAGTTAGAAGAAAATTCAGAACCAGTTGT-3'	Real time RT-PCR, Chapter 3.2
<i>arcA</i> fl	5'-CTTGGCTATAGGCGTTTCAGAAC-3'	Real time RT-PCR, Chapter 3.2, 3.3, 3.4
<i>arcA</i> r1	5'-GTCGCCTGCGGATTTTCA-3'	Real time RT-PCR, Chapter 3.2, 3.3, 3.4
<i>argF</i> fl	5'-CTCCGAGGATTTAAAACGAGCTAA-3'	Real time RT-PCR, Chapter 3.2
<i>argF</i> r1	5'-ATGCACATCGCGTTCCTGTAGA-3'	Real time RT-PCR, Chapter 3.2
<i>asp23</i> fl	5'-CAAGAACAATAAAGAGCCTCAAT-3'	Real time RT-PCR, Chapter 3.1
<i>asp23</i> r1	5'-CTTACGTCAGCGATACCA-3'	Real time RT-PCR, Chapter 3.1
<i>atl</i> fl	5'-GCATATTTAGCTGTACCTGCTGCAC-3'	Real time RT-PCR, Chapter 3.4
<i>atl</i> r1	5'-AGTCGTTTGTGGTTTAGTAACAGTATAAGC-3'	Real time RT-PCR, Chapter 3.4
<i>cap5A</i> fl	5'-TAGATGAGGTGTCAAAGGACTTAAATGATA-3'	Real time RT-PCR, Chapter 3.4
<i>cap5A</i> r1	5'-AGTTGCGTATTTTCTTGGTTTGTAAAT-3'	Real time RT-PCR, Chapter 3.4
<i>cap8C</i> fl	5'-TAACATCACATCACTTACATCCTCGATA-3'	Real time RT-PCR, Chapter 3.4
<i>cap8C</i> r1	5'-GTGCTTGTACTTCTCTAAGCTTTCA-3'	Real time RT-PCR, Chapter 3.4
<i>cidA</i> fl	5'-TCGCAGTCATTATCATAGGAACATGT-3'	Real time RT-PCR, Chapter 3.2
<i>cidA</i> r1	5'-AAGCGTCTACACCTTTACGATGTTTAT-3'	Real time RT-PCR, Chapter 3.2
<i>citB</i> fl	5'-CATTACCACAAGGCGCAACA-3'	Real time RT-PCR, Chapter 3.1, 3.4
<i>citB</i> r1	5'-GAACTCCACAAATTTACCAACAACA-3'	Real time RT-PCR, Chapter 3.1, 3.4
<i>clpB</i> fl	5'-AGTAGCAGTTAGTGAGCCTGATG-3'	Real time RT-PCR, Chapter 3.1, 3.2
<i>clpB</i> r1	5'-TCTATCTTGAATACGCACACCATG-3'	Real time RT-PCR, Chapter 3.1, 3.2
<i>clpC</i> fl	5'-GAAGAAGCAATTCGTTTAAATCATTCA-3'	Real time RT-PCR, Chapter 3.1, 3.4
<i>clpC</i> r1	5'-CTTTCTAATACTTTTGCAGCAATTCCTT-3'	Real time RT-PCR, Chapter 3.1, 3.4
<i>clpC2</i>	5'-ATATGGTACCTGGACTGTTTTATATTATGCTTGC-3'	Molecular cloning, Chapter 3.1
<i>clpP</i> fl	5'-TGACAACGTAGCAAATTCATCGTAT-3'	Real time RT-PCR, Chapter 3.1, 3.2
<i>clpP</i> r1	5'-CACTTCCACCTGGTGAATTAATGTAT-3'	Real time RT-PCR, Chapter 3.1, 3.2
<i>ddh</i> fl	5'-GGTACGCGTGATTATGAGAAAGAGAT-3'	Real time RT-PCR, Chapter 3.2
<i>ddh</i> r1	5'-TTTAATTGATCGACTGTAGCACTTGA-3'	Real time RT-PCR, Chapter 3.2
<i>dfrA</i> fl	5'-CCGAATCGTCGAAATGTTGTAAT-3'	Real time RT-PCR, Chapter 3.4
<i>dfrA</i> r1	5'-TGGCCCGGTAGTTGGTAAATATC-3'	Real time RT-PCR, Chapter 3.4
<i>ermB</i> For	5'-ATGAACAAAAATATAAAATATTCTCAAAAC-3'	Molecular cloning, Chapter 3.1
<i>ermB</i> Rev	5'-TTATTTCCTCCTCCGTTAAATAATAGATAAC-3'	Molecular cloning, Chapter 3.1

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<i>fnbA</i> fl	5'-CCTCTGAGGAATCAAATCCAATTG-3'	Real time RT-PCR, Chapter 3.4
<i>fnbA</i> rl	5'-CAACATCAGCGTGATGTTTTGAAT-3'	Real time RT-PCR, Chapter 3.4
<i>gudB</i> fl	5'-CGTGATAGTTTTGGTACGGTAAACAAAT-3'	Real time RT-PCR, Chapter 3.2
<i>gudB</i> rl	5'-GAAATAGCCGCTGGTACTAAAATGTC-3'	Real time RT-PCR, Chapter 3.2
<i>gyrB</i> fl	5'-GACTGATGCCGATGTGGA-3'	Real time RT-PCR, Internal control
<i>gyrB</i> rl	5'-AACGGTGGCTGTGCAATA-3'	Real time RT-PCR, Internal control
<i>icaA</i> fl	5'-TGCTGGCGCAGTCAATACTA-3'	Real time RT-PCR, Chapter 3.4
<i>icaA</i> rl	5'-GACCTCCCAATGTTTCTGGA-3'	Real time RT-PCR, Chapter 3.4
<i>ilvA</i> fl	5'-TTATTCGTGCGAACACCACTAATTAATC-3'	Real time RT-PCR, Chapter 3.2
<i>ilvA</i> rl	5'-TGAACCTGTAAACTGCATATTTTCAAGT-3'	Real time RT-PCR, Chapter 3.2
<i>KclpC1A</i>	5'-ATATGGATCCAGTAGGAGGTCATTATTTATGTTATT-3'	Molecular cloning, Chapter 3.1
<i>KclpC2Sma</i>	5'-ATATCCCGGGTGGACTGTTTTATAATTATGCTTGC-3'	Molecular cloning, Chapter 3.1
<i>lacD</i> fl	5'-ATGCACGTAACAAAGATTGTGGATTA-3'	Real time RT-PCR, Chapter 3.2
<i>lacD</i> rl	5'-AACGTTTTGCAGACCACTCTACTAAA-3'	Real time RT-PCR, Chapter 3.2
<i>lrgA</i> fl	5'-ATAACATTGCGTTACTCTTCGTACCA -3'	Real time RT-PCR, Chapter 3.2
<i>lrgA</i> rl	5'-TTGTTGAGACGATTATTAGTCCAATGA-3'	Real time RT-PCR, Chapter 3.2
<i>MclpC2Rev</i>	5'-ATATGGATCCCAAATTCACCACGATATTTAGTACC-3'	Molecular cloning, Chapter 3.1
<i>MclpCForw</i>	5'-ATATGGATCCTGTAGAATGATAACTATGGAAGAGG-3'	Molecular cloning, Chapter 3.1
<i>msrA</i> fl	5'-AGGTACGAGTGGTCATTTAGAAACTGTT-3'	Real time RT-PCR, Chapter 3.2
<i>msrA</i> rl	5'-TGTATCTAATGGGTCAATGACTGAGAA-3'	Real time RT-PCR, Chapter 3.2
<i>murA</i> fl	5'-CATCTTTATTAGCTTCTGATAAACCGAGT-3'	Real time RT-PCR, Chapter 3.2
<i>murA</i> rl	5'-TGTATGTAACGTCAGCATTAAAGTTGTT-3'	Real time RT-PCR, Chapter 3.2
<i>nifZ</i> fl	5'-TCCGTCCGTATTAGAGGTTGTAAGATA-3'	Real time RT-PCR, Chapter 3.2
<i>nifZ</i> rl	5'-TTCTTTGAAGTGTCTAAGTTAATACTGCC-3'	Real time RT-PCR, Chapter 3.2
<i>nupC</i> fl	5'-ACAGGAAAAATATTCGCTGGAAATAT-3'	Real time RT-PCR, Chapter 3.4
<i>nupC</i> rl	5'-CCAATAACCTTGATTCCCATTGTAG-3'	Real time RT-PCR, Chapter 3.4
<i>odhB</i> fl	5'-CAGAAAGAAAACAGCTGCCAAAA-3'	Real time RT-PCR, Chapter 3.2
<i>odhB</i> rl	5'-GCAATTCATAACATTTGTCATGTC-3'	Real time RT-PCR, Chapter 3.2
<i>relA</i> fl	5'-CGGATTACCATAACATTATGCATCCT-3'	Real time RT-PCR, Chapter 3.4
<i>relA</i> rl	5'-TCAATTACATCATGCAAAAAACCTG-3'	Real time RT-PCR, Chapter 3.4
<i>SA0774</i> fl	5'-TGGGACACCCAAGCTATGAAGTA-3'	Real time RT-PCR, Chapter 3.2
<i>SA0774</i> rl	5'-TGCATTGCCAAGAATAATCCTG-3'	Real time RT-PCR, Chapter 3.2
<i>SA1272</i> fl	5'-GCAGAATTATTAGCGCCAATGA-3'	Real time RT-PCR, Chapter 3.2
<i>SA1272</i> rl	5'-ACTAAAGTACCTTGACCACCATGTTG-3'	Real time RT-PCR, Chapter 3.2
<i>sulA</i> fl	5'-GTCAACAATGGTATTCATGGATTCATA-3'	Real time RT-PCR, Chapter 3.2
<i>sulA</i> rl	5'-GGTATAGGTGCAGTTAAATTAACGGAC-3'	Real time RT-PCR, Chapter 3.2
<i>thyA</i> fl	5'-CGCTTTGACTTATCTAAAGGATTTCC-3'	Real time RT-PCR, Chapter 3.4
<i>thyA</i> rl	5'-GTATCTCCTTTAATGAACCATAATAATTCG-3'	Real time RT-PCR, Chapter 3.4
<i>tyrS</i> fl	5'-AATGCGTCGTATGTATGGTCAAAAC-3'	Real time RT-PCR, Chapter 3.2
<i>tyrS</i> rl	5'-CAGCACCTGACTCAGACTTACCAA-3'	Real time RT-PCR, Chapter 3.2

### 2.3.2 Construction of *S. aureus clpC* mutant (HCH001) (using transduction)

#### ■ Preparation of Staphylococcal phage lysate

*S. aureus* DSM20231 (donor strain) was grown in 20ml BHI medium in 100ml flask o/n with shaking (230 rpm) at 37°C. Next morning, 1M CaCl<sub>2</sub> was added to the bacterial culture to an end concentration of 5mM CaCl<sub>2</sub> (for a 20 ml culture, 100 µl CaCl<sub>2</sub> of the stock solution). For preparing phage buffer, 1 ml LB was transferred to a 1.5 ml tube and 5µl, 1M CaCl<sub>2</sub> was added. The transducing phage lysate was diluted in phage buffer (10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup>, 10<sup>-4</sup>) (20 µl phage, 180 µl phage buffer). Next, 300 µl of the bacterial culture was transferred

## 2. Experimental Procedures

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to 1.5 ml tube (when using non-isogenic strain, to be heated for 2 min at 52°C in order to inactivate restriction system) and 100µl of phage lysate was added to each tube. This mixture was incubated at room temperature for 15 min to allow adsorption of the phage. The bacteria/phage mixture was then transferred to the pre-warmed glass tube (55°C). 20 ml of warm LB softy (LB + 0.6 % agar) was transferred to 50 ml falcon tube and 100 µl CaCl<sub>2</sub> was added. 4 ml of this warm LB softy was added to each falcon tube, mixed and poured over blood agar plate. The plates were incubated o/n at 30°C (when using phage 85, incubate at 37°C). Next morning, the first plate with confluent lysis (*the blood agar should be brown to indicate that the bacteria could grow and the softy layer should be transparent because all the bacteria were lysed*) was taken. The softy was overlaid with 2 ml LB and 5 mM CaCl<sub>2</sub>. The softy layer was scraped off with a bent Pasteur pipette into a plastic tube and was briefly vortexed. The tube was centrifuged at 9000 rpm for 10 min to remove bacterial debris and agar. The supernatant was taken and filtered through a 0.45 µm Millipore filter. The phage lysate was stored at 4°C.

### ▪ Transduction

*S. aureus* SH1000 (recipient strain) was grown in 20 ml BHI medium with shaking (230 rpm) at 37°C o/n. Cells were collected by centrifuging at 5000 x g for 10 min and then re-suspended in 1 ml of BHI. 500 µl of the cell suspension was added to 500 µl of LB (Sambrook *et al.*, 2001) with 5 mM CaCl<sub>2</sub>. To this, 500µl of the previously prepared phage lysate was added and incubated with shaking (150 rpm) at 37°C for 20 min. 1 ml ice-cold 20mM sodium citrate was added. Cells were then centrifuged (5000 x g ; 5 min) and re-suspended in 1 ml 20mM sodium citrate. 100µl of the cell suspension was spread on BHI-agar plates containing 20 mM sodium citrate and selective antibiotics. Plates were then incubated at 37°C for 24 h - 36 h. Single-colony-purify transductants twice on BHI-agar plates with CaCl<sub>2</sub> to eliminate contaminating phage. Finally, transductants were checked to determine if they have become lysogenic for transducing phage.

### **2.4 Construction of the *S. aureus sigB* mutant (MB290) and the *S. aureus clpC sigB* mutant (MB288)**

This work was done by M. Bischoff, Zürich, Switzerland. Strains MB290 and MB288 were obtained by phage 80α- 15 mediated transduction of the *tet(L)*-tagged *sigBI*(Am)

mutation of GP266 (Bischoff and Berger-Bachi, 2001) into DSM20231 or PBM001, respectively, selecting for tetracycline resistance.

### 2.5 Complementation of the *S. aureus clpC*-mutant (MGM001)

This work was done by M. Grundmeier, Münster, Germany. In brief to create a *clpC* expression vector for complementation, the *clpC* gene from *S. aureus* DSM20231 was amplified by PCR. Additional restriction sites with primers *KclpC1A* (*Bam*HI) and *KclpC2Sma* (*Sma*I) (**Table. 1B**) were introduced. The vector part was derived from the expression vector pCX19 (Hussain *et al*, 2001) by excision of the lipase gene with *Bam*HI and *Sma*I. The restricted PCR-product was ligated into the restricted pCX19 vector. The restriction negative strain *S. carnosus* TM300 (Schleifer and Fischer, 1982) was transformed with this ligation product (pCX19*clpC*) by protoplast transformation (Götz and Schumacher, 1987). After analyzing the expression vector pCX19*clpC* by sequencing, the *clpC*-mutant (PBM001) was transformed with pCX19*clpC* by electroporation resulting in the transformant MGM001. Transformants were verified by plasmid preparation and subsequent restriction.

### 2.6 Real-time RT-PCR

For RNA isolation from culture, *S. aureus* was grown in BHI medium to the desired growth phase (2, 6, 16, 48, 72, and 96 h). Bacteria were mechanically disrupted (Fast Prep FP120 instrument, Qbiogene, Heidelberg, Germany), and RNA isolated (RNeasy mini kit, Qiagen, Hilden, Germany). After treatment with RNase-free DNase I (Qiagen), total RNA samples were amplified in an ABI PRISM<sup>®</sup> 7000 SDS using SYBR<sup>®</sup> Green PCR Master Mix (Applied Biosystems, Weiterstadt, Germany) and *gyrB* primers (**Table. 1B**) to check for absence of gDNA. Previously transcribed cDNA served as a positive control. RNA was then reversely transcribed (High Capacity cDNA Archive Kit, Applied Biosystems). cDNA was used for real-time amplification with specific primers (**Table. 1B**) and 100 ng of cDNA per reaction. The level of mRNA expression of the different genes was normalized against the internal control *gyrB* expression which is constitutively expressed (Wolz *et al*, 2002). The amount of different transcripts was expressed as the n-fold difference relative to the control gene ( $2^{-\Delta C_T}$ , where  $\Delta C_T$  represents the difference in threshold cycle between the target and control genes).

### 2.7 Determination of stationary-phase survival

Single colonies of *S. aureus* strains were inoculated into 1-liter flasks containing 100 ml of BHI, grown at 37°C, and aerated by shaking them at 230 rpm for up to 1 week. Aliquots (200 µl) were taken at different time intervals (23, 42, 48, 65, 70, 96, 120, 144 and 168 h), and the CFU per milliliter were determined on MHA plates.

### 2.8 Determination of glucose, acetate and other metabolites in cultural supernatants

Aliquots of bacteria (2 ml) were centrifuged for 5 min at 21,000×g at 4 °C at the indicated time points. The culture supernatant were removed and adjusted to pH 8 with KOH. The concentrations of glucose, acetate, ammonia, ethanol, and lactate were determined with kits purchased from R-Biopharm AG (Darmstadt, Germany). The concentrations of free amino acids were determined with a Beckman amino acid analyser by aminoNova AG (Berlin, Germany). Assays were performed as described previously (Somerville *et al*, 2002).

### 2.9 Aconitase activity assay

The assay was performed as previously described with slight modifications (Kennedy *et al*, 1983). Briefly, aliquots (4 ml) were harvested at the different time points (24, 48, 72, and 96 h) and resuspended in 1.5 ml of ACN buffer containing 90 mM Tris (pH 8.0) and 100 µM fluorocitrate. Bacteria were mechanically disrupted (Fast Prep) as stated above. The lysate was centrifuged for 5 min at 21,000 × g at 4°C. Aconitase activity was assayed in the resulting cell-free lysate by the method described previously (Kennedy *et al*, 1983). One unit of aconitase activity is defined as the amount of enzyme necessary to yield a  $\Delta A_{240} \text{ min}^{-1}$  of 0.0033 (Baughn and Malamy, 2002). Protein concentrations were determined by the method of Lowry (Lowry *et al*, 1951).

### 2.10 Glutamate dehydrogenase activity assay

The assay was performed as previously described with slight modifications (Lu and Abdelal, 2001). Briefly, aliquots (4 ml) were harvested at the different time points (2, 7, 9, 11, 19, 23, and 28 h) and resuspended in 1.5 ml of GDH-buffer containing 20 mM HEPES (pH 8.2), 100 mM sodium chloride and 1 mM EDTA. Bacteria were mechanically disrupted (Fast Prep 120) at a speed of 6.0 for 20 secs. The lysate was centrifuged for 5 min at 20,000 × g at

4°C. The deamination activity of NAD-glutamate dehydrogenase was determined at room temperature by monitoring the absorbance at 340 nm in a Helios (Unicam) spectrophotometer. The reaction mixture (2 ml) contained 100 mM HEPES (pH 8.2), 2.5 mM NAD<sup>+</sup>, and 50 mM L-glutamate unless otherwise indicated. The amination activity of NAD-glutamate dehydrogenase was measured by monitoring the increase in absorbance at 340 nm. The reaction was initiated by the addition of enzyme. One enzyme unit catalyses the oxidation of 1 μmol of NAD coenzyme per minute per mg of protein to NADH. Protein concentrations were determined by the method of Lowry (Lowry *et al.*, 1951) with bovine serum albumin as the standard.

### 2.11 Scanning Electron Microscopy

Bacterial cells were harvested at different time points (24 h, 48 h, 72 h, 120 h and 192 h). The pellet was resuspended in a mixture of 1% formaldehyde, 1% glutaraldehyde, 0.1% picric acid in 0.1 M phosphate buffer (pH 7.2) at room temperature then stored at 4° C. All formaldehyde solutions were prepared from freshly depolymerized paraformaldehyde. Cell pellets were washed with phosphate buffer then prepared for scanning electron microscopy. A dense suspension of washed cells was transferred on grids. Cells were dehydrated by use of an ethanol gradient, and then subjected to critical-point drying. Subsequently, samples were mounted on aluminium sample holders and sputter coated with platinum. Finally, cells were inspected with an ESEM XL 30 (FEI, The Netherlands) scanning-electron microscope at 20-30 kV.

### 2.12 Measurement of membrane potential

Membrane potential was estimated by H.-G. Sahl, Bonn, Germany, as previously described (Pag *et al.*, 2004). Briefly, cells were grown in BHI medium at 37°C to an absorbance of 1 at 600 nm, centrifuged and resuspended 1:3 in fresh medium. To monitor the membrane potential, 1 μCi/mL of [<sup>3</sup>H]-tetraphenylphosphonium bromide (TPP<sup>+</sup>; 26 Ci/mMol) was added. TPP<sup>+</sup> is a lipophilic cation which diffuses across the bacterial membrane in response to a trans-negative Δψ. The culture was treated with 0.1 % (vol/vol) ethanol after 16 min to estimate the effect of VLEC<sup>+</sup> on membrane potential and samples were filtered and washed as described above. Counts were corrected for unspecific binding of [<sup>3</sup>H]TPP<sup>+</sup> by subtracting the radioactivity of 10% butanol-treated cell aliquots. For calculation of the

membrane potential ( $\Delta\psi$ ), TPP<sup>+</sup> concentrations were applied to the Nernst equation [ $\Delta\psi = (2.3 \times R \times T/F) \times \log (TPP^+_{in}/TPP^+_{out})$ ].

### 2.13 Gas chromatography

This experiment was performed in collaboration with H. H. Maurer, Homburg/Saar, Germany. From the incubation culture (brain heart infusion medium with 0.1% ethanol) with bacteria or without bacteria (n = 2 each), 0.1 mL of sample were taken at time 0, 2, 4, 7, and 24 h. The samples were analyzed by head-space gas chromatography (80°C, column 0.1% SP-1000/Carbopak C) with flame-ionization detection for ethanol quantification or mass selective detection for identification of ethanol and acetaldehyde (Maurer *et al.*, 2002).

### 2.14 Preparative 2D gel electrophoresis

For preparation of cells extracts, bacteria were grown in BHI under conditions mentioned in *Section 2.1.1*. At different time points, cells from 50 ml of culture were separated from the supernatant by centrifugation (7,000 x g) for 10 min at 4° C, washed twice with Tris-EDTA buffer, and resuspended in Tris-EDTA buffer containing 2 mM phenylmethyl-sulfonyl fluoride. After incubation for 10 min on ice with lysostaphin (50 µg/ml), the cells were disrupted with a French press. The lysate was centrifuged for 10 min at 9,000 x g (in order to remove cell debris) and then for 30 min at 21,000 x g at 4°C (in order to remove insoluble and aggregated proteins that disturbed the isoelectric focusing of the proteins). The supernatant was stored frozen (Gertz *et al.*, 1999; Kohler *et al.*, 2003).

Preparative 2D gel electrophoresis was performed by using the immobilized pH gradient technique described by Bernhardt *et al.* The protein samples were separated by using immobilized pH gradient strips (Amersham Pharmacia Biotech, Piscataway, N.J.) in the pH range from 4 to 7. Dual-channel image of 2D gels of cytoplasmic proteins were produced with Delta2D software (Decodon, GmbH). For identification of proteins by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), protein samples (500 µg) were separated on preparative 2D gels, and the proteins were stained with Coomassie blue R-250. The resulting peptide mass fingerprints were analyzed by using the MS-Fit software (<http://prospector.ucsf.edu>), GPMW 4.10 (Lighthouse data), and genome sequences of *S. aureus* N315, Mu50, and COL. Database searches were done by using the



## 2. Experimental Procedures

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BLAST program (Altschul *et al*, 1990). Difference of protein pattern were confirmed with real time RT-PCR analysis (*Section 2.6*) of transcript levels of the corresponding genes of the wild-type *S. aureus* strain DSM20231 and the *clpC* mutant.

## **Chapter 3**

# **RESULTS**

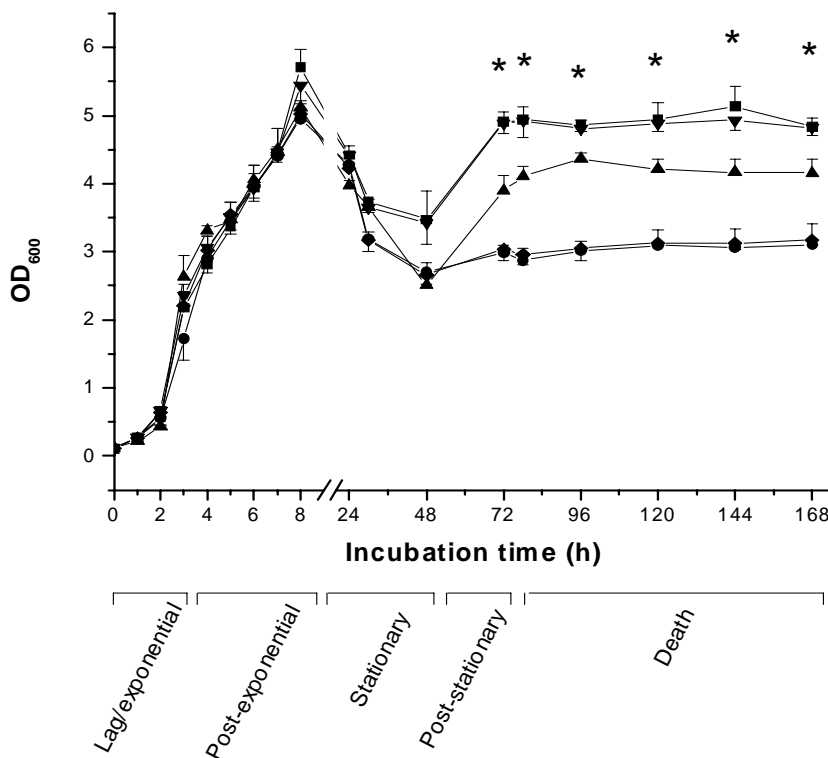
## Chapter 3.1

***Staphylococcus aureus* ClpC ATPase is required for stress resistance, aconitase activity, growth recovery and death**

### 3. Results

#### 3.1 *Staphylococcus aureus* ClpC ATPase is required for stress resistance, aconitase activity, growth recovery and death

***clpC* inactivation impairs post-stationary phase recovery under aerobic conditions.** Long-term survival of the *S. aureus* DSM20231 wild type (WT) as well as of the *S. aureus clpC* mutant strain (PBM001) was determined up to 7 days under aerobic conditions. Both strains grew at similar rates during the exponential growth phase. Between 24 h and 48 h (stationary phase), cell density (OD<sub>600</sub> nm) decreased (stationary phase) in all strains. At 72 h, cell densities of WT again increased until 96 h indicating a recovery in this post-stationary phase, and remained constant up to 7 days while PBM001 was unable to grow following the stationary phase (**Fig. 3.1.1A**).

