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**Effect of simulated microgravity on the virulence properties of  
the opportunistic bacterial pathogen *Staphylococcus aureus***

**Thesis submitted in accordance with the requirements of the School of  
Pharmacy, University of London for the degree of Doctor of Philosophy**

**Microbiology Group, Department of Pharmaceutics  
School of Pharmacy, University of London**

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## PLAGIARISM STATEMENT

This thesis describes research conducted in the School of Pharmacy, University of London between October 2005 and June 2009 under the supervision of Professor Peter W. Taylor. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Helena Isabel Venancio Rosado

Signature

01/12/2009

Date

## ABSTRACT

Microbiological monitoring of air and surfaces within the International Space Station (ISS) has indicated that bacteria of the genus *Staphylococcus* are found with high frequency. *Staphylococcus aureus*, an opportunistic pathogen with the capacity to cause severe debilitating infection, constitutes a significant proportion of these isolates. Ground-based studies of *Salmonella enterica* have provided evidence that bacterial virulence is increased under the influence of simulated microgravity. These studies, and others linking space flight to reduced immune competence, provide clear evidence that extended missions may be compromised by increased risk of infection. The effects of simulated microgravity on the virulence properties of *S. aureus* were therefore examined.

The methicillin-susceptible *S. aureus* (MSSA) isolates RF1, RF6 and RF11 were grown in a Synthecon High Aspect Ratio Vessel (HARV) under low shear modelled microgravity (LSMMG) and compared with cells grown under normal gravity (NG). There were no significant differences in the growth rate, antibiotic susceptibility or cell morphology of MSSA grown under LSMMG compared to NG. Growth in a modelled microgravity environment had an impact on a number of factors associated with the virulence of *S. aureus*. Pigment production and haemolysin secretion was significantly reduced in all three isolates under LSMMG.

Global gene expression was determined by DNA microarray analysis and protein secretion examined using two-dimensional gel electrophoresis. LSMMG elicited large reductions in protein secretion by the three isolates; in particular isolate RF6 displayed a fivefold reduction in protein secretion. In total, 40 proteins were found to be down-regulated under LSMMG in a highly reproducible fashion. LC-MS/MS identified these proteins to be involved mainly with cell metabolism, including protein biosynthesis, folding and transport. DNA microarray identified significant changes in gene regulation; these were in the main associated with cell metabolism, transport, stress and virulence. For isolate RF6, the expression of a major virulence gene, *hla*, and the virulence regulatory system *saeRS* was found to be reduced two- and fivefold respectively.

These data provide strong evidence that growth of *S. aureus* under modelled microgravity leads to a reduction in expression of virulence determinants. This observation raises the possibility that pharmacological modulation of the “microgravity trigger” that produces this avirulent phenotype would “disarm” the pathogen and resolve staphylococcal infections. This work constitutes the first step in a search for inhibitors that would prevent the secretion of a family of proteins necessary for infection to take place; the attenuated phenotype generated by such pharmacological intervention would not survive systemically and invading bacteria are likely to be removed by host immune surveillance. In contrast to conventional antibiotics, such therapeutic agents would modify rather than kill the target pathogen and consequently apply less direct selective pressure on bacterial populations.



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# TABLE OF CONTENTS

<b>CHAPTER ONE - Introduction .....</b>	<b>15</b>
<b>1.1 The exploration of space.....</b>	<b>16</b>
<b>1.2 The effects of space flight on the human body .....</b>	<b>18</b>
<b>1.3 Opportunistic pathogens in space.....</b>	<b>21</b>
<b>1.4 <i>Staphylococcus aureus</i>: an opportunistic pathogen.....</b>	<b>24</b>
1.4.1 Treatment of staphylococcal infections .....	25
1.4.2 Pathogenicity and virulence .....	28
<b>1.5 Simulating microgravity on Earth.....</b>	<b>31</b>
<b>1.6 Effects of microgravity on microorganisms.....</b>	<b>41</b>
1.6.1 Growth kinetics .....	45
1.6.2 Antibiotic susceptibility and cell morphology .....	46
1.6.3 Stress resistance .....	47
1.6.4 Production of secondary metabolites .....	48
1.6.5 Virulence.....	49
1.6.6 Protein production.....	51
1.6.7 Gene expression.....	52
<b>1.7 Aims.....</b>	<b>53</b>
 <b>CHAPTER TWO - Effect of LSMMG on the growth kinetics, antibiotic susceptibility and cell morphology of <i>S. aureus</i> .....</b>	 <b>58</b>
<b>2.1 Introduction .....</b>	<b>59</b>
2.1.1 Effect of microgravity on bacterial growth kinetics .....	60
2.1.2 Effect of microgravity on bacterial antibiotic susceptibility and morphology.....	62