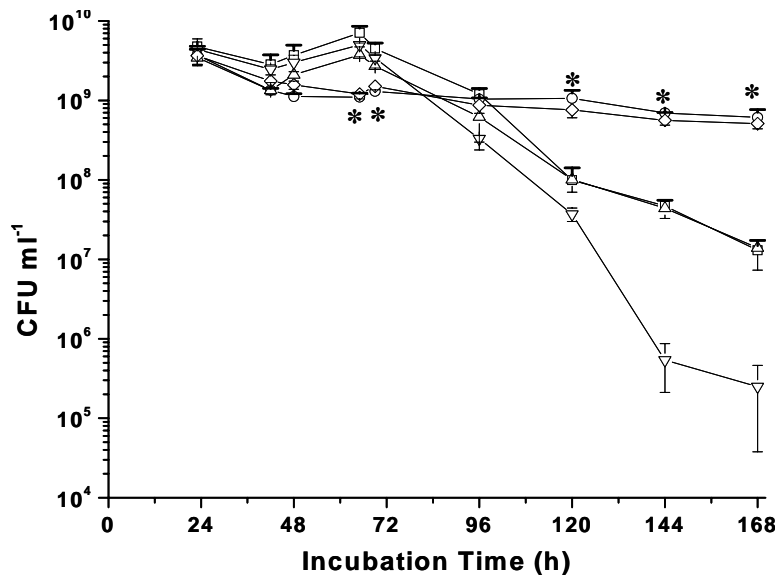


**Fig. 3.1.1A. Growth analysis of WT, *clpC* and *sigB* mutants.** Growth curves (OD<sub>600</sub>) of WT *S. aureus* DSM20231 (■), *clpC* mutant (PBM001; ●), *sigB* mutant (MB290; ▼), and the *clpC sigB* double mutant (MB288; ◆) were determined in BHI medium. For analysis of the *clpC* complemented strain in PBM001 (

### 3. Results

MGM001;  $\blacktriangle$ ), medium was supplemented with xylose (1%). Shown data are mean ( $\pm$  SEM) of values obtained in three independent experiments \*  $P < 0.001$  compared to WT (t-test).

Additionally, we assessed stationary phase survival of the *clpC* mutant and WT. Single colonies were inoculated into BHI and grown aerobically for 1 week, and viable counts were determined daily. CFU values of WT coincided with growth curve results, clearly demonstrating a decrease in viable counts during the stationary phase (24 h - 48 h) followed by a significant increase during the recovery in the post-stationary phase (48 h - 72 h) and finally entering the death phase. In contrast, the *S. aureus clpC* mutant lacked recovery during post-stationary phase but survived to a significantly larger extent during prolonged times of growth ( $>72$  h) (**Fig. 3.1.1B**).

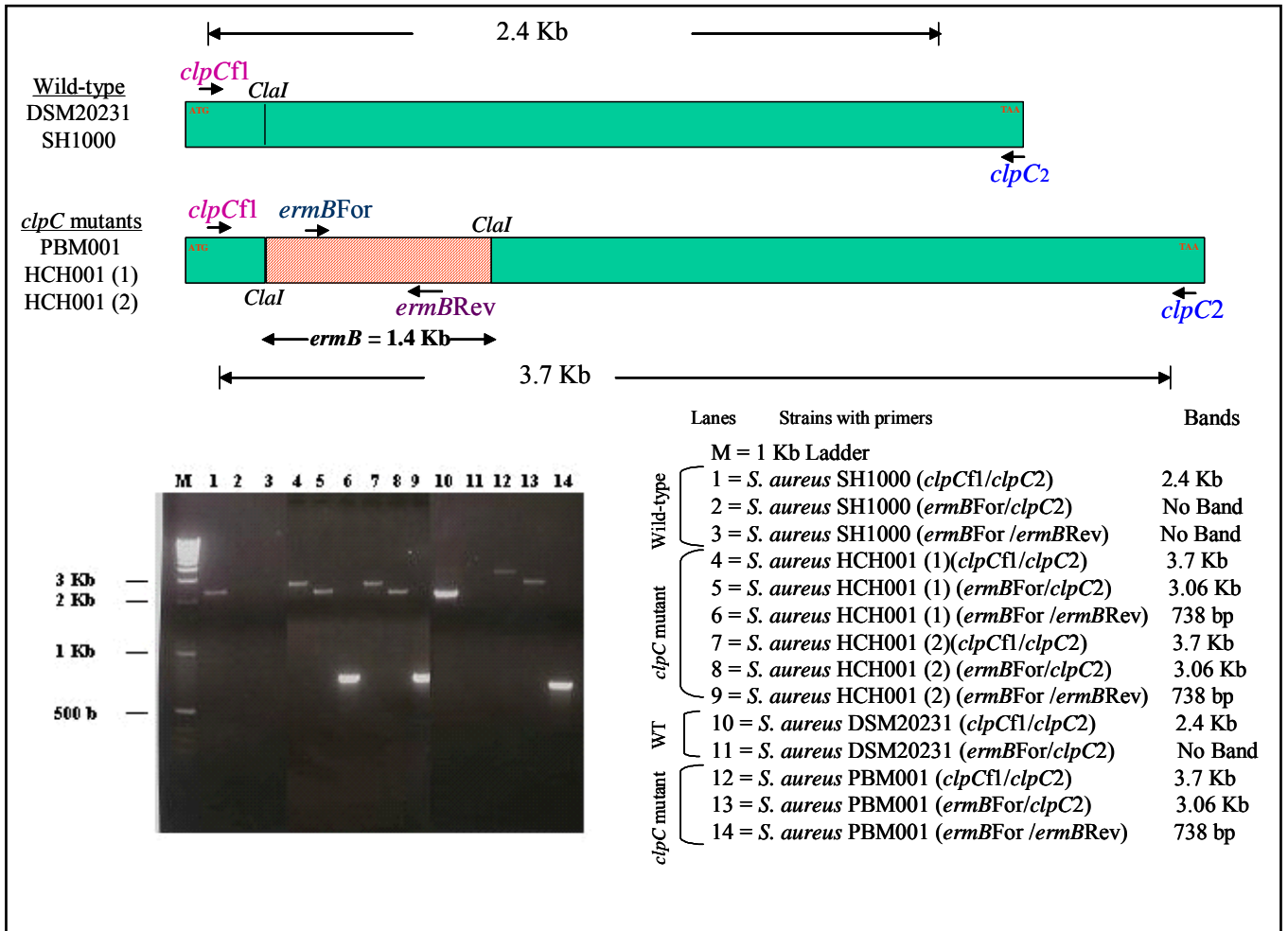


**Fig. 3.1.1B. Inactivation of *clpC* enhances stationary-phase survival.** Single colonies of WT *S. aureus* DSM20231 (□), *clpC* mutant (PBM001; ○), *sigB* mutant (MB290; ▽), and the *clpC sigB* double mutant (MB288; ◇) were inoculated into BHI medium, grown at 37°C and aerated by being shaken at 230 rpm for upto 1 week. At different intervals, aliquots were removed and CFU per millilitre were determined in triplicate. For analysis of the *clpC* complemented strain in PBM001 (MGM001;  $\Delta$ ), medium was supplemented with xylose (1%). Shown data are mean ( $\pm$  SD) of values obtained in two independent experiments \*  $P < 0.05$  compared to WT (t-test).

This observation was further extended by introducing the *clpC* mutation in a different *S. aureus* background by phage 85 mediated transduction of the *ermB*-tagged *clpC* insertion-

### 3. Results

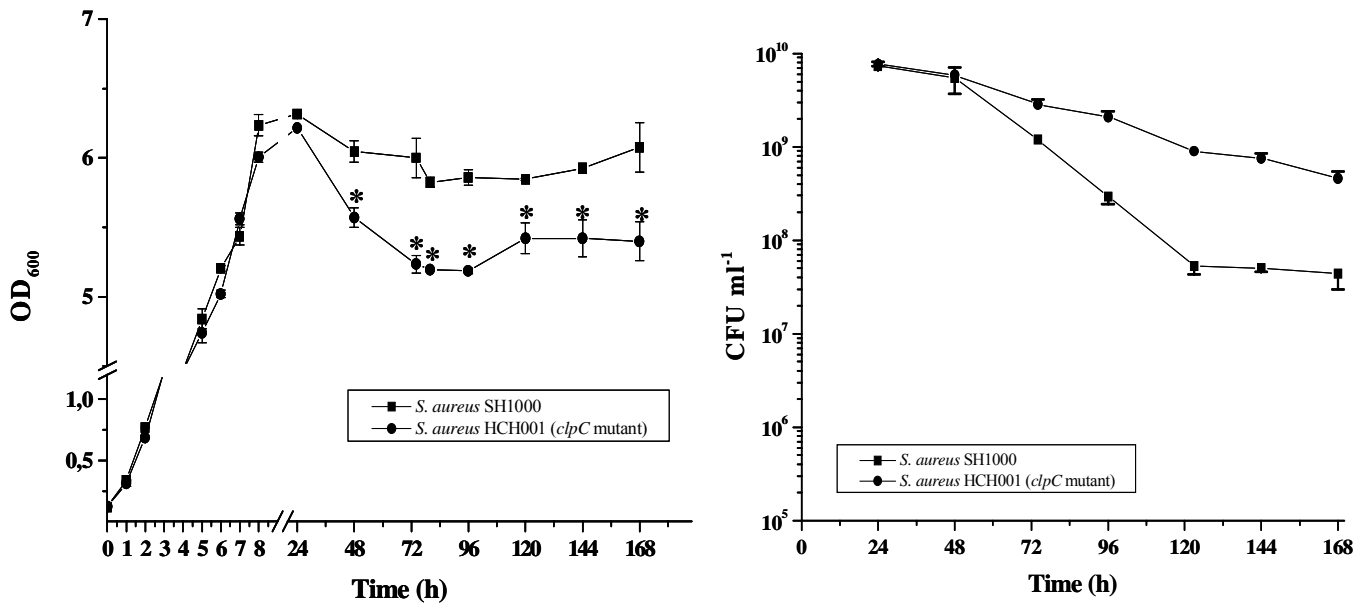
mutation of PBM001 into *S. aureus* SH1000 (an *rsbU*<sup>+</sup> derivative of 8325-4 Horsburgh *et al*, 2002) and selecting for erythromycin resistance (**Fig. 3.1.1C**).



**Fig. 3.1.1C.** Construction and analysis of *S. aureus* SH1000 *clpC* mutant (HCH001) using phage transduction (described in Experimental procedures).

This SH1000 *clpC* mutant (designated as HCH001) also survived during extended times of growth (>72 h) (**Fig. 3.1.1D**).

### 3. Results



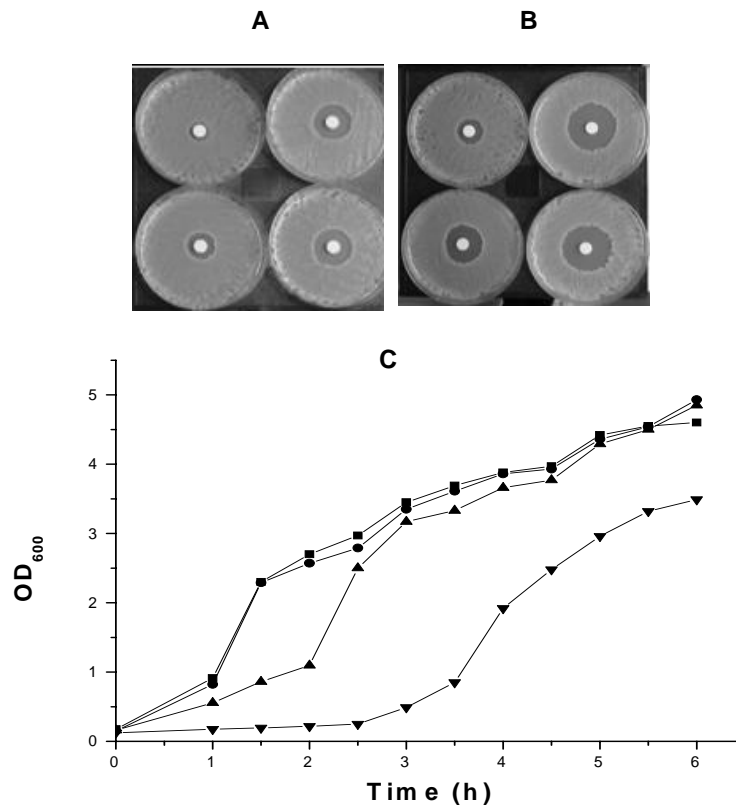
**Fig. 3.1.1D. Growth analysis and stationary phase survival of *clpC* mutant in a different *S. aureus* background (SH1000).** Growth curves (OD<sub>600</sub>) of WT *S. aureus* SH1000 (■) and its *clpC* mutant (HCH001; ●) were determined in BHI medium (left side figure). Shown data are mean (+/- SEM) of values obtained in three independent experiments \* P < 0.001 compared to WT (t-test). Analysis of stationary phase survival of *S. aureus* SH1000 (■) and its *clpC* mutant (HCH001; ●) (right side figure). Shown data are mean (+/- SD) of values obtained in two independent experiments.

The abrogated post-stationary phase recovery in PBM001 was partially restored in the complemented mutant, MGM001, with an increase in cell density (**Fig. 3.1.1A**) and viable counts (**Fig. 3.1.1B**) at 72 h attaining intermediate cell density levels during the post-stationary phase recovery followed by entering the death phase. The pH changes were almost identical for all strains and mutants (pH 5.9-6.0 at 6 h-8 h; effect of glycolysis), followed by catabolism of the L-amino acids in the medium leading to increase in the pH (8.5-9.0 by 168 h). The alternative sigma factor  $\sigma^B$  has been recognized as a general stress-responsive sigma factor (Gertz *et al*, 2000; Kazmierczak *et al*, 2003). Therefore, we compared the  $\sigma^B$ -negative phenotype to a ClpC-negative phenotype by constructing a *sigB* mutant and a *clpC sigB* double mutant (MB290 and MB288, respectively). The growth pattern of MB290 was similar to that of the wild type strain while that of MB288 was identical to that of the *clpC* mutant strain (**Fig. 3.1.1A and 3.1.1B**).

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These data clearly demonstrate that the *S. aureus clpC* mutant and the *clpC sigB* double mutant both failed to grow after stationary phase and failed to enter into the death phase. As Clp analogues have been demonstrated to be of importance in resistance to oxidative stress (Frees *et al*, 2003) as well as to thermotolerance and osmotic stress (Frees *et al*, 2004), and in order to confirm functionality of the *clp* gene system in our WT and the mutants, we compared the stress sensitivity at various conditions.

***clpC* inactivation causes increased sensitivity to oxidative stress.** A disc diffusion assay with two different concentrations of hydrogen peroxide (15 % and 30 %) was performed a previously described (Frees *et al*, 2003). After inoculation of tryptic soy agar (TSA) plates with stationary grown cells (24 h; 37°C; 230 rpm), H<sub>2</sub>O<sub>2</sub> impregnated discs (10 µl) were placed, and plates were incubated (37°C, 18 h). Both *clpC* (PBM001) and *sigB* (MB290) mutant cells displayed increased sensitivity to both hydrogen peroxide concentrations tested while the WT was more resistant to oxidative stress (**Fig. 3.1.2A; 3.1.2B**).



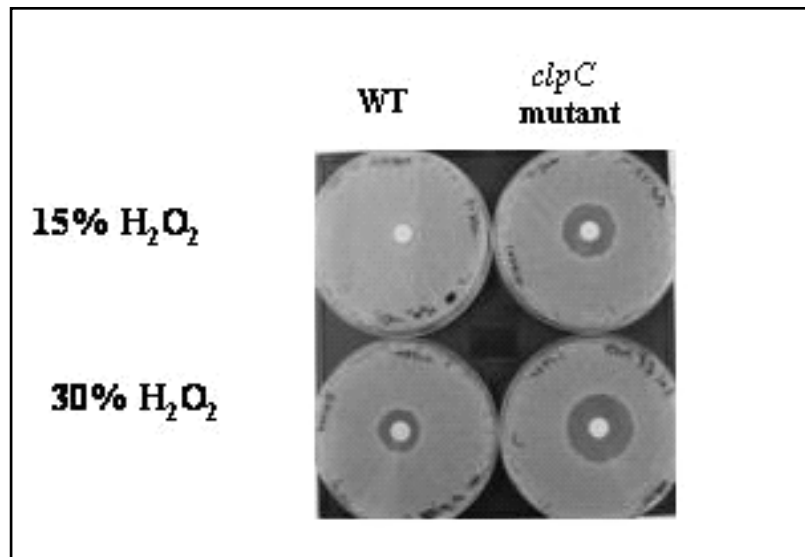
**Fig. 3.1.2. Sensitivity of *clpC* or *sigB* mutants to oxidative stress.** (A-B) Cells of WT (upper left plates), PBM001 (*clpC* mutant; upper right plates), MB290 (*sigB* mutant; lower left plates) and MB288 (*clpC sigB*



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double mutant; lower right plates) grown for 24 h (in TSB) were plated on TSA. A disc containing 10  $\mu$ l of hydrogen peroxide at a concentration of either 15 % (A), or 30 % (B) was placed, then plates were incubated (37°C, 18 h). (C) 50 ml cultures of wild type and the *clpC* mutant were grown to an OD<sub>600</sub> of 0.1. The cultures were split, to one half 7.5 mM of hydrogen peroxide was added, then both halves were incubated (37°C). Shown are OD<sub>600</sub> values obtained at the indicated time points (■, WT without H<sub>2</sub>O<sub>2</sub>; ▲, WT with H<sub>2</sub>O<sub>2</sub>; ●, PBM001 without H<sub>2</sub>O<sub>2</sub>; ▼, PBM001 with H<sub>2</sub>O<sub>2</sub>). Shown are representative results of at least two independent experiments.

The *clpC sigB* double mutant (MB288) did not display any additional increase in inhibition zone size compared to PBM001. Similar results were obtained when the oxidative stress response of SH1000, and its *clpC* mutant HCH001 were examined (Fig. 3.1.2D).



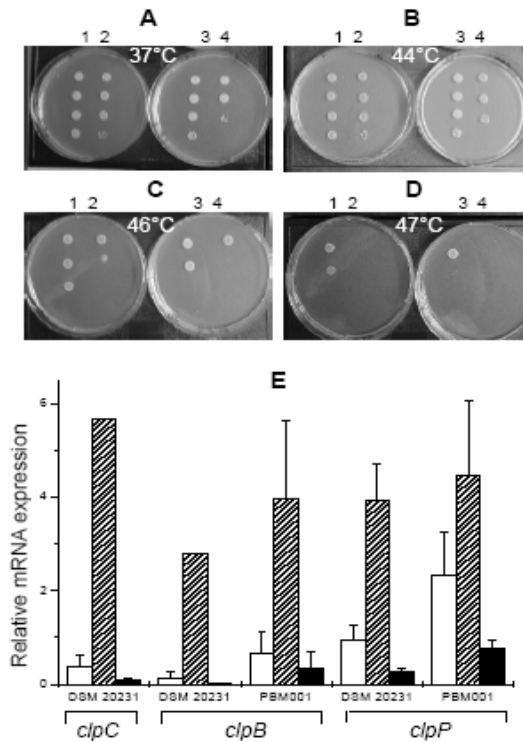
**Fig. 3.1.2D. Sensitivity of *clpC* mutant (HCH001) in a different *S. aureus* background (SH1000) to oxidative stress.** Cells of WT and *clpC* mutant were grown for 24 h (in TSB) and plated on TSA. A disc containing 10  $\mu$ l of hydrogen peroxide at a concentration of either 15 % or 30 % was placed, then plates were incubated (37°C, 18 h).

In addition to the plate assay, growth of WT and PBM001 was also determined in liquid BHI medium with or without hydrogen peroxide supplementation (Fig. 3.1.2C). In agreement with the results obtained in the disc diffusion assay, PBM001 displayed increased susceptibility to hydrogen peroxide with clear growth retardation by approximately 2.5 h.

**Heat shock reduces growth in the *clpC* mutant and enhances *clpC*, *clpB* and *clpP* transcript expression.** Generally, *clp*-homologues are responsive to heat shock (Hecker *et al*, 1996; Chastanet *et al*, 2003; Frees *et al*, 2003; Frees *et al*, 2004). It has previously been reported that *clpB*, *clpC*, *clpL* and *clpP* genes are induced by heat shock in *S. aureus* COL (Gertz *et al*, 2000), RN4220 (Chastanet *et al*, 2003) and 8325-4 (Frees *et al*, 2003). Thus, we compared gene expression of *clpC*, *clpP*, *clpB*, *clpX* and *clpL* in *S. aureus* WT as well as in the mutants grown either at 37°C or exposed to 55°C for 15 min. Real-time quantitative PCR was used to examine the modulation of gene expression in *S. aureus*. The level of mRNA expression of the different genes was normalized against the constitutively expressed internal control *gyrB* (Wolz *et al.*, 2002). A 15-fold up-regulation of *clpC* transcript was noted 5 min after heat shock in the WT followed by a rapid repression of the transcript by 30 min after heat shock (**Fig. 3.1.3E**). No detectable expression was found for the corresponding genes in the samples without heat shock (data not shown). Similarly to *clpC*, following heat shock expression of *clpB* and *clpP* was up regulated in the WT and the *clpC* mutant (**Fig. 3.1.3E**) as well as in the other mutant strains (MB290 and MB288 - not shown) then decreased. Similar to previously published data (Frees *et al*, 2003), *clpX* was not induced after heat shock at detectable levels in either strain (data not shown). In contrast to results using *S. aureus* strain COL (Gertz *et al*, 2000), we found that heat stress did not induce the expression of *clpL* (data not shown).

The increased expression of *clpC* following a heat shock suggested that the *clpC* mutant strain would be sensitive to heat stress. To test this hypothesis, we subjected the WT, *clpC* (PBM001), *sigB* (MB290), and *clpC sigB* (MB288) mutant strains to elevated temperatures and examined the effects on growth (**Fig. 3.1.3A-D**). At 37°C, WT, PBM001, and MB290 grew to a similar extent; however, at elevated temperatures, the viability of the mutant strains was significantly reduced (approximately 100-fold reduction) relative to the WT strain (**Fig. 3.1.3D**).

### 3. Results

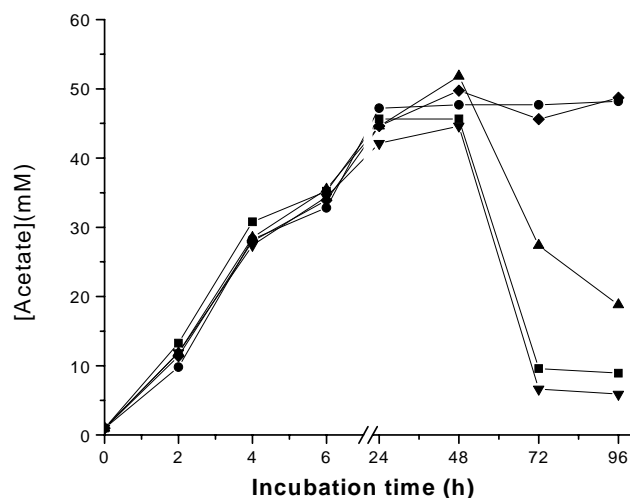


**Fig. 3.1.3. Growth and *clp* gene expression after heat shock.** (A-D) Cultures of WT (1), PBM001 (2), or MB290 (3) and MB288 (4) were grown to the exponential phase in TSB at 37°C. At OD<sub>600</sub> of 0.25-0.30, cultures were diluted 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup> and 10<sup>-4</sup> fold, respectively, and 10 µl of each dilution (top to bottom, respectively) were spotted on TSA plates. The plates were then incubated at 37°C (A), 44°C (B), 46°C (C), and 47°C (D) for 24 h. (E) Real time RT-PCR quantification of *clp* gene expression after heat shock at 55°C for 15 min. mRNA concentrations of *clpC*, *clpP* and *clpB* in WT (DSM 20231) and the *clpC* mutant (PBM001) after heat shock at 0 min (□), 5 min (▨) and 30 min (■) were determined as described in experimental procedures. Shown are transcript quantities relative to control (*gyrB*) transcript and expressed as fold-increase. Data are means ± standard deviation of triplicate determinations.

### 3. Results

**ClpC is required for acetate catabolism and aconitase activity.** Previously we have shown that *S. aureus* post-exponential growth requires an intact TCA cycle to facilitate the catabolism of secondary metabolites (*e.g.*, acetate) (Somerville *et al*, 2002; Somerville *et al*, 2003a). Based on these observations and on the fact that WT strain DSM20231 grew after a brief stationary phase (**Fig. 3.1.1A**), we hypothesized that the post-stationary phase recovery was dependent upon the catabolism of secondary metabolites. To test this hypothesis, we i) analyzed the levels of growth metabolites (glucose and acetate) of the TCA cycle in the supernatant of the *S. aureus* mutants as well as expression of *acsA* encoding the acetate-utilizing enzyme acetyl CoA-synthetase, ii) determined the gene expression levels of *asp23*, *citB* and *clpC*, and iii) performed an aconitase activity assay.

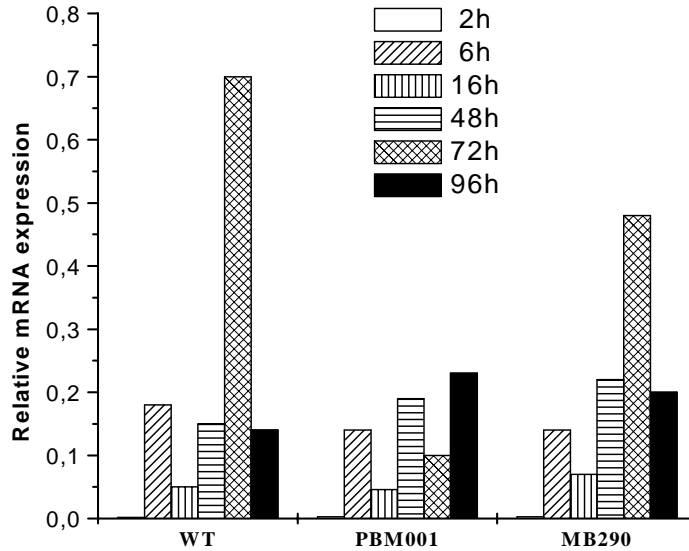
In accordance with published data (Somerville *et al*, 2003a), glucose was catabolized in the exponential phase of growth in all strains and was completely consumed by 4 h (<0.05 mM). The *clpC* functional strains DSM20231, MGM001 (complemented mutant), and MB290 (*sigB* mutant) started to catabolize acetate after 48 h, and by 96 h, the acetate was depleted from the culture medium (**Fig. 3.1.4A**). Elevated levels of *acsA* expression in *clpC*-positive strains (WT and MB290) concur with this observation (**Fig. 3.1.4B**). In striking contrast, the two *clpC* defective mutants PBM001 and MB288 failed to catabolize acetate (**Fig. 3.1.4A and 3.1.4B**), suggesting that *clpC* inactivation impaired TCA cycle function.



**Fig. 3.1.4A. Determination of acetate in the culture supernatant.** After indicated time intervals, supernatants of WT (■), PBM001 (●), MGM001 (▲), MB290 (▼) and MB288 (◆), cultivated in BHI

### 3. Results

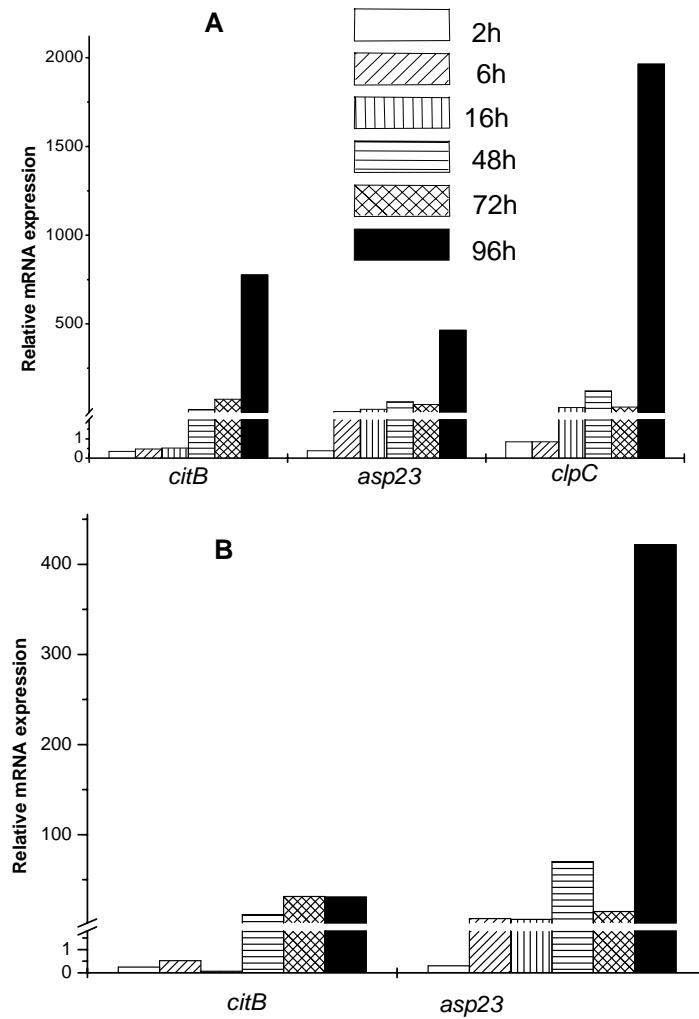
medium, were analyzed for acetate concentrations as described in Methods. Shown are representative results of at least two independent experiments.



**Fig. 3.1.4B. Expression of acetyl-CoA synthetase (*acsA*) in WT, PBM001 and MB290.** Gene expression of *acsA* was determined in WT, PBM001 (*clpC* mutant) and MB290 (*sigB* mutant) by real-time RT-PCR at different time intervals as described in *figure 3.1.3*.

Accordingly, we examined the mRNA abundance of *citB* encoding the TCA cycle enzyme aconitase and *clpC* in the WT, *clpC*, and *sigB* mutant strains (**Fig. 3.1.5A and 3.1.5B**). Additionally, as a control we examined the expression of *asp23* whose transcription is exclusively controlled by the level of free  $\sigma^B$  (Gertz *et al*, 1999; Giachino *et al*, 2001). As predicted, the transcription of *citB* increased in the WT strain coinciding with the decrease in acetate levels (**Fig. 3.1.4A**). Transcript levels of *citB* in PBM001 also increased at 48 h albeit to a lesser extent, and remained stable at later time points. *asp23* expression in WT and PBM001 and *clpC* expression in WT showed a pattern similar to *citB* expression, with an increase after the stationary phase. In the *sigB* mutant, as anticipated, *asp23* expression was very low throughout the growth cycle.

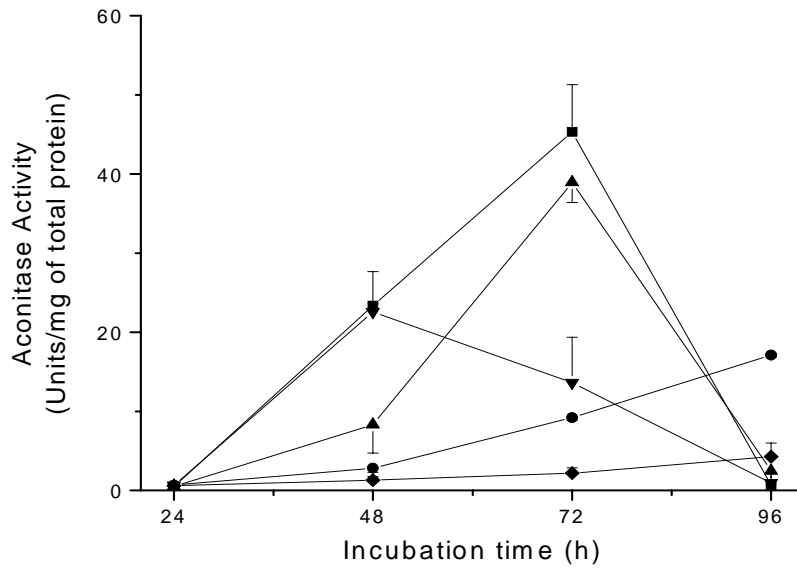
### 3. Results



**Fig. 3.1.5. Expression of *citB*, *asp23*, and *clpC*.** Gene expression of WT (A) and the *clpC* mutant PBM001 (B) was determined by real-time RT-PCR as described in figure 3.1.3. *clpC* was not determined in PBM001.

The *citB* gene expression data were extended by functional assays. Aconitase activity was determined throughout the growth cycle (Fig. 3.1.6) and found to be maximal at 72h in WT. In contrast, the *clpC* mutants (PBM001 and MB288) showed greatly reduced activity throughout the culture period. Actonitase activity in the *sigB* mutant initially increased; however, this activity was not sustained after 48h.

### 3. Results



**Fig. 3.1.6. Aconitase activity of WT and *clpC* or *sigB* mutants.** WT (■), PBM001 (●), MGM001 (▲), MB290 (▼), and MB288 (◆) were grown as described in figure 1 for 24 h, 48 h, 72 h and 96 h. Aconitase activities were determined as described in Experimental procedures in triplicate analysis. Results are means  $\pm$  standard error of two independent experiments.

## **Chapter 3.2**

### **Further characterization of the *Staphylococcus aureus* heat shock protein *clpC* mutant: proteomic analysis and post-stationary phase antibiotic tolerance**



### **3.2 Further characterization of the *Staphylococcus aureus* heat shock protein *clpC* mutant: proteomic analysis and post-stationary phase antibiotic tolerance**

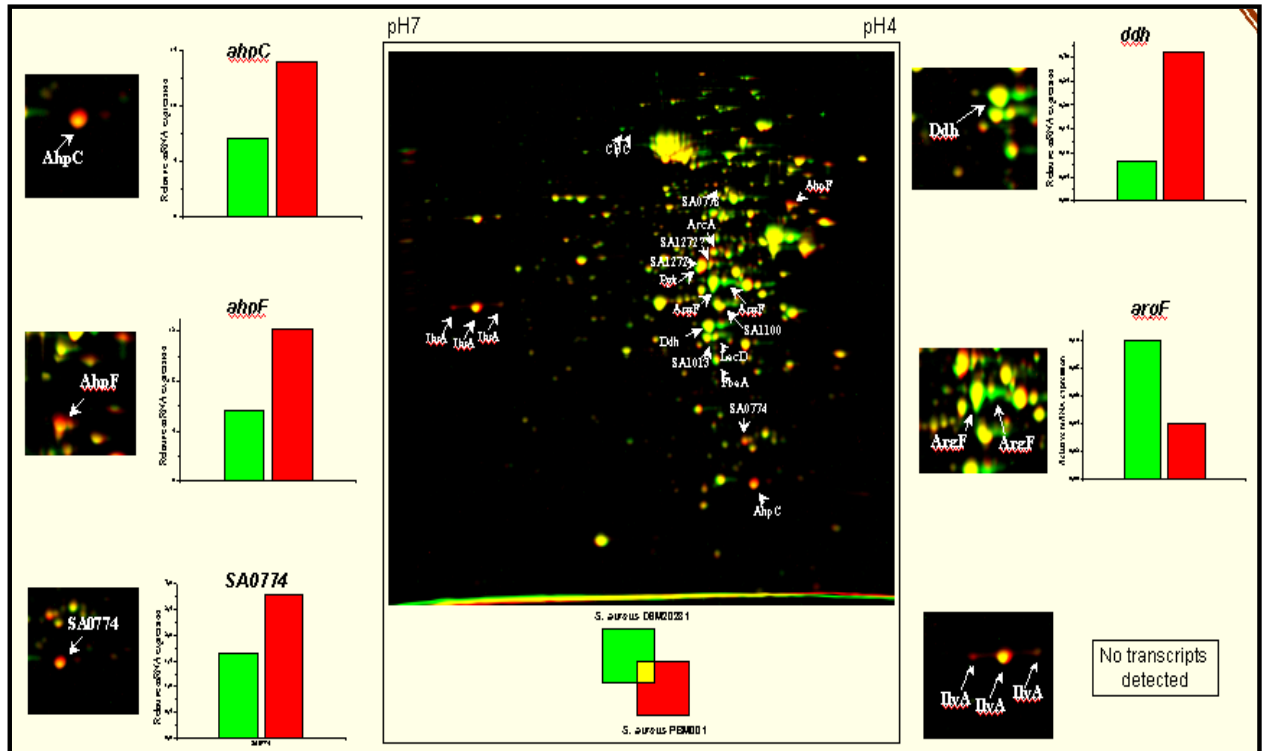
In the previous chapter (*Chapter 3.1A*), we could describe the characterization of a *S. aureus* mutant deficient in the ATP-dependent chaperone ClpC with respect to growth and survival during late growth phases (> 48 h), to expression and activity of enzyme of the tricarboxylic acid cycle, acetate catabolism and entry into death phase. In the first part of this chapter, we have further characterized the *clpC* mutant using proteomic analysis and determine proteins which are differentially expressed in the *clpC* mutants, thereby indicating a probable broader influence of ClpC in staphylococcal survival and metabolism. In the second part of this chapter, we determined the effect of various concentrations of a fluoroquinolone, moxifloxacin (MFX) on the *clpC* mutant during the enhanced post-stationary phase of survival.

#### **A) Proteomic analysis of the *clpC* mutant:**

High resolution two-dimensional (2D) protein electrophoresis technique combined with MALDI-TOF MS (Kohler *et al*, 2003) used for identification of proteins whose levels were changed by a mutation in *clpC*. Cytoplasmic protein extracts were obtained from the mutant & the wild-type (DSM20231) growing in complex medium (in brain heart infusion medium). Protein samples were extracted from two different stages of growth, post-exponential phase (8 h) (**Fig. 3.2.1**) and post-stationary phase (80 h) (**Fig. 3.2.2**).

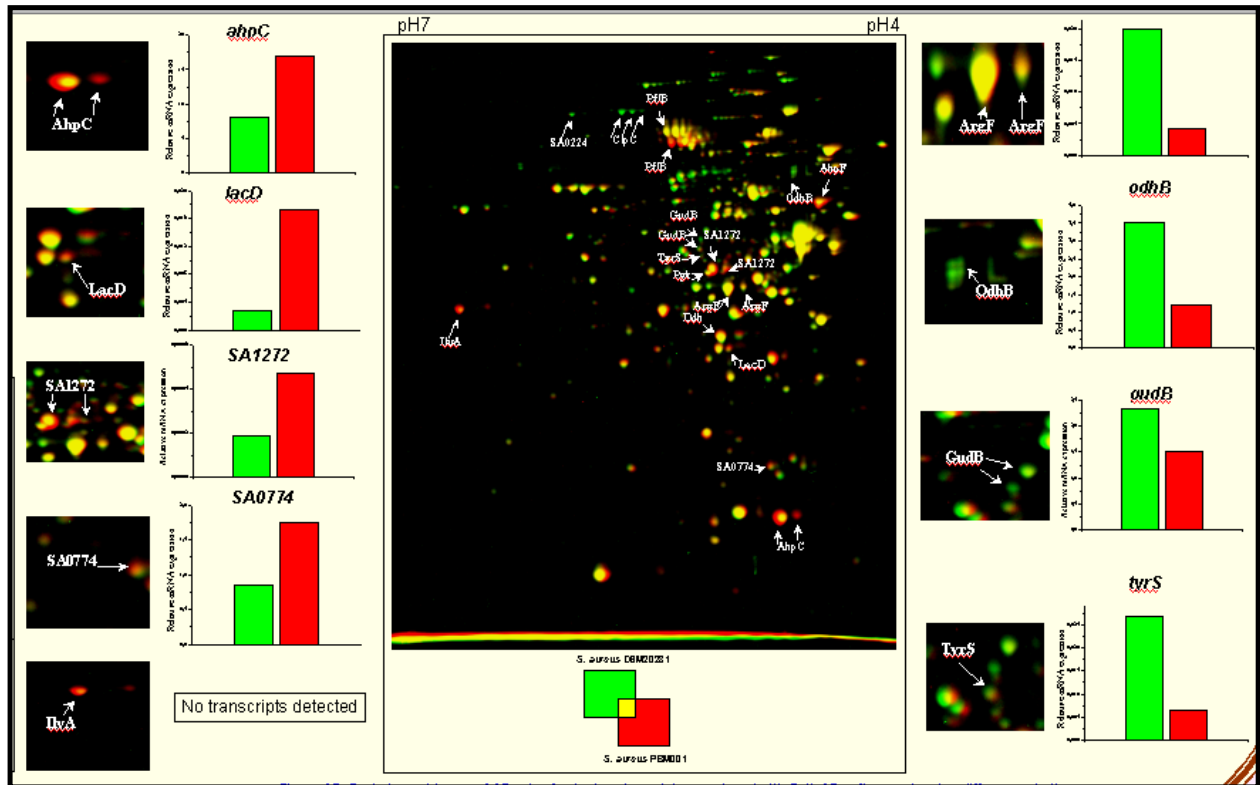
Transcriptional analysis of both *ahpC* and *ahpF*, encoding alkyl hydroperoxide reductase subunit C and subunit F, were also up-regulated. Similarly, *lacD* (tagatose 1,6-diphosphatealdolase), *SA0774* (ABC transporter ATP-binding protein homologue) & *SA1272* (alanine dehydrogenase) were also up-regulated. No transcripts were detected for *IlvA* (threonine deaminase homologue) under both growth phases while equal amount of transcript were detected for *arcA* expressed during post-exponential phase of growth.

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**Fig. 3.2.1.** Dual-channel image of 2D gels of cytoplasmic proteins produced with Delta2D software, showing differences in the protein patterns along with real time RT-PCR analysis of transcript levels of the corresponding genes of the wild-type *S. aureus* strain DSM20231 (green) and the *clpC* mutant (red) at post-exponential phase (8 h).

### 3. Results



**Fig. 3.2.2.** Dual-channel image of 2D gels of cytoplasmic proteins produced with Delta2D software, showing differences in the protein patterns along with real time RT-PCR analysis of transcript levels of the corresponding genes of the wild-type *S. aureus* strain DSM20231 (green) and the *clpC* mutant (red) at post-stationary phase (80 h).

One of the proteins up-regulated in the *clpC* mutant during both post-exponential phase and post-stationary phase (**Fig. 3.2.1 and 3.2.2**) was the oxidative stress proteins, AhpC and AhpF (alkylhydroperoxide reductase subunit C and subunit F). Another protein whose levels were increased was of IlvA (threonine dehydratase, iso-branched chain fatty acids biosynthesis) (**Fig. 3.2.1 and 3.2.2**). These increase probably relates to increased stationary phase oxidative stress and also stability of the cell membrane (Martin *et al*, 1999) in the *clpC* mutant. An increase in iso-branched fatty acids decreases membrane fluidity and subsequently, a non-fluid membrane is more stable and rigid, which would preferably favour late stationary survival of the *clpC* mutant (*Chapter 3.1A*) as it is more resistant to unsaturated fatty acids (UFA), lipids, and oxidant stress (Martin *et al*, 1999).

### 3. Results

Interestingly, no transcripts were detected by real time RT-PCR, probably due to low transcript stability. Given below is a summary of all the proteins induced in a *clpC* mutant and in the wild-type (**Table 2A and 2B**).

**Table 2A. Cytoplasmic proteins identified on 2D gels in *S. aureus clpC* mutant.**

<b>PROTEINS</b>	<b>FUNCTION</b>	<b>Locus in <i>S. aureus</i> N315</b>
<b><u>Induced by a <i>clpC</i> mutation</u></b>		
AhpC (8h, 80 h)	<b>Oxidative stress response:</b> ( <i>alkyl hydroperoxide reductase subunit C</i> )	SA0366
AhpF (8h, 80 h)	<b>Oxidative stress response:</b> ( <i>alkyl hydroperoxide reductase subunit F</i> )	SA0365
ArcA (8h)	<b>Energy metabolism :</b> amino acids and amines ( <i>arginine deiminase</i> )	SA2428
IlvA (8h, 80 h)	<b>Energy metabolism :</b> branched chain amino acid ( <i>threonine deaminase homolog</i> )	SA1271
LacD (80 h)	<b>Energy metabolism :</b> lactose metabolism ( <i>tagatose 1,6-diphosphate aldolase</i> )	SA1994
SA0774 (8h, 80 h)	<b>ABC transposter :</b> ( <i>ABC transporter ATP-binding protein homologue</i> )	SA0774
SA0778 (8h)	<b>Iron sulphur metabolism :</b> ( <i>conserved hypothetical protein similar to Fe-S assembly protein SufB</i> )	SA0778
SA1272 (8h, 80 h)	<b>Energy metabolism :</b> L-alanine biosynthesis ( <i>alanine dehydrogenase</i> )	SA1272

### 3. Results

**Table 2B. Cytoplasmic proteins identified on 2D gels in *S. aureus* DSM20231 (Wild-type).**

<b>PROTEINS</b>	<b>FUNCTION</b>	<b>Locus in <i>S. aureus</i> N315</b>
<u>Induced in the Wild-type</u>		
ArgF (8h, 80 h)	<b>Energy metabolism :</b> amino acids and amines ( <i>ornithine carbamoyltransferase</i> )	SA1012
OdhB (80 h)	<b>Energy metabolism :</b> 2-oxoglutarate dehydrogenase metabolism ( <i>dihydrolipoamide succinyltransferase</i> )	SA1224
GudB (80 h)	<b>Energy metabolism :</b> amino acids and amines ( <i>NAD-specific glutamate dehydrogenase</i> )	SA0819
TyrS (80 h)	<b>Transcriptional antiterminator :</b> amino acids and amines ( <i>tyrosyl-tRNA synthetase</i> )	SA1550
SA0224 (80 h)	<b>Energy metabolism :</b> beta-oxidative pathway ( <i>hypothetical protein, 3-hydroxy-acylCoA dehydrogenase</i> )	SA0224

Together these results suggest that heat shock protein ClpC ATPase might be involved in a broader aspect of staphylococcal metabolism and stress regulation. ClpC might be further involved in regulation of important physiological & metabolic pathways, involving energy metabolism and stress regulation, as indicated by the protein profile of the 2D gel analysis.

#### **B) Stationary phase tolerance of *S. aureus clpC* mutant in presence of moxifloxacin (MFX)**

In the previous chapter, it was shown that the *clpC* mutant has enhanced post-stationary phase survival. It has been long known that the stationary phase surviving cells would have enhanced resistance to antibiotics (Mayhall and Apollo, 1980; Rice *et al*, 2005). In order to show this, we decided to determine the antibiotic sensitivity (moxifloxacin) of the *clpC* mutant by exposing stationary phase surviving cells (120 h/ 5 days) to increasing concentration of MFX (3 x MIC, 30 x MIC and 300 x MIC; Fig. 3.2.3B). The MICs of both strains used were 0.03 µg/ml (as per NCCLS guidelines). As control, we also exposed both wild-type and *clpC* mutant from fresh culture to 30 x MIC of MFX (**Fig 3.2.3A**). As evident from the result (**Fig. 3.2.3B**), *clpC* mutant cells were tolerant to increasing concentration of

### 3. Results

MFX, compared to wild-type and to tolerance in exponential phase growing cells (Fig. 3.2.3A).

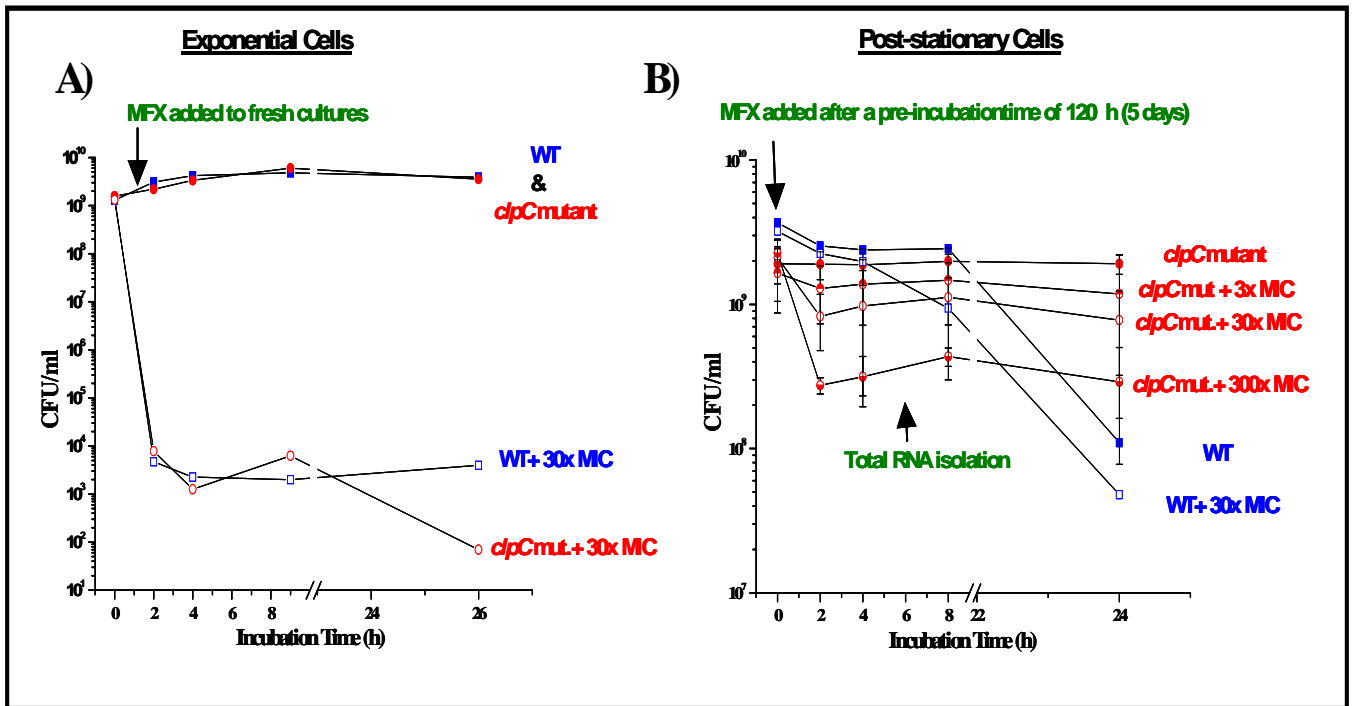
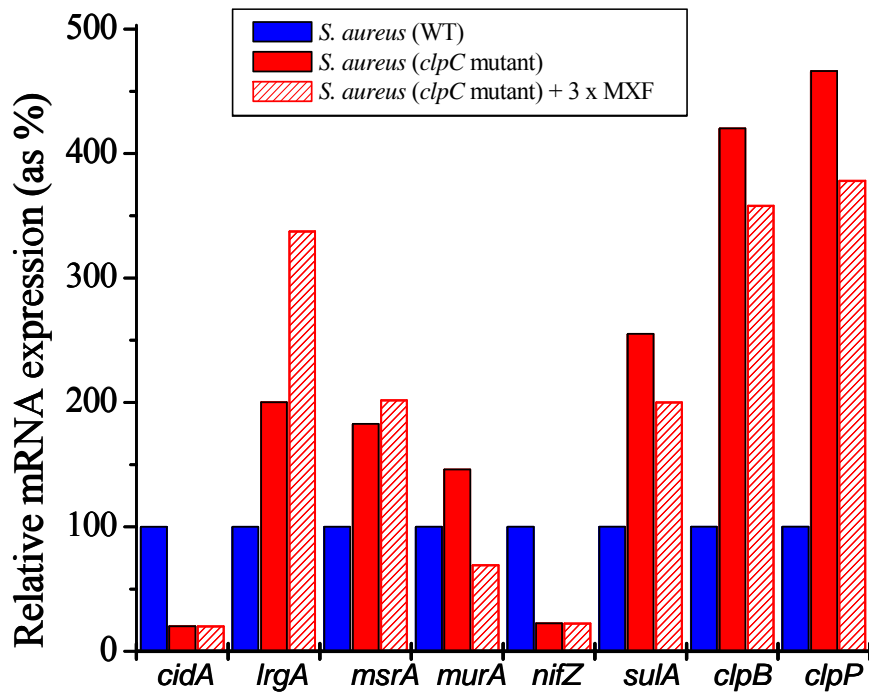


Fig. 3.2.3. Stationary phase tolerance of *S. aureus* ClpC mutant to increasing concentration of MFX.

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Note: Relative mRNA expression of wild-type strain was set to 100 % (Control)

**Fig. 3.2.4.** Relative transcript expression of cell wall and stress response genes in *clpC* mutant during post-stationary phase of growth (-/+ 0.1  $\mu\text{g/ml}$  of MFX) .

The enhanced stationary phase survival of the *clpC* mutant might well have been due to the slow-growth of the bacterial population (during stationary phase) as the fluoroquinolones (MFX) have their antibacterial effect by targeting the gyrase and the topoisomerase machinery units of bacteria. In order to further clarify the transcription levels of the *clpC* mutant under antibiotic stress during post-stationary phase of growth, transcript analysis of select genes were performed. (**Fig 3.2.4**) Total RNA was isolated 6 h after adding 3x MIC of MFX from stationary phase growing *clpC* mutant & wild-type staphylococcal cells. The time at which MFX was added to stationary phase culture is indicated by an arrow (denoted in **Fig 3.2.3B**).

The *Staphylococcus aureus* *lrg* and *cid* operons encode homologous proteins that regulate extracellular murein hydrolase activity and antibiotic tolerance in a diametrically opposing manner (Rice *et al*, 2005), in line with this observation, *cidA* was down-regulated and *lrgA* was up-regulated, both of which might contribute to enhanced MFX tolerance (**Fig 3.2.4**). *msrA* (encoding methionine sulfoxide reductase), an antioxidant repair enzyme, previously found to be strongly up-regulated by antibiotic in *S. aureus* (Utaiida *et al*, 2003), was also upregulated in *clpC* mutant. Also cell-wall related genes like *murA* (encoding UDP-N-

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acetylglucosamine 1-carboxyvinyl transferase 1) and *sulA* homologue (cell division inhibitor) were also up-regulated in the mutant. Finally, transcription of other heat shock proteins *clpB* and *clpP* were also strongly induced during these phases of growth.

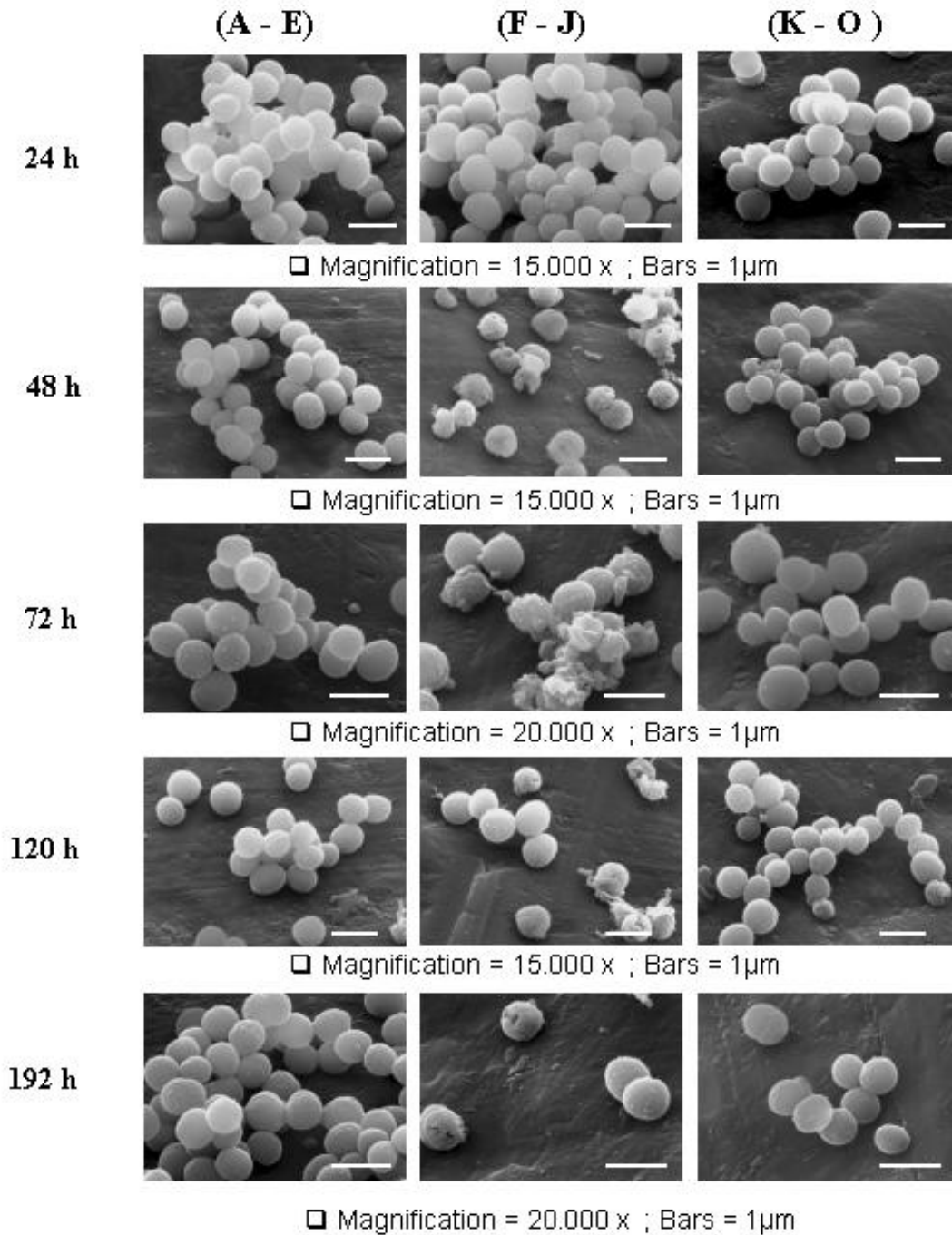


## **Chapter 3.3**

**Very low ethanol concentrations affect viability and growth recovery in post-stationary *Staphylococcus aureus* populations**

#### **3.3 Very low ethanol concentrations affect viability and growth recovery in post-stationary *Staphylococcus aureus* populations**

**Electron microscopy analysis of *S. aureus* cells grown in the presence of ethanol under microaerobic conditions.** High concentrations of ethanol are bactericidal; however, bacteria can grow in the presence of low concentrations of ethanol (Knobloch *et al*, 2001; Knobloch *et al*, 2002). These observations led us to question whether morphological changes would be induced upon growth of *S. aureus* at such conditions. Thus, we examined *S. aureus* grown in VLEC+ conditions using scanning electron microscopy at different time points throughout the growth cycle (**Fig. 3.3.1**). No morphological differences were observed (**Fig. 3.3.1 A,F,K**) until between 48 h and 192 h post inoculation, when striking changes could be seen in *S. aureus* grown in VLEC+ (**Fig.3.3.1G-J**). The presence of collapsed and broken cells, cell debris and indentation of the cell surface in these cells suggested a possibility of a weakened cell wall. In contrast, cells grown in absence of ethanol had more intact cells and a normal smooth spherical appearance (**Fig. 3.3.1A-E**). Interestingly, the effects of ethanol only occur when the bacterial cultures are grown under microaerobic conditions (data not shown). Taken together, these data suggest the affect of VLEC on growth and/or viability of *S. aureus* is delayed.



**Fig. 3.3.1. Effect of VLEC and arginine on micromorphology of *S. aureus* DSM20231.**

Shown are representative scanning electron micrographs of *S. aureus* DSM20231 in unsupplemented media (A-E, top to bottom) in VLEC+ conditions (F-J, top to bottom), or in VLEC+ conditions and supplemented with 5 mm arginine (K-O, top to bottom) grown for various time intervals (24 h, 48 h, 72 h, 120 h and 192 h).

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**Ethanol delays post-stationary phase recovery.** VLEC did not alter the exponential growth rate (Fig. 3.3.2A and 3.3.2B); however, it slightly decreased the growth yield (24 h). Between 48 h - 72 h in culture, the cell density for bacteria grown in the absence of ethanol increased suggesting post-stationary phase growth. In contrast, bacteria grown in VLEC+ conditions lysed between 24 - 48 h reaching a nadir in cell density (Fig. 3.3.2A) and CFU (Fig. 3.3.2B) at 96 h and increasing cell density after 120 h. These findings were consistent with the morphological observations (Fig. 3.3.1). The effect of ethanol on the late stationary phase was dependent on the ethanol concentration (Fig. 3.3.2.C).

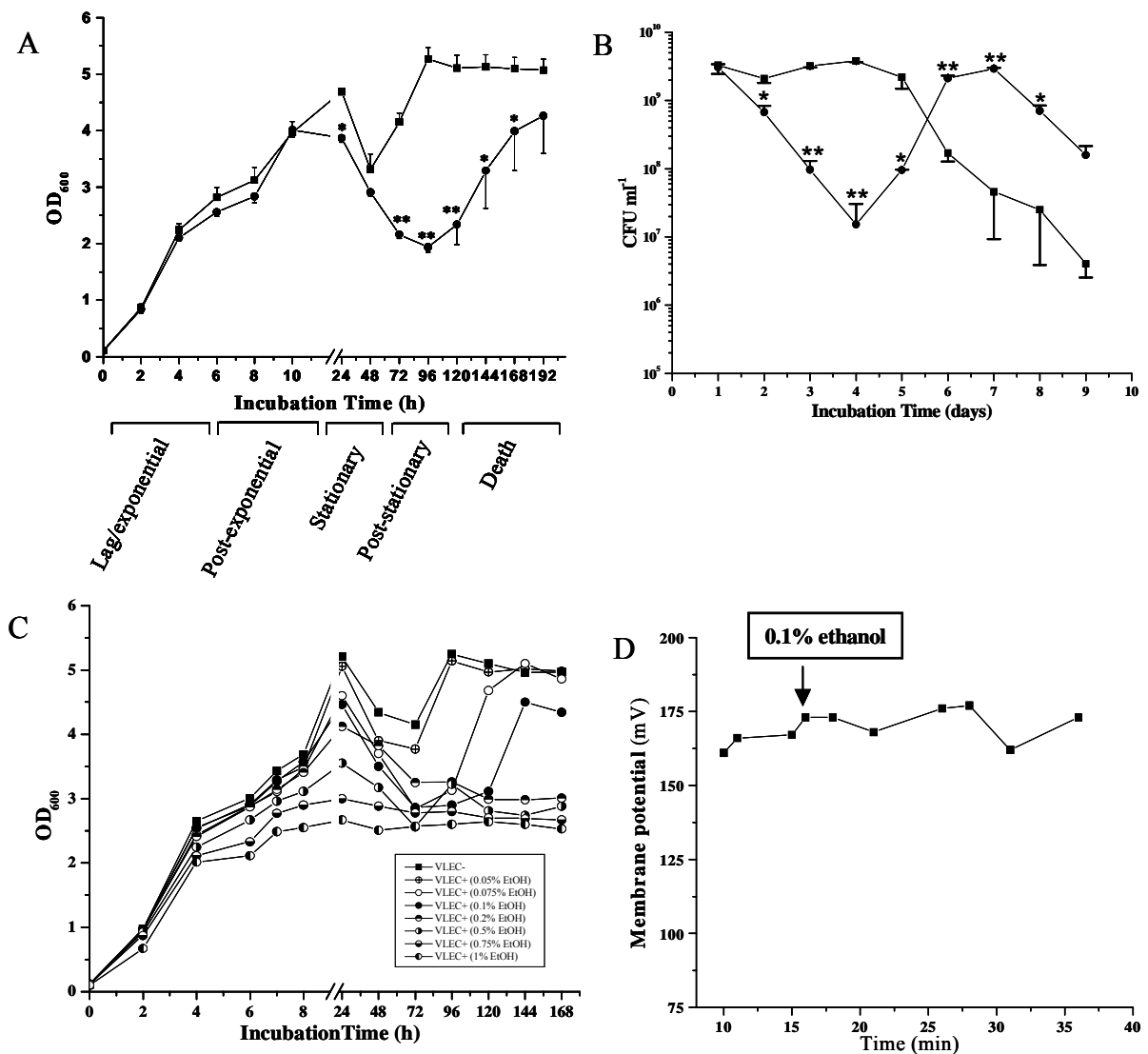


Fig. 3.3.2. Analysis of long-term growth, stationary phase survival and membrane potential of *S. aureus*. A. Growth analysis (OD<sub>600</sub>) of *S. aureus* DSM20231 in VLEC- (■) and in VLEC+ conditions (●)

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determined in BHI medium. Single colonies were inoculated into BHI in absence (■) or in presence of 0.1% (vol/vol) ethanol (●), and incubated at 37°C at microaerophilic conditions for up to 8 days. Shown data are means +/- standard errors of the means of values obtained in three independent experiments \*,  $P < 0.05$ ; \*\*,  $P < 0.001$  ( $t$  test). **B.** Viability of *S. aureus* DSM20231. After growth for indicated time interval VLEC- (■) or VLEC+ (●) conditions, aliquots were removed and CFU/ml was determined in triplicate. Data are means +/- standard deviations of values obtained in two independent experiments \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  ( $t$  test). **C.** Ethanol concentration-dependent delayed recovery. **D.** Membrane potential measurement of *S. aureus* DSM20231. Addition of ethanol (0.1%) is indicated by an arrow. Data are presented as representative of two independent experiments.

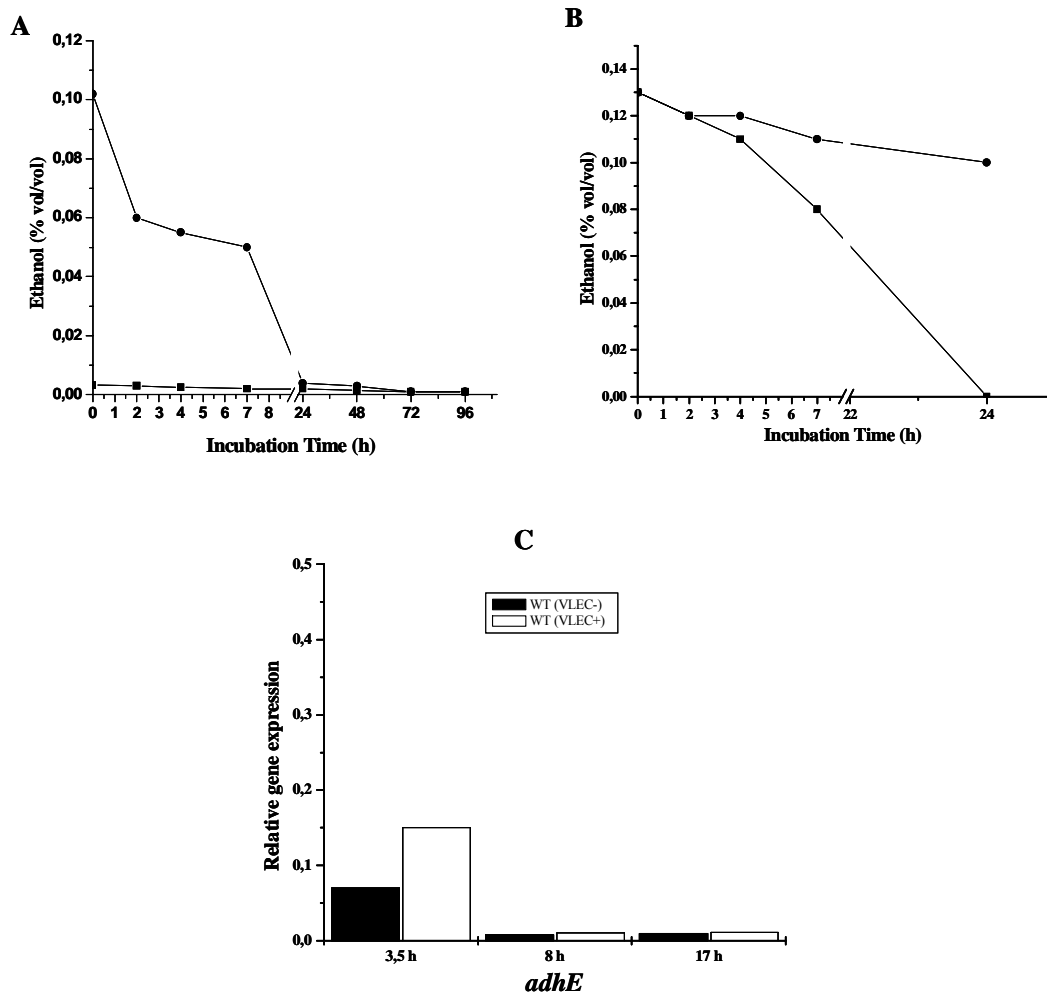
Post-stationary growth was delayed at concentrations between 0.075% vol/vol and 0.1% vol/vol while at more elevated concentrations post-stationary growth was inhibited, and exponential growth (at 24 h) was also affected. In contrast, a concentration of 0.05% vol/vol showed post-stationary growth characteristics indistinguishable from VLEC- conditions. *S. aureus* grown in unsupplemented media entered the final death phase (defined as the loss of viable counts without concomitant reduction in optical density) immediately after the post-stationary growth (at 96 - 120 h) (5 - 6 days). In contrast, the VLEC+ treated *S. aureus* entered the final death phase much later (168 h). To determine if these results were due to strain specific factors, an identical experiment was performed using strain *S. aureus* SH1000. The effect of VLEC on strain SH1000 was nearly identical to that of strain DSM20231 (data not shown), suggesting that the response of strain DSM20231 to VLEC is common to *S. aureus* strains of different genetic backgrounds.

A possible explanation for the delayed post stationary phase recovery in the VLEC+ population is the emergence of escape mutants with reduced susceptibility to ethanol. To test this hypothesis, we performed an ethanol susceptibility assay of late stationary phase bacteria. VLEC+ grown staphylococci (both DSM20231 and SH1000 strains) were grown for 12 h, from post-stationary phase (120 h), in media containing various concentration of ethanol (0%, 1%, 5%, 10%, 25% and 50%). These bacteria were equally sensitive to ethanol as were bacteria obtained from fresh overnight cultures or organisms grown under VLEC- conditions (data not shown). A second possible explanation for the prolonged recovery time of VLEC treated bacteria might be due to inefficient membrane repair. To address this possibility, we determined the membrane potential of *S. aureus* in the presence of VLEC (**Fig. 3.3.2D**). We were unable to detect any difference in membrane potential in *S. aureus* DSM20231 after

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addition of 0.1% ethanol relative to the untreated control. Taken together, these data suggest VLEC does not facilitate the generation of escape mutants or significantly alter the membrane potential.

**Ethanol is rapidly removed from the culture medium.** Ethanol is a volatile organic alcohol (flash point 13°C), thus it was surprising that the effects of VLEC on post-stationary phase recovery persisted until 120 h (5 days) into the growth cycle. We speculated that ethanol would be lost due to evaporation and/or catabolism well before 120 h; hence, we determined the concentration of ethanol in the culture medium throughout the growth cycle. As expected, the concentration of ethanol in the culture medium began to decrease immediately after inoculation, and by 24 h no ethanol remained (**Fig. 3.3.3A**).



**Fig. 3.3.3.** Analysis of ethanol in the cultural supernatant and effect of alcohol-aldehyde dehydrogenase (*adhE*) in VLEC exposure. **A.** Determination of ethanol in the cultural supernatant of *S. aureus* DSM20231 in

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VLEC- (■) and VLEC+ conditions (●) at the indicated time points. **B.** Estimation of the non-enzymatic loss of ethanol from the culture supernatant with (■) or without (●) bacteria under VLEC+ conditions using gas chromatography. **C.** Expression of alcohol-aldehyde dehydrogenase (*adhE*) in *S. aureus* VLEC at different time points. Data are presented as representative of two independent experiments.

To assess if ethanol evaporated or was enzymatically catabolized, VLEC+ supernatants were examined by gas chromatography at various time points after supplementation of ethanol both in the presence and in the absence of *S. aureus* (**Fig. 3.3.3B**). In the absence of microorganisms, the concentration of ethanol in the medium remained stable over 24h, while in the presence of *S. aureus* ethanol was depleted from the culture medium by 24h, suggesting the bacteria were catabolizing the ethanol. Concomitantly, in VLEC+ conditions transcription of *adhE* (alcohol-acetaldehyde dehydrogenase gene) was elevated early (3.5 h) when compared to VLEC-, indicating a contribution of alcohol dehydrogenase to ethanol catabolization (**Fig. 3.3.3C**). Most importantly, these data demonstrate that the effects of VLEC on late stationary growth and survival persist long after ethanol has been depleted from the culture medium, and suggest that recovery from ethanol-induced alteration is a delayed process.

**Ethanol delays acetate catabolism and ammonia accumulation.** In VLEC+ cultures, the onset of post-stationary phase growth was delayed suggesting that VLEC impaired the metabolism of non-preferred carbon sources. To determine if VLEC affects metabolism, DSM20231 was grown in VLEC+ medium, and the pH was measured; pH is an indicator of organic acid production. During the first 10 h of incubation, the pH of the culture medium was nearly identical, irrespective of the presence of ethanol (**Fig. 3.3.4A**). In the absence of ethanol, the pH of the culture medium began to increase at 24 h post-inoculation, and by 192 h (8 days), it was alkaline (pH 9.1). In contrast, in VLEC+ conditions the pH values remained acidic (pH 5.5) until nearly 120 h (5 days). An ethanol-induced inhibition of medium alkalinization can be caused by either a decreased catabolism of organic acids and/or a decreased accumulation of ammonia. To determine which of these two possibilities was responsible for the observed pH difference, we measured the concentrations of glucose, acetate, ethanol, lactic acid and ammonia in the culture medium. During the exponential phase of growth, the catabolization of glucose was unaffected by the presence of ethanol (**Fig. 3.3.4B**). Similarly, VLEC did not affect the accumulation or depletion of lactic acid in the culture medium (maximum lactate concentrations in VLEC-, 5.77 mM; VLEC+, 6.8 mM).

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The accumulation of acetate in the culture medium was also found to be unaffected by VLEC; however, in VLEC+ treated cultures the depletion of acetate was greatly delayed (Fig. 3.3.4C). Additionally, VLEC+ delayed the accumulation of ammonia (Fig. 3.3.4D) until after 144 h, coinciding with the onset of acetate catabolism and the recovery of viable counts and cell density.

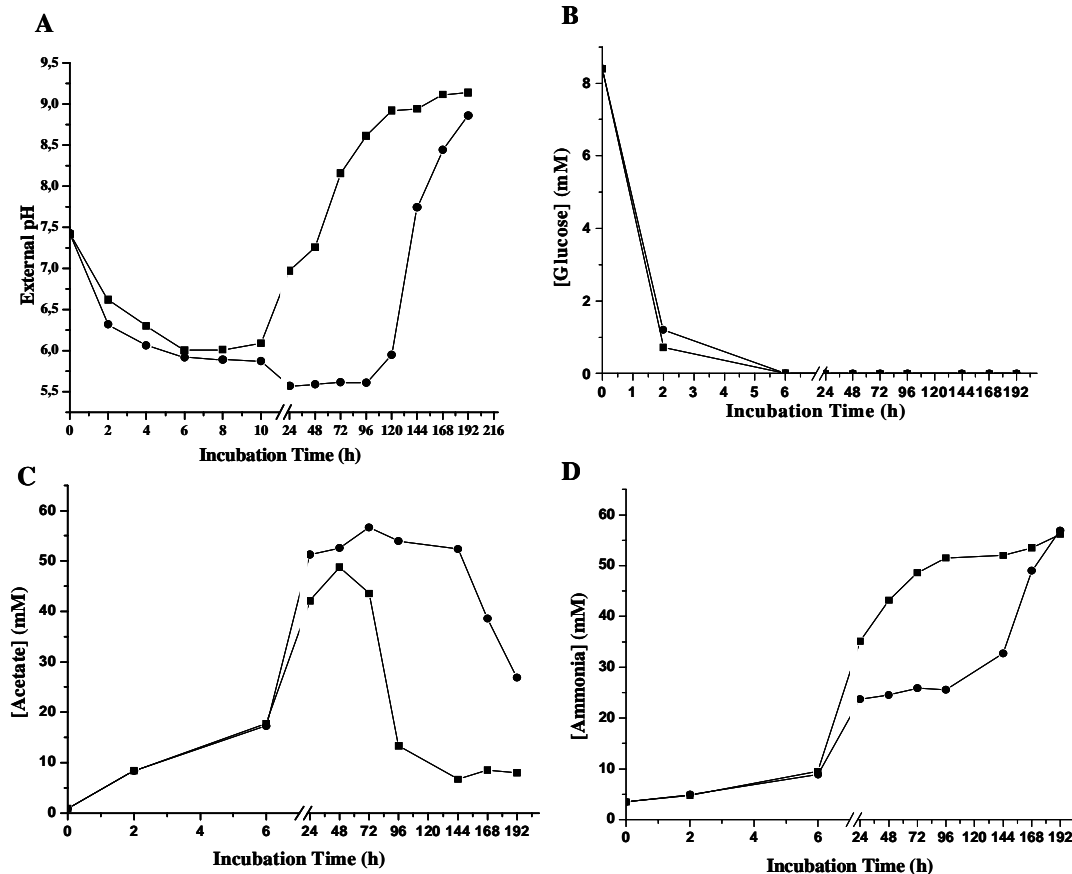


Fig. 3.3.4. Analysis of external pH, and metabolites of the cultural supernatant. Determination of external pH (A), glucose (B), acetate (C), and ammonia (D) in the cultural supernatant of *S. aureus* DSM20231 in VLEC- (■) and VLEC+ conditions (●) at the indicated time points. Data are presented as representative of two independent experiments.

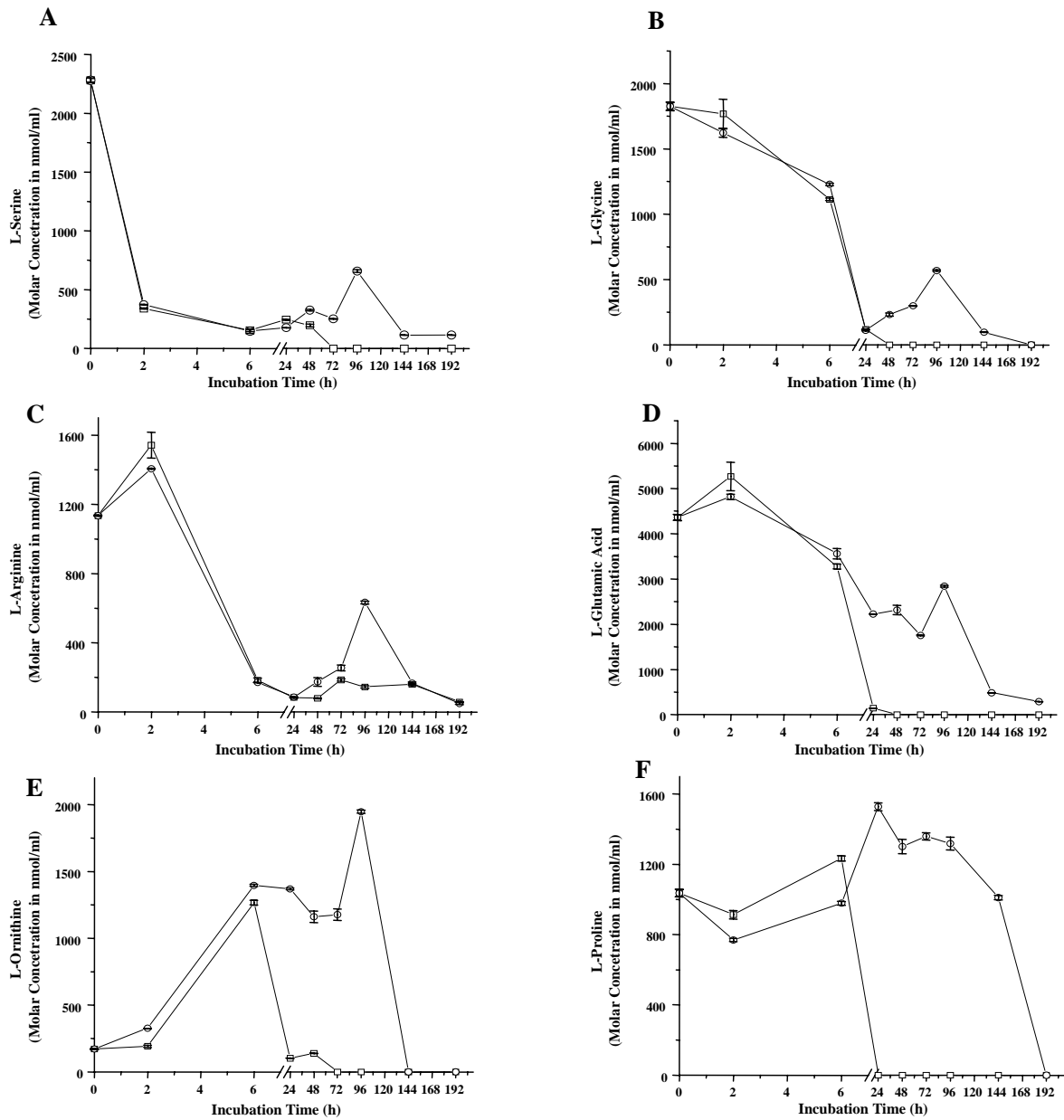
#### Ethanol affects bacterial uptake of specific amino acids from the culture medium.

The accumulation of ammonia in the culture medium is an indication of amino acid catabolism. As stated above, VLEC+ conditions reduced the accumulation of ammonia in the



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culture medium until after 144 h (**Fig. 3.3.4D**), leading us to hypothesize that low concentrations of ethanol affect amino acid catabolism. To test this hypothesis, the concentrations of select free amino acids in the culture medium were determined during

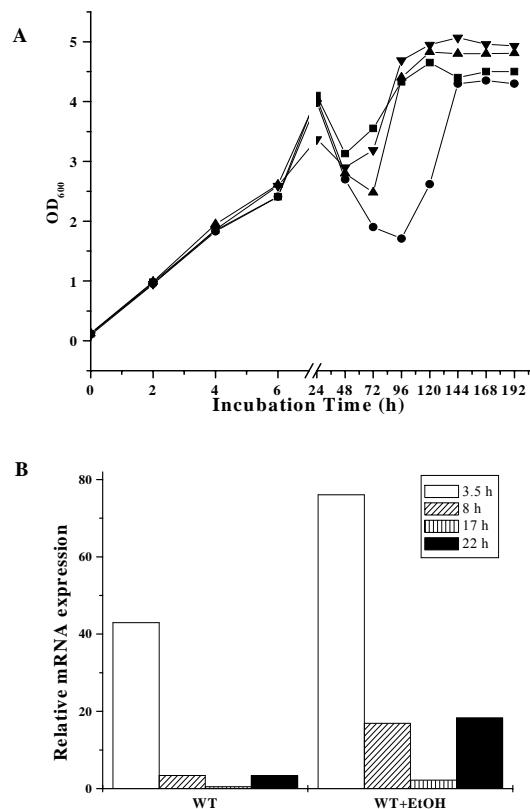


**Fig. 3.3.5. Depletion of free amino acids from the BHI medium.** Concentrations of free amino acids, (A) L-serine, (B) L-glycine, (C) L-arginine, (D) L-glutamic acid, (E) L-ornithine and (F) L-proline, in BHI culture medium of *S. aureus* DSM20231 grown in VLEC- (□) and VLEC+ conditions (○). Shown data are mean molar concentrations (nmol/ml)  $\pm$  SD of two independent experiments.

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growth in VLEC+ and compared to respective determinations in VLEC- cultures. Serine, glycine, and arginine were depleted from the growth medium irrespective of the presence of ethanol (**Fig. 3.3.5A, 3.3.5B and 3.3.5C**). In contrast, glutamic acid, ornithine and proline (**Fig. 3.3.5D, 3.3.5E and 3.3.5F**) were depleted from the culture medium only after growth resumed, resulting in the delayed accumulation of ammonia. In contrast to the other amino acids tested, ornithine accumulated in the medium during growth. Staphylococci use an arginine-ornithine antiporter to transport arginine into the cell; hence, ornithine concentrations increase as arginine concentrations decrease. As the availability of carbon and/or nitrogen becomes limited, staphylococci can catabolize ornithine. VLEC+ conditions delayed the catabolism of ornithine relative to VLEC- conditions. Surprisingly, the difference in amino acid uptake was only detectable during or after the stationary phase of growth after the ethanol was gone (**Fig. 3.3.3A**), while exponential phase amino acid catabolism was independent of ethanol. Taken together, these data indicate that the effect of low ethanol concentrations can persist long after the ethanol had been consumed.

**Arginine restores post-stationary phase recovery in VLEC+ conditions.** Amino acid catabolism is an important source of carbon and energy. The selective depletion of amino acids from the culture medium (**Fig. 3.3.6**)

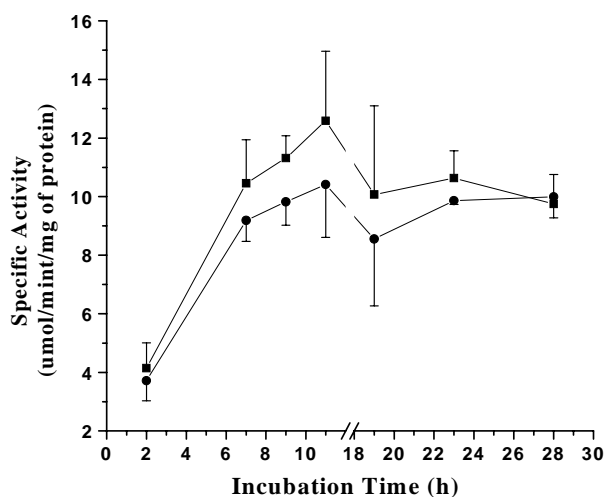


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**Fig. 3.3.6A. Effect of L-arginine supplementation.** *S. aureus* DSM20231 were grown in VLEC- (■), or in VLEC+ (●) conditions, or in VLEC+ supplemented with 2 mM L-Arginine (▲), or in VLEC+ with 5 mM L-Arginine (▼) in BHI medium, and cell densities were determined as described in figure 2. Data are presented as representative of two independent experiments. **B. Real time RT-PCR quantification of microaerobic *arcA* gene expression.** Gene expression of *arcA* in *S. aureus* DSM20231 was determined in VLEC- or VLEC+ grown populations by real-time RT-PCR at different time intervals as described in Methods. Shown are transcript quantities relative to internal control (*gyrB*) transcript, expressed as fold-increase.

led us to speculate that supplementation of the culture medium with a depleted amino acid would restore post-stationary phase growth. We tested this hypothesis by supplementation of VLEC cultures with single amino acids at a concentration of 2 mM and assessed the growth and viability. Interestingly, only arginine restored the post-stationary phase recovery and viability (**Fig. 3.3.6A** and data not shown). The catabolism of arginine usually involves the arginine deiminase (ADI) pathway. To ascertain if VLEC+ conditions resulted in increased transcription of genes of the ADI pathway, we determined the relative concentration mRNA for the *arcA* gene (encoding arginine deiminase) by real-time RT-PCR (**Fig. 3.3.6B**). Consistent with our hypothesis, *arcA* transcript levels were significantly greater at 3.5 h, 8 h, 17 h and 22 h in staphylococci grown in VLEC+ relative to VLEC- conditions.

**Glutamate dehydrogenase activity is reduced under VLEC+ conditions.** Glutamate can be catabolized to the TCA cycle intermediate  $\alpha$ -ketoglutarate by glutamate dehydrogenase.



**Fig. 3.3.7.** Glutamate dehydrogenase activity in *S. aureus* DSM20231 without (■) and with 1 % (vol/vol) ethanol (●) were grown as described in figure 1 for 2 h, 7 h, 9 h, 11 h, 19 h, 23 h and 28 h. Glutamate

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dehydrogenase activities were determined as described in Experimental procedures in triplicate analysis. Results are means  $\pm$  standard error of three independent experiments.

VLEC+ selectively inhibited the depletion of glutamate, proline and ornithine from the culture medium, leading us to hypothesize that a reduction in glutamate dehydrogenase activity might contribute to, or result, in persistently elevated concentrations of the 'glutamate family amino acids'. Consistent with our hypothesis, glutamate dehydrogenase activity was reduced ( $\approx$  15-20 %) in the staphylococcal culture supplemented with ethanol (**Fig. 3.3.7**). Thus, the activity of glutamate dehydrogenase was also influenced during the growth of *S. aureus* DSM20231 in presence of ethanol.

## **Chapter 3.4**

**Transcription analysis of thymidine-dependent  
*Staphylococcus aureus* small colony variants (SCV) at  
different phases of growth using real-time RT-PCR**

#### **3.4 Transcription analysis of thymidine-dependent *Staphylococcus aureus* small colony variants (SCV) at different phases of growth using real-time RT-PCR**

In previous studies, the electron transport chain deficient SCVs had been characterized (*hemB* mutant, Kohler *et al*, 2003); *menD* mutant, (Bates *et al*, 2003) and their persistence were found to be partly due to noticeable differential gene expression pattern. The transcriptional basis for the thymidine-dependent SCV phenotype is yet unknown, but these might be significantly contributing different changes seen in these organisms. This SCV subpopulation is more resistant to antibiotics, grows very slowly, and demonstrates unusual colony morphology (Kahl *et al*, 2003a; Kahl *et al*, 2005). Hence, these strains can be easily being resistant to conventional antibiotic therapy. In order to verify if differential gene expression is one of the many factors leading to the thymidine-dependent SCV phenotype, real time RT-PCR analyses was performed with total RNA harvested from cells at two different stages of growth (early log phase and stationary phase).

##### **A) Transcriptional analysis of select genes in thymidine-dependent SCV during early log phase.**

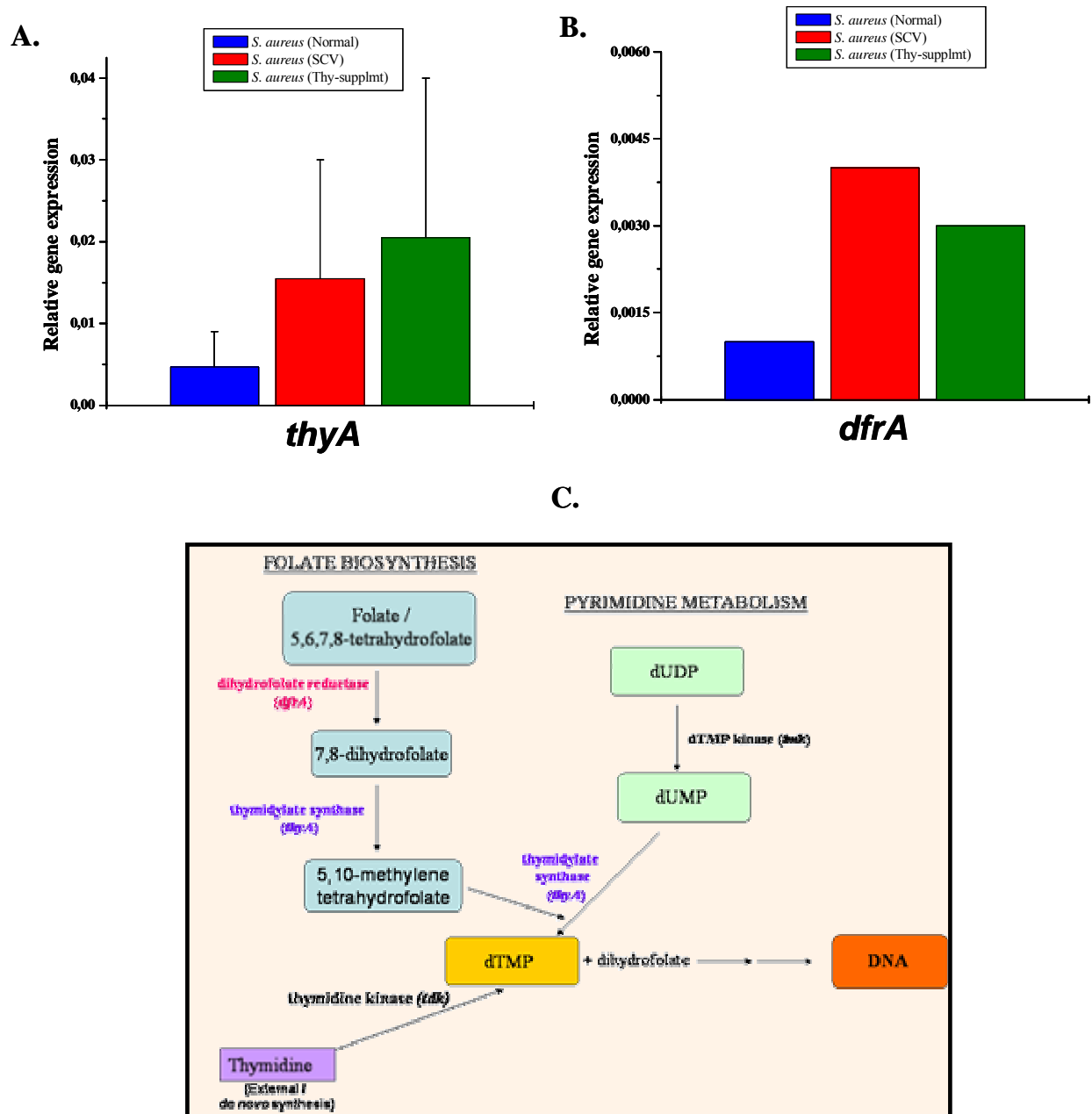
A range of genes were selected involving different aspects of *S. aureus* physiology, metabolism, virulence determinants and stress response, all of which might be directly or indirectly involved in the observed characteristics of thymidine-dependent SCVs.

##### **a) Genes involved in pyrimidine metabolism/ deoxyribonucleotide biosynthesis. (one carbon pool by folate):**

Two important genes involved in pyrimidine metabolism through the one-carbon folate pool, *thyA* (thymidylate synthase; annotated as SA1260 in *S. aureus* N315 genome) and *dfrA* (dihydrofolate reductase; annotated as SA1259 in *S. aureus* N315 genome), were selected. As expected, thymidine dependency of the SCVs was associated with increased transcription of both these *thyA* and *dfrA* during the early log phase of growth (**Fig. 3.3.1.A and 3.3.1.B**). The gene expression of the SCVs supplemented with external thymidine into medium also had these genes up-regulated, behaving similar to the SCVs

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without external thymidine in medium. This was similar to the growth characteristics of these strains under similar time points (refer to Fig 1A presented in Kahl et al, 2005).



**Fig. 3.3.1.** Real time RT-PCR quantification of gene expression of pyrimidine metabolism during early phase of growth. mRNA concentrations of (A) *thyA* and (B) *dfrA* in clinical *S. aureus* strain (normal), SCV and thymidine-supplemented SCV were determined as described in Experimental procedures. Shown are transcript quantities relative to control (*gyrB*) transcript and expressed as fold-increase. Data are means  $\pm$  standard deviation of triplicate determinations. (C) Schematic representation showing folate biosynthesis and pyrimidine metabolism (<http://www.genome.jp/kegg/pathway.html>).

The reaction catalyzed by dihydrofolate reductase (*dfrA*) represents an essential step for DNA precursor synthesis for the conversion of dUMP to dTMP while thymidylate synthase (*thyA*) essentially catalyzes the reduction of dUMP to yield dTMP (**Fig. 3.3.1.C**) and thus provides the sole *de novo* source of dTMP for DNA biosynthesis. A presumptively disruptive hit in *M. pneumoniae* knockout experiments (Castellanos *et al*, 2004) showed this gene is non-essential, but as the authors point out, there are several possibilities why this might not be true: cells contain a functional duplicate gene, function supplied by other compounds from the medium, or crossfeeding. Additionally, an *E. coli* thymidylate synthase mutant, strain \_2913, requires thymidine supplements, because the deleted gene is essential for survival (Castellanos *et al*, 2004). Up-regulation of both these genes (probably co-transcribed) might denote that during the initial phases of growth, SCVs try to utilize the normal pathway for synthesis of DNA, presumably due to compensation of thymidine deficiency. Also under present conditions, the thymidine-dependent SCVs were unable to grow during early log phase, even in presence of externally supplemented thymidine.

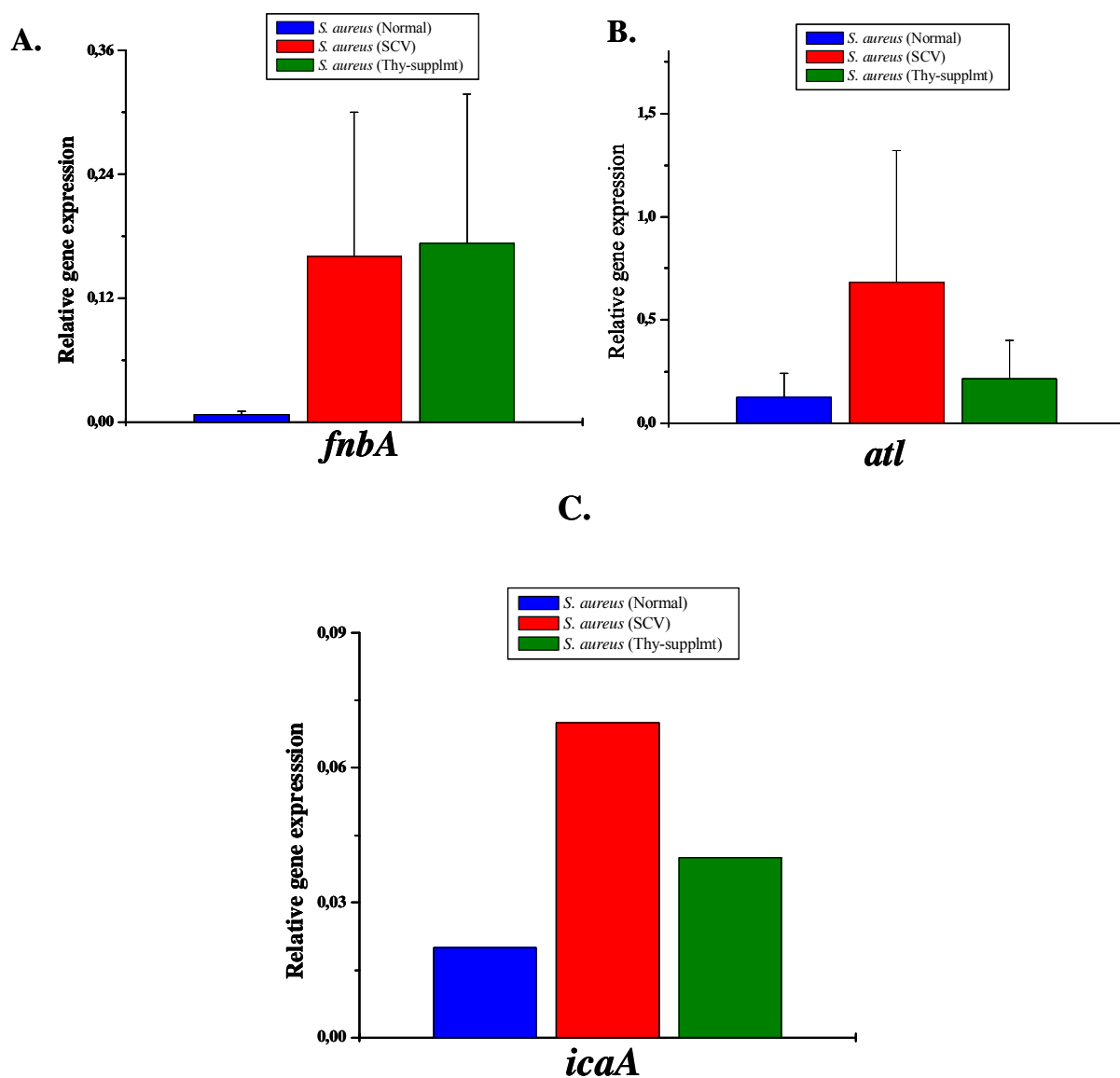
It has been shown that TMP-SMX inhibits the synthesis of tetrahydrofolate (THF) (Gilligan *et al*, 1987). Also, THF acts as a co-factor for thymidylate synthase (*thyA*), which converts dUMP to dTMP, therefore these expression data would serve as important clue to determine the yet unknown causes of the mechanism of thymidine-dependent SCV.

#### **b) Genes involved as putative virulence determinants:**

*S. aureus* has the capability to synthesize a variety of extracellular and cell-wall bound proteins, many of which are involved in pathogenesis (Kahl *et al*, 2005). In one of our recent study (Kahl *et al*, 2005); we could demonstrate that the transcription patterns of important regulators (*sarA*, *sigB* and *agr*) and virulence genes (*hla* and *spa*) of long-term persisting thymidine-dependent *S. aureus* SCV, where in transcription of *sarA*, RNAlII and *hla*, were repressed especially in the late phases of growth while *spa* was upregulated. In the present study, we decided to determine the transcriptional regulation of other important virulent factors involved in pathogenicity of *S. aureus* in the early log phase. These were the fibronectin-binding protein A (*fnbA*), major autolysin (*atl*) and intercellular adhesion protein A (*icaA*).



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**Fig. 3.3.2.** Real time RT-PCR quantification of transcript expression of genes for virulent determinants during early phase of growth. mRNA concentrations of (A) *fnbA*, (B) *atlA* and (C) *icaA* in clinical *S. aureus* strain (normal), SCV and thymidine-supplemented SCV were determined as described in Experimental procedures. Shown are transcript quantities relative to control (*gyrB*) transcript and expressed as fold-increase. Data are means  $\pm$  standard deviation of triplicate determinations.

Fibronectin-binding proteins (FnBPs) appear to play a critical role in *S. aureus* pathogenesis (Menzies, 2003), by facilitating adherence to fibronectin and has been shown to be expressed during the exponential phase of growth. On line with these observations, *fnbA* was upregulated during the early log phase in both the SCVs (-/+

thymidine) (**Fig. 3.3.2.A**). Presence of external thymidine was not able to restore the level of gene expression of *fnbA* in SCV to the normal phenotype.

Also, pathogenesis of *S. aureus* is attributed to the combined effects of many invasive properties such as adherence and biofilm formation. Accordingly, we could show that the transcript levels of *icaA* were upregulated in the SCV (**Fig. 3.3.2.C**). The polysaccharide intracellular adhesions (PIA), encoded by *ica* operon (*icaADBC*), has been shown to be required for biofilm formation by staphylococci (Gotz, 2002). It has been also shown that biofilm formation was associated with an enhanced primary attachment probably owing to an increased autolysin activity (Fournier and Hooper, 2000). Accordingly, the gene expression of the major autolysin *atl*, was also upregulated in the SCV (**Fig. 3.3.2.B**).

It was interesting to note that *icaA* transcripts were detected only in one of the normal/SCV strain pair (*Janning*), which was also confirmed by the biofilm assay. The 2<sup>nd</sup> normal/SCV strain pair (*Schmidt*) lacked *icaA* transcripts and also had negative biofilm assay. Finally, the 3<sup>rd</sup> normal/SCV strain pair (*Mallone*) also lacked *icaA* transcripts but did give a positive biofilm assay (where the normal strain had higher biofilm than the SCV strain). Thus, in strain pair 3, the normal strain accumulated higher levels of PIA than the SCV strain, whereas the transcriptional levels of *icaA* both in normal and the SCV were absent. A similar discrepancy between the accumulation of PIA and the transcription of the *icaADBC* operon has also been described in *S. epidermidis* growing in presence of glucose (Dobinsky *et al*, 2003). Thus, it seems that the thymidine-dependent SCV possessed both *ica*-positive/PIA-positive (strain 1) and *ica*-negative/PIA-positive phenotypes (strain 3). Alternatively, it could be that as soon as enough PIA is accumulated, bacteria may repress the synthesis of *ica* transcription as part of some kind of negative-feedback regulatory mechanism.

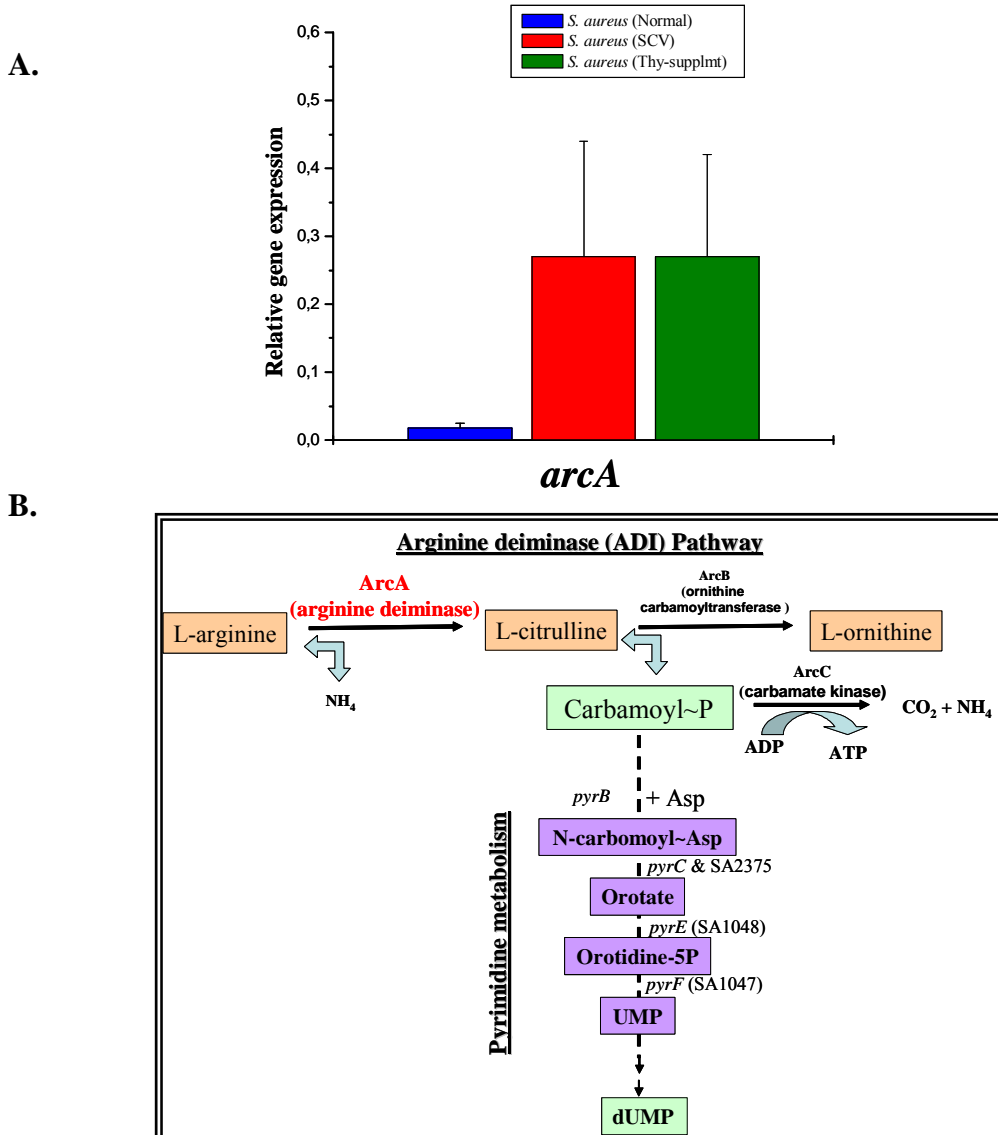
Altogether, the upregulation of all these virulent determinants would lead to an increased adherence of the SCVs compared to the normal strain, paving way for long-term persistence in later stages of growth.

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#### c) Genes involved in arginine deiminase (ADI) pathway:

The arginine deiminase pathway (ADI) is of critical importance in many gram-positive bacteria. This system occurs in a variety of bacteria and appears to be induced by arginine under anaerobic conditions (Cunin *et al.*, 1986). The ADI consists of three enzymes. Arginine is hydrolyzed by arginine deiminase (ArcA) to generate citrulline and ammonia. Citrulline is then converted to ornithine and carbamoylphosphate via ornithine carbamoyltransferase (ArcB). Finally, carbamate kinase (ArcC) transfers a phosphate from carbamoylphosphate to ADP, yielding ATP, which provides a source of energy for the cells.

Accordingly, *arcA* gene was markedly upregulated during the early log phase in the thymidine-dependent SCVs (Fig. 3.3.3.A). Presence of external thymidine was unable to complement the *arcA* upregulation.



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**Fig. 3.3.3.** Real time RT-PCR quantification of gene expression of arginine metabolism during early phase of growth. mRNA concentrations of (A) *arcA* in clinical *S. aureus* strain (normal), SCV and thymidine-supplemented SCV were determined as described in Experimental procedures. Shown are transcript quantities relative to control (*gyrB*) transcript and expressed as fold-increase. Data are means  $\pm$  standard deviation of triplicate determinations. (B) Schematic representation associating arginine deiminase pathway with pyrimidine metabolism (<http://www.genome.jp/kegg/pathway.html>).

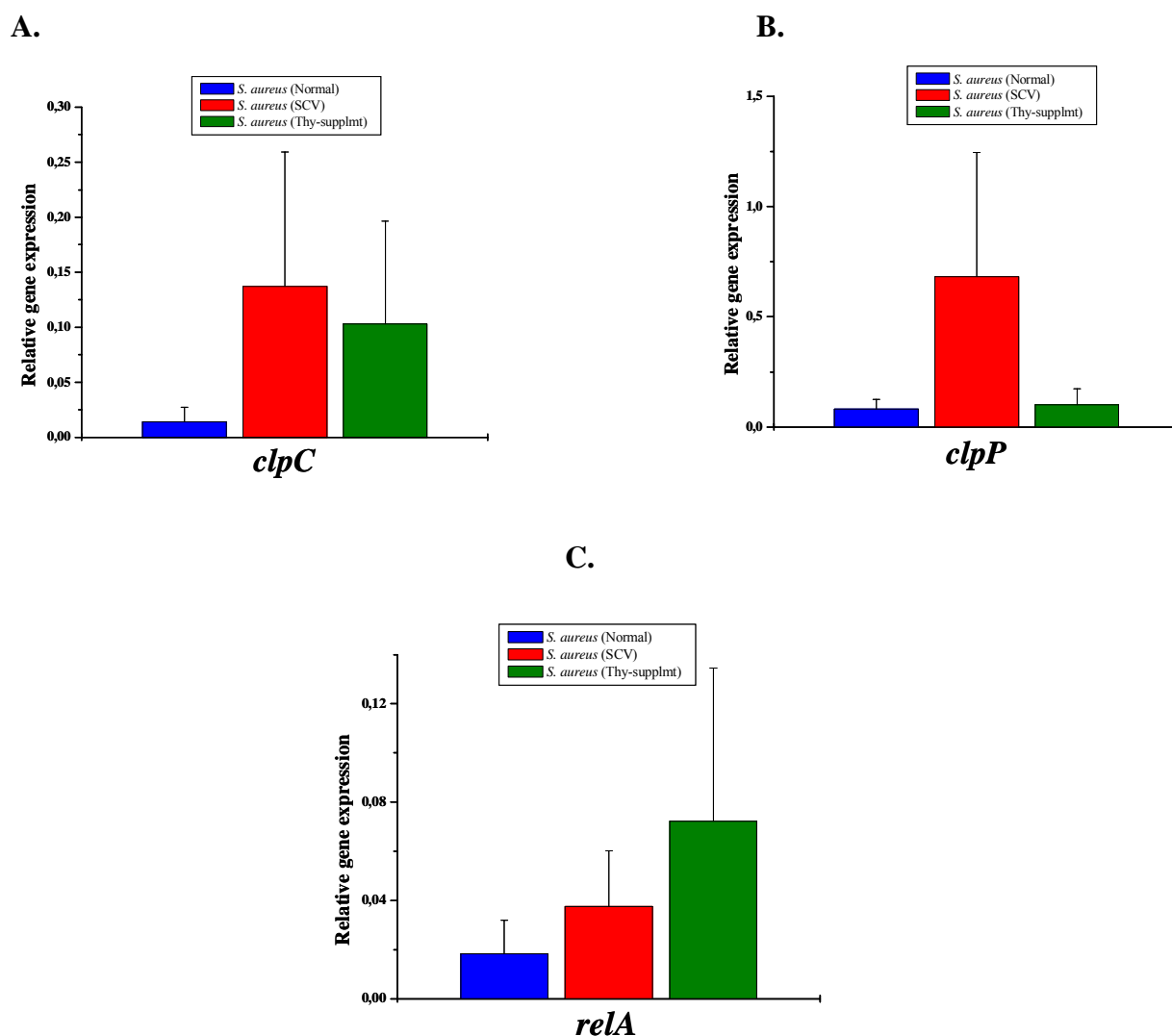
Thymidine-dependent SCVs exhibit a long lag phase (refer to Fig 1A presented in Kahl *et al*, 2005), denoting lack of energy and/or lack of metabolism of critical metabolites required for growth of the cells. Under these conditions, upregulation of the arginine deiminase pathway might serve two purposes. Firstly, as mentioned previously, activation of this pathway allows bacteria to grow anaerobically in the presence of arginine (Maghnouj *et al*, 1998; Kohler *et al*, 2003) producing energy in form of ATP (Fig. 3.3.3.B). A similar situation was also observed in the electron transport deficient (hemin auxotroph) SCV or the *hemB* mutants (Kohler *et al*, 2003). Secondly, activation of the ADI pathway might also lead to biosynthesis of pyrimidine (UMP/dUMP) through carbomoyl~P (Fig. 3.3.3.B), a step which is critical for the physiology of the thymidine-dependent SCV. Thus, upregulation of the *arcA* would be of crucial importance for the survival for the thymidine-dependent SCV during the early phases of growth.

#### d) Genes involved in stress (heat shock proteins) and stringent responses:

During the early phases of growth, stress response genes belonging to class III group of heat shock proteins (HSP100 family) were also upregulated in the thymidine-dependent SCVs. These were *clpC* and *clpP* (Fig. 3.3.4.A and 3.3.4.B), both being under the transcriptional repression of *ctsR*. Clp (caseinolytic protease) protein complexes (eg. ClpCP) play a crucial role in energy-dependent proteolysis, a common mechanism in prokaryotic and eukaryotic cells for intracellular homeostasis and regulation, particularly under stress conditions (Wu *et al*, 1999). The Clp complex is composed of a proteolytic subunit, ClpP, which associates with a Clp ATPase. The latter comprise a protein family designated HSP100 proteins. While Clp ATPases alone have a substrate-specific chaperone function (*i.e.*, refolding and reactivation of proteins), the Clp ATPase-ClpP complex confers protease activity (Wawrzynow *et al*, 1996). The increase in transcription of the heat shock proteins reflects a state of stress within the thymidine-dependent SCV, probably due to the inability to synthesize deoxyribonucleic acid due to defect in

### 3. Results

pyrimidine metabolism. As seen in expression of few other genes, presence of external thymidine was unable to fully restore the expression pattern of wild-type, in the SCVs.



**Fig. 3.3.4.** Real time RT-PCR quantification of relative gene expression of genes for stress and stringent response during early phase of growth. mRNA concentrations of (A) *clpC*, (B) *clpP* and (C) *relA* in clinical *S. aureus* strain (normal), SCV and thymidine-supplemented SCV were determined as described in Experimental procedures. Shown are transcript quantities relative to control (*gyrB*) transcript and expressed as fold-increase. Data are means  $\pm$  standard deviation of triplicate determinations.

A major mechanism which regulates cellular metabolism under starvation-stress conditions is the stringent response (Mechold *et al*, 2002), which involves rapid alterations in gene expression including restriction of stable RNA synthesis, stimulation of certain amino acid biosynthetic pathways and induction of stationary phase-specific

genes. In line with this observation, there was also upregulation of the gene *relA* (**Fig. 3.3.4.C**) coding for GTP pyrophosphokinase (SA1460 according to the *S. aureus* N315 genome designation). Rel of *S. equisimilis* was known to have (p)ppGpp hydrolase activity and, under certain conditions, (p)ppGpp synthetase activity and was involved in the stringent response. In *E. coli*, inhibitors of cell wall or lipid synthesis were reported to increase the amount of intracellular guanosine 3',5'-bispyrophosphate (ppGpp) and overproduction of ppGpp resulted in penicillin tolerance (Rodionov and Ishiguro, 1995). Moreover, it has been shown that accumulation of ppGpp and ppGp in *Staphylococcus aureus* 8325-4 is followed by nutrient starvation (carbon- and amino acid) (Crosse *et al*, 2000).

Though it is not clear whether a stringent response is linked to tolerance in *S. aureus*, it is very interesting that *relA*, thought to be involved in a stringent response, may also be involved in TMP-SMX resistance in thymidine-dependent SCVs, triggered by thymidine limited and low energy conditions.

#### **B) Transcriptional analysis of select genes in thymidine-dependent SCV during stationary phase.**

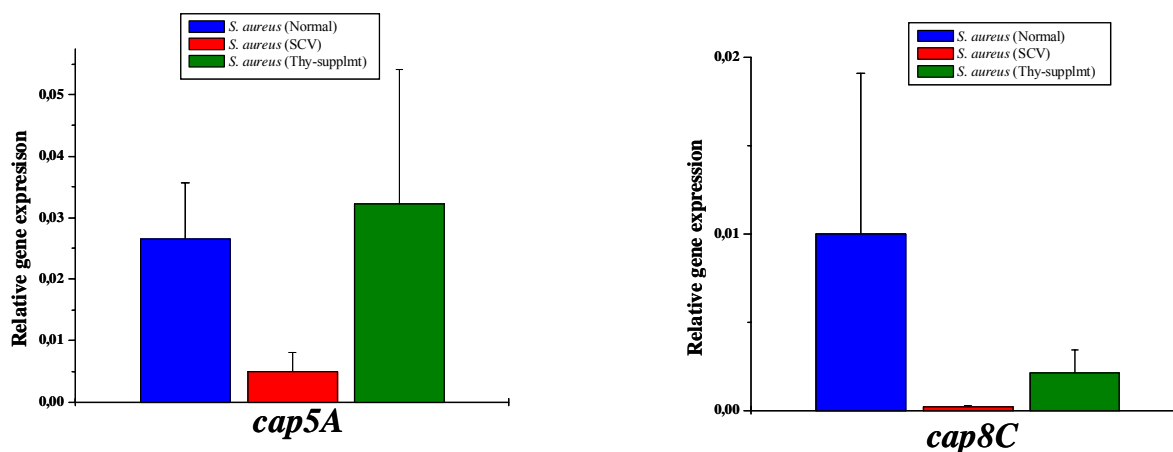
Similar to the previous section, *S. aureus* capsular polysaccharide synthesis, transport of pyrimidines, physiologic and metabolic, and virulence determinant genes were chosen for transcriptional analysis, all of which might be directly or indirectly involved in the observed characteristics of thymidine-dependent SCVs.

##### **a) Genes involved in capsular polysaccharides (CP) biosynthesis:**

The gene expression of two gene clusters, *cap5A* and *cap8C*, involved in the synthesis of *S. aureus* type 5 and type 8 capsular polysaccharides (CPs), respectively were determined. The expression of antiphagocytic polysaccharide capsules (CP) is an important pathogenetic step in establishing staphylococcal infections. They enhance staphylococcal virulence by impeding phagocytosis, resulting in bacterial persistence in the bloodstream of infected hosts and are shown to be regulated by different environmental conditions (culture medium, iron limitation, solid media broth, alkaline and anaerobic growth conditions) and regulators (*agr*, *sae*) (see *reviewed* by O'Riordan and Lee, 2004). In accordance to the published data, expression of both *cap5A* and *cap8C* were found to be increased in the normal strain during the stationary phase of growth

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(Fig.3.3.5.A & B). In contrast, expression of the CPs in the thymidine-dependent SCVs was drastically downregulated (Fig.3.3.5.A & B). Supplementation with thymidine (100µg/ml) in medium could efficiently restore the transcription of CPs in the thymidine-dependent SCVs.



**Fig. 3.3.5.** Real time RT-PCR quantification of gene expression of CP biosynthesis during stationary phase of growth. mRNA concentrations of (A) *cap5A* and (B) *cap8C* in clinical *S. aureus* strain (normal), SCV and thymidine-supplemented SCV were determined as described in Experimental procedures. Shown are transcript quantities relative to control (*gyrB*) transcript and expressed as fold-increase. Data are means  $\pm$  standard deviation of triplicate determinations.

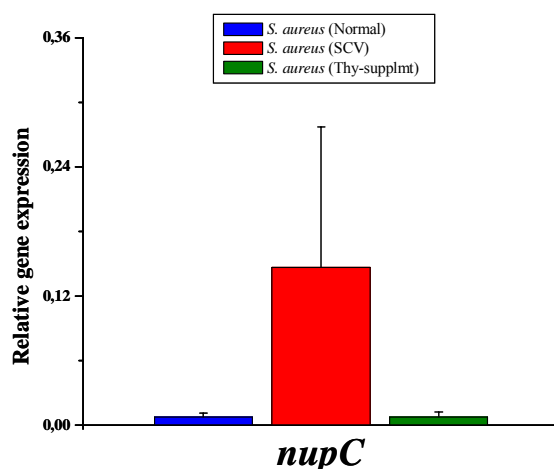
Capsular expression has been shown to vary under different expression conditions *in vivo* and *in vitro*. Studies have shown that CP5 expression was not detected *in vivo* in several staphylococcal infections. Minimal expression of CP5 was observed in either lung tissue or nasal polyp tissue obtained from two cystic fibrosis patients infected with *S. aureus* (Herbert *et al.*, 1997; 1997A). Similarly, when rats were challenged with a serotype 5 *S. aureus* strain in the granuloma pouch model,  $\leq 5\%$  of the cells harvested from the pouch exudates were CP5 positive (Herbert *et al.*, 1997). In both of these tissues, the absence of CP5 expression correlated with elevated CO<sub>2</sub> levels ( $\geq 4\%$ ). As discussed above, CO<sub>2</sub> and/or anaerobiosis has been shown to be an environmental signal that down-regulates CP5 expression *in vitro*. Moreover, capsular polysaccharide expression has been shown to be positively controlled by the global regulator *agr* (Dassy *et al.*, 1993). The *agr* locus is a complex multigene operon in *S. aureus* that acts as a two-component quorum-sensing system. Genes under the control of *agr* include secreted virulence factors and adhesions that are regulated in a growth phase-dependent manner.

Thus, the decrease in the transcriptional level of the CPs may contribute to the characteristics of the thymidine-dependent SCVs which tend to persist in lungs of cystic fibrosis patients (anaerobic and high CO<sub>2</sub> environment) and have the *agr* operon significantly down-regulated.

#### b) Gene involved in pyrimidine nucleoside transport:

We also determined the transcription pattern of a Na<sup>+</sup> dependent pyrimidine nucleoside transporter designated in the *S. aureus* N315 genome as *nupC* (annotated as SA0479). The logic behind selection of this transporter was that lack of dTMP synthesis in the thymidine-dependent SCVs (see *result & figure 3.3.1*) might up-regulate the expression of genes, which are involved in uptake of external thymidine present in the growth medium. The transcription of *nupC* was up-regulated during the early phase of growth in normal, SCVs and the thymidine-supplemented (100µg/ml) SCVs (data not shown). Interestingly, this increase in transcription level was not able to facilitate the growth in the SCV and/or thymidine-supplemented SCVs, during this phase of growth.

The scenario during the stationary phase of growth was entirely different with increased transcription levels of *nupC* presumably due to compensation of thymidine deficiency (**Fig.3.3.6**). This was also evident from the over low growth yields (see *growth curve* in *fig 1A* in Kahl *et al*, 2005) of the thymidine-dependent SCV. When supplemented with external thymidine, the SCVs could grow after a long lag phase. It seems that



**Fig. 3.3.6** Real time RT-PCR quantification of gene expression of *nupC* during stationary phase of growth. mRNA concentrations in clinical *S. aureus* strain (normal), SCV and thymidine-supplemented



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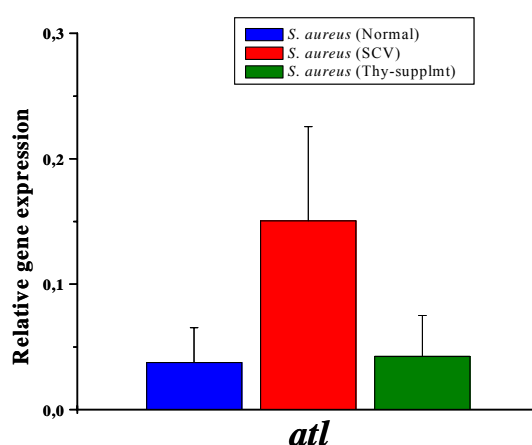
SCV were determined as described in Experimental procedures. Shown are transcript quantities relative to control (*gyrB*) transcript and expressed as fold-increase. Data are means  $\pm$  standard deviation of triplicate determinations.

external thymidine could complement both growth and the expression pattern of most of the genes during the stationary phase of growth.

It has been shown that many of the CF isolates are thymidine auxotrophs, predominantly from patients receiving SXT treatment and as pus is especially rich in thymidine, thymidine-dependent SCVs could uptake thymidine (released from pus by DNase) by an energy dependent process (through membrane spanning NupC) (R. A. Proctor, *personnel communications*). Thus, the transport of external thymidine could act as an important point for the survival/persistence of the thymidine-dependent SCVs.

#### c) Gene for major staphylococcal autolysin:

The staphylococcal *atl* gene products are involved in cell separation, roughened cell wall and penicillin-G induced lysis of the cells and cell wall turnover (Takahashi *et al*, 2002). Accordingly, an *atl* null mutation showed phenotypic alteration of the cell wall structure and thickness, as seen also in the thymidine-dependent SCVs (Kahl *et al*, 2003a). Thereby, we expected a down-regulation of the *atl* transcription in the thymidine-dependent SCVs. In contrast to our expectation, the transcription of *atl* in the thymidine-dependent SCVs was found to be up-regulated (**Fig.3.3.7**).



**Fig. 3.3.7** Real time RT-PCR quantification of transcript expression of *atl* during stationary phase of growth. mRNA concentrations in clinical *S. aureus* strain (normal), SCV and thymidine-supplemented SCV were determined as described in Experimental procedures. Shown are transcript quantities relative to

### 3. Results

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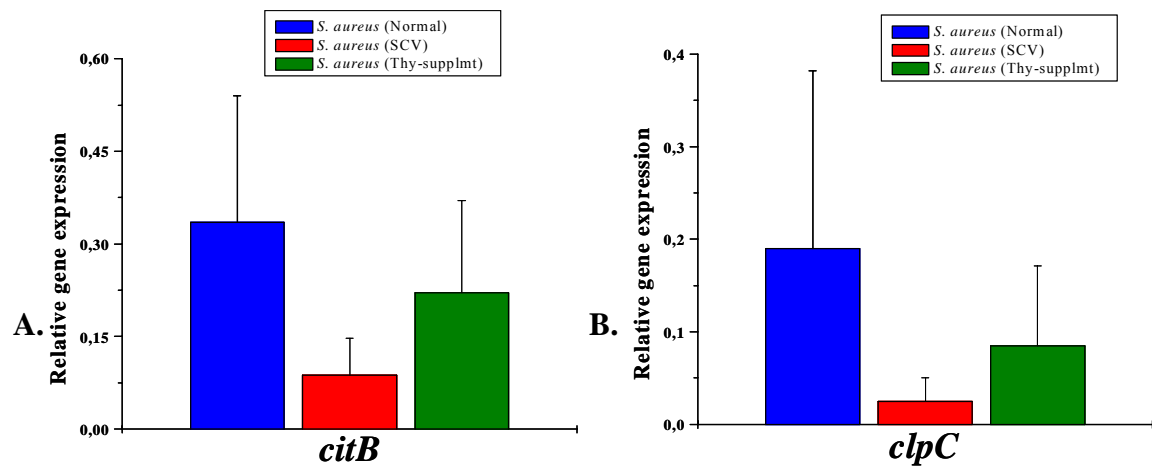
control (*gyrB*) transcript and expressed as fold-increase. Data are means  $\pm$  standard deviation of triplicate determinations.

Possible involvement of *atl* or its related proteins in pathogenesis of staphylococci has been suggested by several groups (Heilmann *et al*, 1997; Hell *et al*, 1998). In *S. epidermidis*, the AtlE (the Atl equivalent of *S. epidermidis*) defective mutant showed loss of initial attachment processes to polystyrene surface (Heilmann *et al*, 1997). AtlE (Heilmann *et al*, 1997), Aas (Hell *et al*, 1998) (the Atl homologue of *S. saprophyticus*), and Ami (Milohanic *et al*, 2001)(Atl like bacteriolytic protein of *Listeria monocytogenes*) have been demonstrated to possess properties equivalent to an adhesin. AtlE was found to bind to vitronectin whereas Aas was found to bind fibronectin (Hell *et al*, 1998). Ami was shown to bind to cultured cells. By analogy, Atl may also possess adhesive property since it too possesses the common cell-wall anchoring motif, which would benefit the thymidine-dependent SCVs in a late stage adhesion [along with decreased levels of toxins (*hla*)]. However, it is difficult to speculate about any direct function of Atl as adhesin without performing further experiments.

#### **d) Regulation of TCA cycle by heat shock protein:**

Recently, we could that ClpC is required for growth in the post-stationary phase as a result of acetate utilization and restoration of transcription and protein activity of TCA enzyme aconitase (*citB*) and involved in the death of *S. aureus* cells during late growth phases (Chatterjee *et al*, 2005 and *Chapter 3.1*). In line with this observation, we could see that the transcription of both *clpC* (encoding for class III heat shock protein ClpC ATPase) and *citB* (encoding for the TCA cycle enzyme aconitase) were down-regulated during the stationary phase of growth (**Fig.3.3.8A** and **3.3.8B**). This was a very interesting observation as lack of TCA cycle metabolism, due to down-regulation *clpC*, would eventually result the thymidine-dependent SCVs to an enhanced stationary survival phase (or lack of death phase as seen in the *S. aureus clpC* mutant) thereby increasing their persistence within the cells. Further experiments (stationary phase survival assay, glucose and acetate assays, metabolic enzyme activity assays) need be performed in future projects in order to confirm these hypotheses.

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**Fig. 3.3.8.** Real time RT-PCR quantification of transcript expression of (A) *citB* and (B) *clpC* during stationary phase of growth. mRNA concentrations in clinical *S. aureus* strain (normal), SCV and thymidine-supplemented SCV were determined as described in Experimental procedures. Shown are transcript quantities relative to control (*gyrB*) transcript and expressed as fold-increase. Data are means  $\pm$  standard deviation of triplicate determinations.

## **Chapter 4**

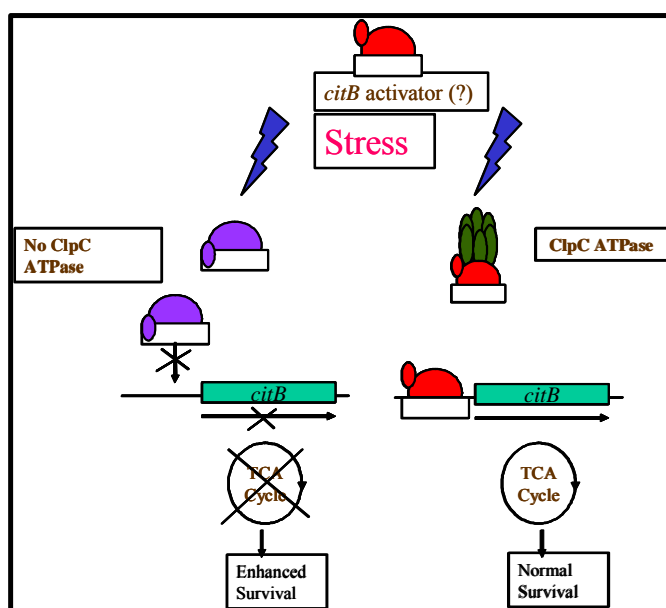
# **DISCUSSION**

## 4. Discussion

### 4.1 *Staphylococcus aureus* ClpC ATPase and late-stationary phase persistence

In *Chapter 3.1*, a role of ClpC on post-stationary growth recovery and death is described, and these effects could be related to the activity of aconitase resulting in a functional TCA cycle (**Fig. 4.1.1**). Furthermore, a  $\sigma^B$ -independent effect of ClpC on stress resistance could be demonstrated. To our knowledge, this is the first report implicating a *S. aureus* chaperone as being necessary for metabolism and growth following the stationary phase. An appraisal of these findings includes the following discussion.

During the exponential phase of growth, *S. aureus* preferentially catabolizes glucose via glycolysis to generate pyruvate (Kohler *et al*, 2003; Somerville *et al*, 2003a). Under aerobic growth conditions, pyruvate is catabolized to acetyl-CoA, and subsequently into acetyl-phosphate (Gardner and Lascelles, 1962; Somerville *et al*, 2003a). Acetyl-phosphate is used for substrate level phosphorylation generating ATP and the by-product acetate. Consistent with these observations, our data demonstrate a rapid depletion of glucose from the culture medium and a concomitant increase in the concentration of acetate.



**Fig. 4.1.1** Hypothetical model for the regulation of the TCA cycle enzyme aconitase activity by ClpC.

## 4. Discussion

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Derepression of the TCA cycle occurs upon depletion of the readily catabolizable carbon source(s) and/or glutamate and coincides with the depletion of acetate from the culture medium. It is thought that the depletion of rapidly catabolizable carbon sources or possibly multiple-nutrient limitations as well as an acidic pH contributes to the decrease in the number of viable cells during the stationary phase (Watson *et al.*, 1998). As expected, the cell densities and the viable count of WT and mutant strains decreased upon entry into stationary phase. Subsequently, *S. aureus* may display growth recovery (Patton *et al.*, 2005). The prevention of growth recovery and the stationary-phase survival in the *clpC* mutant, however, can be related to the findings of our previous study using a *S. aureus citB* mutant (Somerville *et al.*, 2002) which demonstrated stationary-phase survival similarly enhanced as the *clpC* mutant and *clpC sigB* mutant in this study. This should be seen in context with previous reports on stationary phase survival in *B. subtilis*: the lack of expression of *citB* (Craig *et al.*, 1997) or *clpP* (Msadek *et al.*, 1998) in respective *B. subtilis* mutants was shown to account for the loss of post stationary phase growth and/or survival. However, important species differences in metabolism or the differential role of *clpP* vs. *clpC* might account for the apparent discrepancies with our findings in *S. aureus*. In further extension to our earlier work (Somerville *et al.*, 2002), it therefore seems that TCA cycle inactivation due to lack of ClpC completely prevents post-stationary phase recovery in a complex medium and that TCA cycle is essential for entry into the death phase.

In the stationary phase, bacteria are exposed to a variety of environmental stress factors such as nutrient limitation and accumulation of toxic bacterial metabolic products, particularly reactive oxygen species (ROS) (Becerra and Albesa, 2002). Clp protein complexes are important for reactivation and refolding damaged proteins under stress conditions (Hecker *et al.*, 1996). The importance of the *S. aureus* heat shock proteins, ClpX and ClpP on stress tolerance and virulence has been demonstrated (Frees *et al.*, 2003). Interestingly, in this report the absence of the ClpX ATPase conferred enhanced growth at either elevated temperatures or higher puromycin concentrations. It was suggested that a Clp ATPase different from ClpX cooperates with ClpP in the degradation of stress-damaged proteins, and that ClpX antagonizes this cooperation. Our data demonstrate that inactivation of ClpC enhances sensitivity to heat shock, suggesting that ClpC may be the protein cooperating with ClpP ATPase. This contention is supported by

the observation that following a heat shock *clpP* and *clpC*, but not *clpX* expression were enhanced.

In contrast to a previous report (Gertz *et al*, 2000), we found that heat stress did not induce the expression of *clpL*. This apparent contradiction might be explained as follows:  $\sigma^B$  dependent gene expression in response to heat shock is known to be transient, being highest shortly after the heat stress is applied (within 3 to 6 min) and decreasing thereafter to or below the uninduced level (> 12 min) (Gertz *et al*, 1999; Gertz *et al*, 2000). In our studies, the heat shock was applied for 15 min, a period that might have been too long to detect heat shock-mediated  $\sigma^B$ -dependent effects on *clpL* expression. Alternatively, culture media and growth conditions might have caused the lack of heat shock effect on *clpL* (Gertz *et al*, 1999 and M. Bischoff, unpublished results).

Further, it was reported that *S. aureus* resistance to hydrogen peroxide was not dependent on ClpC (Frees *et al*, 2004). These experiments were performed with exponential-phase SH1000 and 8325-4, and we could confirm these results using both SH1000 and DSM20231. However, when using stationary phase organisms, ClpC indeed was important for growth in presence of hydrogen peroxide as shown in this manuscript. Thus, the growth phase appears to be crucial for the role of ClpC in stress resistance towards hydrogen peroxide.

The alternative sigma factor  $\sigma^B$  has been shown to be important for responding to environmental stimuli (*e.g.*, pH, heat shock), growth phase-specific signals (*e.g.*, stationary phase of growth) and ATP- limitation conditions in *B. subtilis* (Hecker *et al*, 1996) and in *S. aureus* (Kullik *et al*, 1998). Therefore, in addition to the analysis of ClpC we examined the role of  $\sigma^B$  on post- stationary phase recovery. In fact, in contrast to data obtained for *clpC*, these results do not suggest a role of  $\sigma^B$  during these phases of growth. Furthermore, the impact on survival after oxidative and heat stress was more pronounced in the *clpC* mutant when compared to the *sigB* mutant. Thus, we conclude that the effect of ClpC on late stationary growth and survival as well as on stress resistance is independent of cooperativity with  $\sigma^B$ .

## 4. Discussion

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In addition to its role on stress resistance in *S. aureus*, ClpC may have a more specific role on the oxygen-dependent bacterial metabolism at late stationary phase. Our finding that *citB* transcript levels were elevated in late stationary phase concurs with our previous reports on a functional aconitase and TCA cycle in *S. aureus* (Somerville *et al*, 2002). Compared to WT, however, *citB* expression during late stationary phase was significantly reduced in the *clpC* mutant. Moreover, while no difference was observed with respect to glucose catabolism, both the *clpC* and the *clpC sigB* mutants (but not the *sigB* mutant) failed to catabolize acetate during post-stationary phase recovery. These findings concur with those from our recent study, where we have shown that a *S. aureus*  $\Delta$ *citB* mutant failed to grow post-exponentially and to catabolize acetate in a rich medium demonstrating that a fully functional TCA cycle is essential for acetate catabolism (Somerville *et al*, 2002; Kohler *et al*, 2003). Our assumption, *i.e.* a functional defect of the TCA cycle activity in the *clpC* mutant, could subsequently be confirmed upon demonstration of a marked reduction of aconitase activity and restoration in the *clpC*-complemented mutant. Taken together, our observations clearly demonstrate the regulation of the TCA cycle enzyme aconitase activity by ClpC. They also indicate that aconitase activity albeit not post-stationary recovery is in part  $\sigma^B$  dependent, an observation in accordance with our previous data (Somerville *et al*, 2003a).

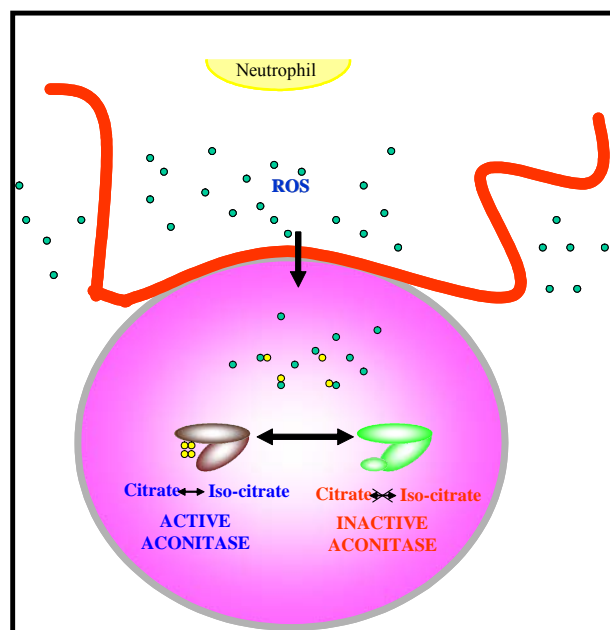
The ability to regulate the TCA cycle in staphylococci forms an important step for its increased survival and persistence. The activity of TCA cycle enzymes is affected by the nutritional status of the cell and a variety of stress-inducing stimuli. TCA cycle activity can be disrupted by certain stress inducing stimuli such as heat (Tomlins *et al*, 1971) and ethanol (Kornmann *et al*, 2003; *Chapter 3.2*). A recent study demonstrated that altering TCA cycle activity would effect PIA production, an important step towards staphylococcal biofilm formation. It was shown that increased PIA production in *S. epidermidis* is associated with decreases in TCA cycle activity (Vuong *et al*, 2005). Biofilms are the leading cause of staphylococcal infection in indwelling medical devices (*eg.* catheters) (Vuong *et al*, 2004). Our previous studies have shown that ClpC ATPase was expressed within biofilm forming population of staphylococci and present study (*Chapter 3.1*) could demonstrate the regulation of the TCA cycle by ClpC. Thus, ClpC could be speculated to forms an important link between the transition from actively growing planktonic cells (increased TCA cycle and decreased PIA production) and static



## 4. Discussion

biofilm forming cells (decreased TCA cycle and increased PIA production), thereby forming an important protein involved in regulation of persistence of *S. aureus*.

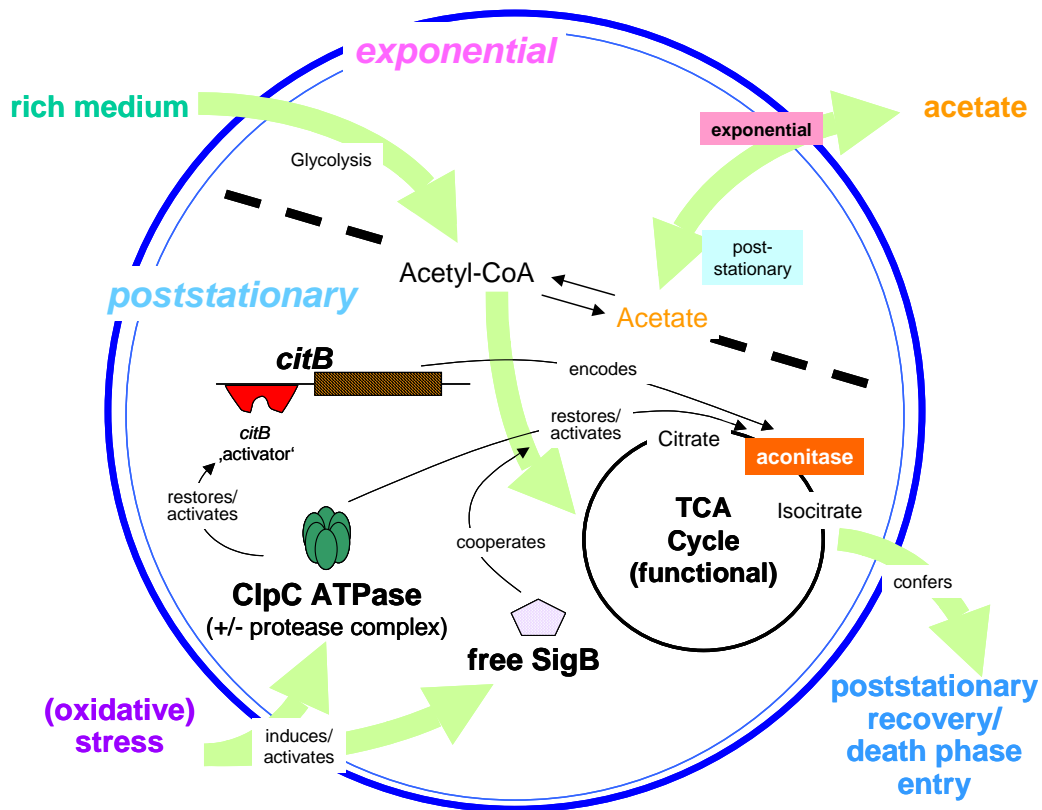
Additionally, the *S. aureus clpC* mutants demonstrated an enhanced stationary phase survival due to the decreased aconitase activity. Inactivation of aconitase has very serious *in vivo* implication for the long-term survival and persistence of *S. aureus*. Aconitase is a TCA cycle enzyme that converts citrate to isocitrate via a cis-aconitate intermediate. The enzymatic function is mediated by an [4Fe-4S] iron-sulfur center that is necessary for binding of the substrate. Three of the iron atoms in the [4Fe-4S] center are coordinately bound by three conserved cysteine residues. The fourth iron atom is held in place by a molecule of substrate and water (Kennedy *et al*, 1987). In the absence of substrate, the fourth iron atom is susceptible to reversible inactivation by oxygen radicals (Gardner and Fridovich, 1992). As part of the innate immune response, phagocytic leukocytes produce reactive oxygen species to limit the growth and dissemination of bacteria and it has been shown that that early in an infection aconitase is reversibly inactivated, resulting in decreased production of formylated  $\delta$ -toxin a chemo-attractant for human leukocytes (Somerville *et al*, 2003b). Taken together, these data points towards that reversible inactivation of aconitase suppress the pro-inflammatory immune response (**Fig. 4.1.2**).



**Fig. 4.1.2** Model for reactive oxygen species (ROS)-mediated aconitase inactivation and the consequences of such inactivation (*reproduced with permission of Greg A. Somerville*).

It has been reported previously that both aconitase (CitB) and ClpC ATPase inactivation significantly enhances stationary-phase survival (Somerville *et al*, 2002; Chatterjee *et al*, 2005). Thus, the fine-tuning of *clpC* expression may not only regulate the metabolic state and proliferation but also influence virulence of a given *S. aureus* population through regulation of aconitase (and there by TCA cycle) which in turn reduces inflammation, decreases the production of virulence factors, and promotes long-term bacterial survival and persistence.

**Possible role of ClpC in post-stationary phase recovery.** The mechanism by which ClpC exerts this profound effect on aconitase activity and the resulting changes in TCA metabolism has yet to be further defined. ClpC might be acting in several ways (**Fig. 4.1.3**). It could be a chaperone for CitB (aconitase), which is a DNA binding protein and could be reversibly inactivated by oxidative stress (due to presence of the [4Fe-4S] cluster). ClpC might also be restoring/activating regulators of *citB* expression and activity (**Fig. 4.1.3**). The observed important transcriptional reduction of the *citB* gene (**Fig. 3.1.5**; 25.3 fold decreased compared to wild type at 96 h) in the *clpC* mutant suggests that the absence of ClpC during the late stationary phase might lead to energy limitation due to compromised TCA cycle activity. Overall, this might explain the demonstrated role of ClpC for the observed energy requiring phenomena of post-stationary phase recovery followed by entry into the death phase.



**Fig. 4.1.3** A hypothetical model of the role of ClpC in oxidative metabolism of *S. aureus*. During exponential growth in rich medium, glucose is rapidly catabolized which finally accumulates as acetate in the medium. During late stationary phase (such as in biofilm populations), the bacterial cell yields free ATP primarily by oxidative metabolism provided by TCA cycle activity. The function of the key TCA cycle enzyme, aconitase, requires ClpC since functional deletion of *clpC* results in a marked reduction in *citB* (aconitase) transcription along with loss of aconitase activity and persistent acetate accumulation. Under oxidative stress conditions, ClpC might be either restoring/activating yet unknown activator(s) of *citB*, which in turn encodes active aconitase, and/or by activating/restoring the activity of aconitase protein. This in turn confers post stationary phase growth followed by entry into death phase. Free  $\sigma^B$  also seems to cooperate in activating/restoring the aconitase activity, through yet unknown pathways.

Our findings may have important implications for the *in vivo* situation. Recently, we have shown that aconitase inactivation not only causes reduced cell densities post-exponentially but also impairs the production of secreted *agr* dependent virulence factors (Somerville *et al.*, 2002; Somerville *et al.*, 2003a). Moreover, impaired aconitase activity may suppress neutrophil migration normally elicited by the aconitase-dependent production of formylated peptides such as  $\delta$ -toxin (Somerville *et al.*, 2003b). Thus, the fine-tuning of ClpC activity, *e.g.* due to modulation of *clpC* expression in established biofilms (Becker *et al.*, 2001), may not only be important for post-stationary phase recovery in inanimate and *in vivo* environments but might also influence a number of *S. aureus* virulence mechanisms. Hence, our findings on ClpC modulation of oxidative metabolism extend our understanding of the overall physiology of this pathogen and can be related to other metabolic adaptive mechanisms resulting in ‘small colony variant’ or persister states.

Apart from the regulation of oxidative metabolism, the *clpC* mutant was found to be involved in regulation of other important physiological & metabolic pathways, involving energy metabolism and stress regulation, as indicated by the protein profile of the 2D gel analysis (Chapter 3.2A). Threonine deaminase homologue (IlvA) was one of the proteins found to be up-regulated in the *clpC* mutant under both growth phases [post-exponential (8 h) and post-stationary phase (80 h)]. The *ilvA* (isoleucine, leucine, valine genes) increase probably relates to cell membrane stability. This increase probably relates to cell membrane stability (Martin *et al.*, 1999) of the *clpC* mutant. An increase in iso-branched fatty acids decreases membrane fluidity and subsequently, a non-fluid membrane is more stable and rigid, which would preferably favour late stationary survival of the *clpC* mutant (Chapter 3.2A) as it is more resistant to unsaturated fatty acids (UFA), lipids, and oxidant stress (Martin *et al.*, 1999).

Another staphylococcal protein involved in oxidative stress response is the alkyl hydroperoxide reductase subunit CF (AhpC and AhpF). These are under the regulation of the PerR regulon. PerR seems to control one of the most essential regulons, the products of which may protect *S. aureus* cells against oxidative stress challenge (Horsburgh *et al.*, 2001). *katA* (catalase), *ahpCF* (alkylhydroperoxide reductase), *mrgA* (Dps-like protein), *bcp* (bacterioferritin co-migratory protein) and *trxB* (thioredoxin reductase) are members of the PerR regulon that are derepressed in a *perR* mutant (Horsburgh *et al.*, 2001). Thus,

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up-regulation of both transcription and protein levels of AhpCF denotes that the *clpC* mutant cell is under constant oxidative stress conditions. A recent article reported that *S. aureus* cells undergoes increased oxidative damage to cellular constituents on entry into stationary phase (Nyström, 2005). Moreover, up-regulation of these proteins also supports the hypothesis of increased oxidative stress incurred by the *clpC* mutant (as discussed above) there by inactivating aconitase, down-regulating the TCA cycle activity followed by enhanced stationary phase survival.

The *clpC* mutant was further shown (*Chapter 3.2B*) to induce enhanced stationary phase tolerance to the fluoroquinolone, moxifloxacin (MFX). This was found to be through differential gene regulation of cell wall and stress related genes. Important among these were:

a) the regulation of the Lrg and Cid gene products are molecular control elements involved in the regulation of programmed cell death (PCD) in *S. aureus* (Bayles, 2003; Rice and Bayles, 2004; Rice *et al*, 2005; Patton *et al*, 2005). Accordingly, we found *cidA* to be down-regulated and *lrgA* to be up-regulated, both of which might contribute to enhanced antibiotic (MFX) tolerance.

b) the up-regulation of two other class III heat shock proteins, *clpB* and *clpP* in the *clpC* mutant during the enhanced stationary phase of survival, which might be involved in an yet unknown and important role in diverting the staphylococcal cells from entering death phase into enhanced stationary phase, under energy deprived conditions, though further experiments could be required to confirm these hypothesis.

Therefore, these new observations (*Chapter 3.2*) on ClpC modulation of oxidative and energy metabolism extend our understanding of the overall physiology of this pathogen and can be related to other metabolic adaptive mechanisms, required for adaptation, survival and persistence under various conditions of stress. The most important observation is that most of these genes were already up-regulated in the *clpC* mutant, and not after addition of the antibiotic. This indicates that the *clpC* mutants undergo a drastic shift in gene regulation in absence of TCA cycle, and while transiting into the post-stationary phases (> 96 h). Broadly, lack of ClpC led to enhanced stationary phase

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tolerance to MFX, most probably by differential gene regulation of cell wall and stress related genes.

In conclusion, our work has provided insight in new adaptative response mechanisms comprising of metabolic enzyme and regulator over-expression in *S. aureus* which may account for a number of direct or indirect mechanisms involved in cell-cell-interaction, long-term survival and persistence. These studies underline the necessity to further characterize the regulation of *S. aureus* grown in a biofilm, particularly with respect to the Clp complex system.

## 4.2 Effects of very low ethanol concentration on adaptation and persistence of *Staphylococcus aureus*

Our results indicate that the transition from primary to secondary metabolite catabolism is delayed by VLEC. *S. aureus* preferentially catabolize glucose to generate for carbon and energy, a process resulting in the accumulation of organic acids in the culture medium (Somerville *et al*, 2002; Kohler *et al*, 2003; Somerville *et al*, 2003a). Our results are consistent with these observations, as glucose is rapidly consumed and the pH of the culture medium decreased due to the accumulation of lactate and acetate. Notably, the consumption of glucose and the acidification of the culture medium were unaffected during exponential phase in VLEC+ conditions; however, VLEC resulted in a delayed transition from glucose catabolism to secondary metabolite catabolism (Somerville *et al*, 2003a; Chatterjee *et al*, 2005). The delayed transition to the catabolism of non-preferred carbons sources also resulted in decreased amino acid catabolism (**Fig. 3.3.5**). In *S. aureus*, acetate catabolism requires tricarboxylic acid (TCA) cycle activity but staphylococci lack the glyoxylate shunt. Hence, for every 2 carbons that enter into the TCA cycle as acetyl-CoA, 2 carbons are lost during the oxidative decarboxylation reactions. That is to say, if any carbons leave the TCA cycle in the form of biosynthetic intermediates, then those carbons must be replaced for the TCA cycle to continue to function. Staphylococci replace lost carbons through the catabolism of amino acids; hence, a decrease in acetate catabolism results in a decrease in amino acid catabolism.

Additionally, VLEC+ conditions selectively inhibited the utilization of amino acids such as glutamate, proline, and ornithine. D-glutamate is found in the second position of the peptidoglycan stem peptides in virtually all species analyzed thus far (Schleifer and Kandler, 1972), and is essential for growth in *E. coli* (Lugtenberg *et al*, 1973) and *S. aureus* (Fass *et al*, 1970; Chatterjee and Young, 1972; Good and Tipper, 1972). The other ‘glutamate family’ amino acids ornithine and proline can be converted into glutamate: ornithine by the ornithine aminotransferase (SA0818) and the  $\Delta^1$ -pyrroline-5-carboxylate dehydrogenase (SA2341), and proline by the proline dehydrogenase (SA1585). Thus, the inability to acquire, or synthesize, glutamate in VLEC+ conditions may contribute to the cell lysis in presence of ethanol.

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Ethanol enhances the ability of staphylococci to form a biofilm (Knobloch *et al*, 2004). Recent transcriptional profiling data on staphylococci growing in biofilms has suggested the bacteria are growing anaerobically (Beeken *et al*, 2004; Resch *et al*, 2005; Yao *et al*, 2005). Consistent with that suggestion, these studies noted an increased expression of the anaerobic alternative energy generating arginine deiminase (ADI) pathway (Beeken *et al*, 2004; Resch *et al*, 2005; Yao *et al*, 2005). The ADI pathway is comprised of three enzymes, arginine deiminase (*arcA*), ornithine transcarbamoylase (*arcB*), and carbamate kinase (*arcC*). Together, these enzymes convert arginine to ornithine, ammonia, and carbon dioxide yielding 1 mol of ATP per mol of arginine consumed. Our data demonstrate that ethanol up-regulates expression of the ADI pathway, leading us to speculate that ethanol enhances biofilm formation, in part, through an alteration of the metabolic flux toward the ADI pathway.

Another potentially interesting point is that even though most of the staphylococci cells in VLEC induce a response similar to autolysis [programmed cell death (PCD)], in order survive the hostile growth conditions, a small fraction of cells remain fully viable after prolonged incubation (day 8). A historical publication in Lancet (Bigger, 1944) demonstrated a similar observation where addition of penicillin to staphylococci produced lysis, even though a small fraction of cells ( $10^{-6}$ ) remained fully viable. This seems to be an “intelligent” strategy to avoid disastrous outcome of PCD based suicide which could eliminate all cells of a clonal population in response to damage (Lewis, 2000). These “persisters” are not mutants as firstly their growth is completely sensitive to VLEC, and secondly their progeny are not more resistant to lysis by VLEC than the original population. The existence of the persisters suggests that a bacterial cell potentially has a choice of whether to live or die; the most interesting point is not why the majority of the cells choose to be killed by VLEC, but how the rare cells survives. This phenomenon of persistence has been recorded in a wide variety of bacterial species treated with a range of deleterious factors (Koch, 1996).

Thus, the role of VLEC on staphylococcal metabolism should be considered on biological and innate surfaces, during de-colonization and disinfection processes, in biological sciences (antibiotic stock solutions). Moreover, the present working model could help us to determine the role of persisters in both planktonic and biofilm populations, even in presence of other damaging factors (including anti-bacterials)



#### 4. Discussion

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(Levy, 1998). Antibiotic-resistant strains have become a major clinical problem, and persisters, especially those found in biofilm cells, may be important intermediates in the development of resistance.

In conclusion, to our knowledge this is the first report demonstrating the effects of VLEC on *S. aureus* growth, viability, metabolism, and cell wall morphology. These effects of VLEC were only evident after the complete depletion of ethanol from the culture medium, suggesting that bacterial recovery from, and adaptation to, ethanol stress is a prolonged process. These observations are incongruent with a prevailing dogma, *i.e.* that bacteria rapidly adapt or die when exposed to disinfectants, and open new perspectives in our understanding of bacterial senescence in the presence of subinhibitory concentrations of antiseptic agents.

### 4.3 Thymidine-dependent small colony variants of *Staphylococci*: A link to persistent infection

*S. aureus* SCVs are highly prevalent in respiratory secretions of CF patients, persist over extended periods, and may contribute to *S. aureus* persistence in CF patients (Kahl *et al*, 1998). These SCVs are associated with persistent, antibiotic resistant, and relapsing infections. The potential of SCVs to cause resistant and recurrent infections may arise from their ability to reside within host cells without causing lysis of the host cells (Balwit *et al*, 1994). The intracellular milieu may offer several benefits to the SCVs. This localization shields the bacteria from host defenses and minimizes the ability of antibiotics to reach these organisms. Moreover, the intracellular milieu provides a biochemical environment that allows the auxotrophs to survive (*eg.* thymidine). Thus, the ability of *S. aureus* to cause infections that are slow to respond to antibiotic therapy and that may relapse after a prolonged disease free interval may be due in part to the intracellular survival of an SCV subpopulation (Proctor *et al*, 1994)

Since many bacterial species form SCVs (von Eiff *et al*, 1997; Bates *et al*, 2003; Kohler *et al*, 2003; Senn *et al*, 2005), the complex transcriptional regulations demonstrated by the thymidine-dependent SCVs may prove to be valuable for understanding the relationships between bacterial metabolism and the expression of virulence factors. Several investigators (Howden, 1981) have described the emergence of TMP-SMX-resistant *S. aureus* isolates in CF patients during or after therapy with this agent. Resistance of these organisms is presumably due to their ability to bypass the folic acid synthetic pathway, which is blocked by TMP-SMX, and directly procure thymidine, a major end product of this pathway, from their environment (Then *et al*, 1982). Because a medium with low thymidine content will not support the growth of these *S. aureus* strains, these organisms are referred to as thymidine-dependent (Gilligan *et al*, 1987). Accordingly, during early log phase of growth, expression of *thyA* and *dfrA* was up-regulated at the same time (probably co-transcribed), due to low thymidine in the external growth medium (*Chapter 3.4*). Virulent determinant genes like *fnbA* and *icaA* were also up-regulated during this period of time, which would probably facilitate adherence of the SCVs. Another important gene up-regulated was the arginine deiminase (*arcA*). Up-regulation of the ADI pathway might provide both energy for the bacteria to grow

anaerobically in presence of arginine, and intermediates for synthesis of the pyrimidines. At the same time, the heat shock proteins *clpC* and *clpP* were also upregulated, denoting a stressful condition due to SCVs inability to synthesize pyrimidines. During the stationary phase of growth, the genes transcribing for CPs were down-regulated in the thymidine-dependent SCVs. This tends them to persist in lungs of CF patients (anaerobic and high CO<sub>2</sub> environment). Along with this, there was also an upregulation of *nupC*, indicating that the thymidine-dependent SCVs were starving for thymidine and were trying to uptake it from the environment. The thymidine starved SCV cells were also found to have a reduced TCA cycle level (reduced *citB* expression) probably regulated by simultaneous reduction of *clpC* expression (as discussed in *Chapter 3.1*). A similar condition was also observed in electron transport chain variant SCV, denoted by a *hemB* mutant (Kohler *et al*, 2003). Thus, the transcriptional analysis of the thymidine-dependent SCVs reflects their entrance into a “persister state” during the stationary phase, which would facilitate its intracellular survival. Moreover, because the intracellular concentrations of thymidine are low, the SCV phenotype can be maintained.

Thus, the transformation of *S. aureus* into an SCV may be a potent strategy for protection against host defenses and antibiotic therapy, and thereby making them candidate for causing some of the more difficult to eradicate human infections, especially in CF patients. To further evaluate the persistence and the virulence of the thymidine-dependent SCVs, we plan to construct a stable *thyA* mutant and perform further investigation of its physiology and metabolic status during growth in complex medium and in an appropriate animal infection model.

### 5. Summary

*Staphylococcus aureus* is a very adaptable organism and can live in a wide variety of environments, many of which will be stressful. Therefore, it needs and has regulatory systems to manage the stress. Previously, it has been demonstrated that Clp homologues are important for a variety of stress conditions, and our laboratory has shown that a *clpC* homologue was highly expressed in the *S. aureus* strain DSM20231 during biofilm formation relative to planktonic cells. Persistence and long-term survival is a hallmark of biofilm-associated staphylococcal infections as cure even in the presence of bactericidal antimicrobials frequently fails.

In the first part of the study [**Chapter 3.1**], the role of *clpC* in this context was determined. Metabolic, gene expression, and long-term growth and survival analyses of DSM20231 as well as an isogenic *clpC* allelic-replacement, a *sigB*, and a *clpC sigB* double mutant were performed. As expected, the *clpC* mutant showed increased sensitivity to oxidative and heat stress. Unanticipated, however, was the reduced expression of the tricarboxylic acid (TCA) cycle gene *citB* (encoding aconitase), resulting in the loss of aconitase activity and preventing the catabolization of acetate during the stationary phase. *clpC* inactivation abolished post-stationary phase recovery but also resulted in significantly enhanced stationary-phase survival compared to the wild-type strain. These data demonstrate the critical role of the ClpC ATPase in regulating the TCA cycle and implicate ClpC as being important for recovery from the stationary phase and also for entering the death phase.

In an effort to further characterize the functions of ClpC ATPase [**Chapter 3.2A**], we performed the proteomic analysis of the *clpC* mutant at two different phases of growth. We could show that apart from the regulation of oxidative metabolism, the *clpC* mutant was found to be involved in regulation of other important physiological & metabolic pathways, involving energy metabolism and stress regulation, as indicated by the protein profile of the 2D gel analysis. Together these results suggest that heat shock protein ClpC ATPase might be involved in a broader aspect of staphylococcal metabolism and stress regulation.

The *S. aureus clpC* mutant had enhanced post-stationary phase survival and it is known that the stationary phase surviving cells would have enhanced resistance to antibiotics. In order to show this [**Chapter 3.2B**], we decided to determine the antibiotic sensitivity (moxifloxacin) of the *clpC* mutant by exposing stationary phase surviving cells (120 h/ 5 days) to increasing concentration of MFX. Thus, we could show that lack of ClpC led to enhanced stationary phase tolerance to MFX, most probably by differential gene regulation of cell wall and stress related genes. Understanding the stationary and post-stationary phase recovery in *S. aureus* may have important clinical implications, as little is known about the mechanisms of long-term persistence of chronic *S. aureus* infections associated with formation of biofilms. These studies help us to illustrate the complexity and interrelationship of the response to Clp systems with those involved in long-term persistence of *S. aureus*. More research is needed into stress responses with particular reference to the role played by other Clp ATPase units.

The third part of this study [**Chapter 3.3**] deals with the effect of very low ethanol concentration (VLEC) on physiology and metabolism of *S. aureus*. Pharmaceuticals, culture media used for *in vitro* diagnostics and research, human body fluids, and environments can retain very low ethanol concentrations (VLEC) ( $\leq 0.1\%$  vol/vol). In contrast to the well-established effects of elevated ethanol concentrations on bacteria, little is known about the consequences of exposure to VLEC. We supplemented growth media for *Staphylococcus aureus* strain DSM20231 with VLEC (=VLEC+ conditions) and determined ultra-morphology, growth, and viability compared to unsupplemented media (VLEC- conditions) for prolonged culture time (up to 8 days). VLEC+ grown late stationary phase *S. aureus* displayed extensive alterations of cell integrity as shown by SEM. Surprisingly, while ethanol in the medium was completely metabolized during exponential phase, a profound delay of *S. aureus* in post-stationary phase recovery (>48 h) was observed. Concomitantly, in VLEC+ conditions the concentration of acetate in the culture medium remained elevated while ammonia was reduced, contributing to an acidic culture medium and suggesting decreased amino acid catabolism. Interestingly, amino acid depletion was not uniformly affected: in VLEC+ conditions, glutamic acid, ornithine, and proline remained in the culture medium while the uptake of other amino acids was not affected. Supplementation by arginine, but not by other amino acids, was able to restore post-stationary growth and viability. Taken together, these data

demonstrate that VLEC has profound effects on the recovery of *S. aureus* even after ethanol depletion, delays the transition from primary to secondary metabolite catabolism, and suggests that the concentration of ethanol needed for bacteriostatic control of *S. aureus* is lower than previously reported.

Since an association of persistence and the presence of *Staphylococcus aureus* small-colony variants (SCVs) and relapsing infection has been reported, a renewed interest in infections due to staphylococcal SCVs has emerged. Device-related infections, skin and soft tissue infections, chronic osteomyelitis, and persistent airway infections in patients with cystic fibrosis have been associated with this naturally occurring subpopulation. All cases reported impressively illustrate the poor clinical and microbiological response to even prolonged antimicrobial therapy in patients infected with these variants. The potential of these variants to persist intracellularly within non-professional phagocytes shields SCVs from host defences and decreases exposure to antimicrobial agents. Consequently, in the last part of this study [**Chapter 3.4**], we determined the gene expression of different clinically isolated *S. aureus* thymidine-dependent SCVs, in order to determine if the persistence in the thymidine-dependent SCV is partly regulated by relative gene expression during different phases of growth. We could show that thymidine-dependent small colony variants had different relative gene expression pattern during early log phase and stationary phase of growth of select genes involving pyrimidine metabolism, virulent determinants, arginine metabolism, stress and stringent response, capsular biosynthesis and gene involving TCA cycle. Thus, these results indicate that the transformation of *S. aureus* into an SCV may be a potent strategy for protection against host defences and antibiotic therapy, and thereby making them candidate for causing some of the more difficult to eradicate human infections, especially in CF patients.

Taken together, these results presented in this thesis work indicate that the fate of staphylococci, or otherwise said, the characteristics how staphylococcal senesce, may be largely influenced by regulatory mechanisms (here exemplified by the adaptive heat shock system). Furthermore, senescence of staphylococci may be altered by subtle environmental changes such as trace amounts of ethanol. Thymidine auxotrophism and a resulting SCV phenotype is yet another example of the unique ability of *S. aureus* to adapt to a changing milieu.

## 5. Summary

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Altogether, our analysis put forward our understanding of the complex interrelationship of metabolic and regulatory functions which characterize the senescing *Staphylococcus* micro-organism. It is expected that based on our and other research groups' work during the recent past, new insights in this complex adaptative response of the ageing staphylococcal cells will be achieved. It is hoped that these insights provide for novel, alternative prophylactic and treatment strategies devised to combat chronic, persistent *S. aureus* infections even given a scenario of sobering predictions of *S. aureus* resistance to classic treatment strategies.

### 6. Zusammenfassung

*Staphylococcus aureus* ist ein äußerst anpassungsfähiger Mikroorganismus, der in der Lage ist, unter sehr unterschiedlichen und für den Erreger zum Teil mit erheblichen Stressbedingungen verbundenen Umgebungsbedingungen zu überleben und sich zu vermehren. Daher hat dieser Erreger unterschiedliche Mechanismen zur Adaptation an Stress entwickelt. Clp homologe Proteine sind bedeutsam für unterschiedliche Stressbedingungen, und in früheren Arbeiten haben wir zeigen können, dass das *clpC* Gen im *Staphylococcus aureus* Stamm DSM20231 unter Biofilmbedingungen im Vergleich zu planktonischen Zellen aufreguliert ist. Persistenz und Langzeitüberleben sind typische Charakteristika biofilmassoziiertes Staphylokokkeninfektionen, die darüber hinaus dadurch gekennzeichnet sind, dass eine Heilung dieser Infektionen auch in Anwesenheit bakterizider antimikrobieller Substanzen häufig nicht zu erzielen ist.

Im ersten Teil dieser Studie [**Kapitel 3.1**] wurde die Bedeutung von *clpC* in diesem Zusammenhang bestimmt. Untersuchungen zum Metabolismus, zur Genexpression und Langzeitwachstum und Überleben von DSM20231 sowie einer isogenen *clpC* allelen Ersatzmutante wurden ebenso durchgeführt wie Untersuchungen mit einer *sigB* Mutante sowie mit einer *clpC sigB* Doppelmutante. Wie erwartet zeigte die *clpC* Mutante erhöhte Sensitivität gegenüber oxidativen und Hitzestress. Unerwartet war hingegen die abgeschwächte Expression des Tricarboxylsäure (TCA)-Zyklus-Gens *citB* (welches für Aconitase kodiert). Die Konsequenz hieraus war ein Verlust der Aconitase-Aktivität und eine Verhinderung des Acetatkatabolismus während der stationären Wachstumsphase. *clpC*-Inaktivierung verhinderte poststationäres Wachstum, resultierte jedoch in einem signifikant erhöhten Überleben während der stationären Phase im Vergleich zum Wildtypstamm. Diese Ergebnisse zeigen die kritische Rolle der *clpC* ATPase für die Regulation des TCA-Zyklus und belegen eine Rolle von ClpC als einem wichtigen Molekül für die Erholung in stationärer Phase und dem (aktiven) Eintritt in die Todesphase.

Im Bestreben, die Funktion der ClpC ATPase weiter zu charakterisieren [**Kapitel 3.2A**], führten wir Proteom-Analysen der *clpC* Mutante während unterschiedlicher Wachstumsphasen durch. Wir konnten zeigen, dass unabhängig von der Regulation des oxidativen Metabolismus die *clpC* Mutante klare Unterschiede in Bezug auf die



Regulation auch anderer wichtiger physiologischer und metabolischer Reaktionswege im Vergleich zum Wildtypstamm aufwies, von denen einige Moleküle aus dem Energiestoffwechsel und der Stressregulation betrafen, wie im Proteinprofil einer 2D-Gel-Analyse gezeigt wurde. Insgesamt sind diese Resultate ein Hinweis dafür, dass das Hitzeschockprotein ClpC ATPase im breiteren Sinne mit Staphylokokkenmetabolismus und Stressregulation assoziiert ist.

Die *Staphylococcus aureus clpC* Mutante zeigte ein gesteigertes poststationäres Überleben und es ist bekannt, dass Bakterienzellen aus diesen poststationären Phasen eine erhöhte phänotypische Resistenz gegenüber Antibiotika aufweisen. Mit dieser Fragestellung untersuchten wir [**Kapitel 3.2B**] die Aktivität von Moxifloxacin auf eine solche *clpC* Mutante durch Exposition von *S. aureus* DSM20231 Zellen aus stationärer Phase gegenüber ansteigenden Konzentrationen von Moxifloxacin. Hier konnten wir zeigen, dass die Abwesenheit von *clpC* zu einer erhöhten Moxifloxacin-Toleranz in der stationären Phase führt und konnten genregulatorische Hinweise darauf finden, dass die Toleranz durch Zellwand und Stress-assoziierte Gene vermittelt wird.

Das Verständnis stationären und poststationären Wachstums in *Staphylococcus aureus* hat möglicherweise wichtige klinische Implikationen, insbesondere da relativ wenig über die Mechanismen zur Langzeitpersistenz chronischer *S. aureus* Infektionen in Verbindung mit der Biofilmproduktion bekannt ist. Diese Untersuchungen helfen uns daher, die Komplexität und die gegenseitige Abhängigkeit in der metabolischen Antwort durch *clpC* Systeme als Ausdruck der Langzeitpersistenz von *Staphylococcus aureus* zu beschreiben. Hier sind weitere Untersuchungen erforderlich, um Stressantworten und insbesondere die Rolle der ClpC ATPase Moleküle weiter zu definieren.

Im 3. Teil dieser Untersuchung [**Kapitel 3.3**] haben wir die Bedeutung von sehr niedrigen Ethanolkonzentrationen („very low ethanol concentrations“, VLEC) auf die Physiologie und den Metabolismus von *Staphylococcus aureus* untersucht. Arzneimittel, Kulturmedien für die bakterielle *in-vitro*-Diagnostik und die bakteriologische Forschung ebenso wie menschliche Körperflüssigkeiten oder verschiedene Umgebungsbedingungen können solche niedrigen Ethanolkonzentrationen (VLEC) ( $\leq 0.1\%$  vol/vol) enthalten. Im Unterschied zu den gut beschriebenen Effekten erhöhter Ethanolkonzentrationen auf lebende Bakterien gibt es keine publizierten Erkenntnisse über die Konsequenzen der

Exposition gegenüber VLEC. Zur Untersuchung des spätstationären Staphylokokkenwachstums haben wir Kulturmedien mit *Staphylococcus aureus* Stamm DSM20231 inokuliert und die Medien vorher mit VLEC supplementiert. Anschließend haben wir die Ultramorphologie, Wachstum und die Überlebensfähigkeit im Vergleich zu un-supplementierten Medien für verlängerte Kulturdauer (bis zu 8 Tage) untersucht. *Staphylococcus aureus*, der unter VLEC+ Bedingung bis zur spätstationären Phase angezogen wurde, wies ausgeprägte Veränderung der Zellintegrität auf, wie wir durch Rasterelektronenmikroskopie darstellen konnten. Ethanol wurde dabei während der exponentiellen Phase im Medium vollständig metabolisiert, dennoch zeigte sich überraschenderweise eine ausgeprägte Verzögerung des *Staphylococcus aureus* Wachstums in der poststationären Phase (>48 h). Gleichzeitig beobachteten wir persistierend erhöhte Konzentrationen von Acetat in VLEC+ Bedingungen, während nur verringerte Konzentrationen von Ammoniak generiert wurden mit der Konsequenz eines sauren Kulturmilieus und damit einer Stoffwechsellage, die insgesamt einen reduzierten Aminosäurenkatabolismus nahe legt. Interessanterweise war die Aminosäurendepletion nicht gleichförmig beeinträchtigt: Unter VLEC+ Bedingungen war insbesondere die Aufnahme von Glutamat, Ornithin und Prolin gestört, während die Aufnahme anderer Aminosäuren durch die Bakterienzellen nicht beeinträchtigt war. Eine Supplementierung durch Arginin konnte dabei in der poststationären Phase Wachstum und Überleben wieder herstellen, während andere Aminosäuren hierzu nicht in der Lage waren. Insgesamt zeigen diese Daten, dass VLEC ausgeprägte Effekte auf die Wachstumserholung von *Staphylococcus aureus* auch nach Ethanoldepletion vermittelt, den Übergang vom primären zum sekundären Metaboliten-Katabolismus verzögert und insgesamt suggeriert, dass die Konzentrationen von Ethanol zur bakteriostatischen Kontrolle von *S. aureus* niedriger sind als ursprünglich mitgeteilt.

Auf Grund der Tatsache, dass persistierende Infektionen mit dem Nachweis von *Staphylococcus aureus* small-colony variants (SCVs) assoziiert worden sind, hat das Interesse an diesen SCVs und ihrer Bedeutung für Staphylokokkeninfektionen allgemein in letzter Zeit wiederum deutlich zugenommen. Fremdkörper-assoziierte Infektionen, Haut- und Weichteilinfektionen, die chronische Osteomyelitis und die chronischen Atemwegsinfektionen bei Patienten mit Mukoviszidose sind alle mit diesen natürlicherweise vorkommenden *S. aureus* Subpopulationen in Verbindung gebracht worden. Alle diese Fälle haben eindrücklich die schlechte klinische und mikrobiologische

Antwort auch gegenüber verlängerter antimikrobieller Therapie in Patienten mit diesen Variantenpopulationen belegt. Es wird davon ausgegangen, dass diese Varianten intrazellulär in nicht professionellen Phagozyten geschützt vor Wirtabwehrmechanismen und bakteriziden Konzentrationen von Antibiotika persistieren können. Um Aufschluss zu gewinnen über Mechanismen, die zu der Ausbildung dieser Variantenpopulationen führen können, haben wir im letzten Teil dieser Arbeit [**Kapitel 3.4**] die Genexpression unterschiedlicher klinischer, Thymidin-abhängiger *S. aureus* SCVs in der Absicht untersucht, eine Abhängigkeit von Thymidin-abhängigen SCVs in unterschiedlichen Wachstumsphasen von der relativen Genexpression zu identifizieren. Wir konnten dabei zeigen, dass Thymidin-abhängige SCVs unterschiedliche relative Genexpressionsmuster während früher Log-Phase und stationärer Wachstumsphase, insbesondere hinsichtlich von Genen, die mit Pyrimidin-Metabolismus, Arginin-Metabolismus, „stringent response“ Virulenzmerkmalen, Kapsular-Biosynthese oder TCA-Zyklus involviert sind, aufweisen. Unsere Ergebnisse zeigen, dass die Transformation von *S. aureus* in einen SCV-Phänotyp eine Protektionsstrategie gegen Wirtabwehrmechanismen und antibiotische Therapie vermittelt und sie damit zu Kandidaten für diese schwierig zu eradizierenden menschlichen Infektionen besonders in CF-Patienten machen.

Zusammen genommen weisen die Resultate dieser Arbeit darauf hin, dass das Schicksal von Staphylokokken, oder anders gesagt, die Charakteristika der Alterung von Staphylokokkenpopulationen wesentlich durch regulatorische Mechanismen vermittelt werden, die hier beispielhaft mit einer Adaptation spezifischer Hitzeschockmoleküle dargestellt werden konnten. Dieser Alterungsprozess der Staphylokokken kann hierbei durch diskrete Umweltbedingungen, wie z.B. Spurenkonzentrationen von Ethanol vermittelt werden. Der Thymidin-Auxotrophismus und der resultierende SCV-Phänotyp ist ein weiteres Beispiel der einzigartigen Fähigkeit von *Staphylococcus aureus* an ein sich wechselndes Umgebungsmilieu zu adaptieren.

Insgesamt erlauben unsere Untersuchungen Fortschritte in unserem Verständnis der komplexen Beziehungen metabolischer und regulatorischer Funktionen der Seneszenz von Staphylokokkenmikroorganismen. Es ist zu erwarten, dass auf der Basis der Ergebnisse unserer und anderer Forschergruppen in den letzten Jahre neue Einblicke in diese komplexen adaptativen Antworten von alternden Staphylokokkenzellen gewonnen werden können. Es bleibt dabei zu hoffen, dass diese Einblicke die Möglichkeit für neue

## 6. Zusammenfassung

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Alternativen prophylaktischer und Therapiestrategien aufzeigen mit dem Ziel, diese chronischen persistierenden Staphylokokkeninfektionen auch in Antizipation einer zunehmenden Resistenz gegen klassisch antibiotische Behandlungsstrategien zu therapieren.

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