<b>2.2 Materials and Methods</b>	<b>64</b>
2.2.1 Bacterial strains	64
2.2.2 HARV preparation and sterilisation	65
2.2.3 Bacterial culture	66
2.2.4 Determination of growth kinetics and final cell density	67
2.2.5 Determination of minimum inhibitory concentration (MIC)	68
2.2.6 Morphologic analysis by electron microscopy - scanning electron microscopy (SEM) and transmission electron microscopy (TEM)	68
<b>2.3 Results</b>	<b>69</b>
2.3.1 Selection of <i>S. aureus</i> isolates	69
2.3.2 Effect LSMMG on <i>S. aureus</i> growth kinetics	70
2.3.3 Effect of LSMMG on susceptibility of <i>S. aureus</i> to antibiotics	72
2.3.4 Effect of LSMMG on <i>S. aureus</i> morphology	74
<b>2.4 Discussion</b>	<b>76</b>
 <b>CHAPTER THREE - Effect of LSMMG on the production of virulence determinants by <i>S. aureus</i></b>	 <b>82</b>
<b>3.1 Introduction</b>	<b>83</b>
3.1.1 Production of carotenoid pigments by <i>S. aureus</i>	83
3.1.2 Secretion of toxins by <i>S. aureus</i>	86
3.1.2.1 Alpha-toxin	87
3.1.2.2 Other staphylococcal toxins	89
<b>3.2 Materials and Methods</b>	<b>90</b>
3.2.1 Extraction and quantification of carotenoid pigments	90
3.2.2 Assays for staphylococcal haemolytic activity	91
3.2.3 Statistical analysis	91
<b>3.3 Results</b>	<b>92</b>
3.3.1 Effect of LSMMG on <i>S. aureus</i> carotenoid pigment production	92
3.3.2 Effect of LSMMG on <i>S. aureus</i> haemolysin secretion	94
<b>3.4 Discussion</b>	<b>97</b>

## **CHAPTER FOUR - Effect of LSMMG on the proteomic profile of *S. aureus* 102**

<b>4.1 Introduction .....</b>	<b>103</b>
4.1.1 Two-dimensional gel electrophoresis (2D electrophoresis).....	103
<b>4.2 Materials and Methods.....</b>	<b>106</b>
4.2.1 Preparation of samples .....	106
4.2.1.1 Preparation of proteins present in the supernatants.....	106
4.2.1.2 Preparation of intracellular proteins .....	107
4.2.1.3 Determination of protein content .....	107
4.2.2 One-dimensional gel electrophoresis .....	108
4.2.3 2D gel electrophoresis.....	108
4.2.3.1 2D sample cleanup .....	108
4.2.3.2 First dimension: Isoelectric focusing (IEF).....	109
4.2.3.2.1 Loading of IPG strips.....	109
4.2.3.2.2 Focusing of IPG strips.....	110
4.2.3.2.3 Equilibration of IPG strips .....	110
4.2.3.3 Second-dimension: SDS-PAGE .....	111
4.2.3.4 Protein detection and analysis .....	111
4.2.3.5 Protein identification by liquid chromatography-mass spectrometry (LC/MS-MS).....	111
4.2.3.6 Statistical analysis .....	113
4.2.4 Determination of protease activity using zymogram gels.....	113
<b>4.3 Results .....</b>	<b>114</b>
4.3.1 Effect of LSMMG on <i>S. aureus</i> supernatant proteins.....	114
4.3.2 Effect of LSMMG on <i>S. aureus</i> supernatant proteome.....	117
4.3.3 Effect of LSMMG on <i>S. aureus</i> intracellular proteome.....	119
4.3.4 Protein identification by LC/MS-MS.....	121
4.3.5 Effect of LSMMG on <i>S. aureus</i> protease activity.....	124
<b>4.4 Discussion.....</b>	<b>124</b>

**CHAPTER FIVE - Effect of LSMMG on the transcriptomic profile of *S. aureus* ..... 139**

**5.1 Introduction ..... 140**

5.1.1 DNA microarrays and transcriptomics technology ..... 140

**5.2 Materials and Methods ..... 144**

5.2.1 RNA extraction ..... 144

5.2.2 Purification of total RNA and DNase digestion..... 145

5.2.3 Microarray analysis..... 146

5.2.4 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)..... 147

**5.3 Results ..... 149**

5.3.1 Microarrays ..... 149

5.3.2 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)..... 154

**5.4 Discussion..... 161**

**CHAPTER SIX - General discussion ..... 171**

**REFERENCES..... 181**

**PUBLICATIONS, PRESENTATIONS AND AWARDS..... 210**

## FIGURES AND TABLES

Fig. 1.1: Key time points in the development and introduction of new classes of antibiotics into clinical practice..	26
Fig. 1.2: Virulence factors of <i>S. aureus</i> and possible local and systemic effects on the host.	29
Fig. 1.3: Parabolic flight illustrating the typical flight parabola of an aircraft (A300).	35
Fig. 1.4: The Rotary Cell Culture System (RCCS)..	36
Fig. 1.5: Operating orientations of the Rotary Cell Culture System (RCCS)..	38
Fig. 1.6: Aims and strategies of the study of the effect of simulated microgravity on the virulence properties of the opportunistic bacterial pathogen <i>S. aureus</i> .....	55
Fig. 1.7: An alternative approach to antibacterial chemotherapy.....	56
Fig. 2.1: Experimental procedures for bacterial growth in the RCCS.....	65
Fig. 2.2: Growth kinetics of <i>S. aureus</i> under NG and LSMMG. ....	71
Fig. 2.3: Final cell density determined as cell dry weight of <i>S. aureus</i> grown under NG and LSMMG after 24h. ....	72
Fig. 2.4: Scanning electron microscopy images of <i>S. aureus</i> grown under NG and LSMMG....	74
Fig. 2.5: Transmission electron microscopy images of sections of <i>S. aureus</i> grown under NG and LSMMG. ....	75
Fig. 2.6: Transmission electron microscopy images of sections of <i>S. aureus</i> isolated from J. L. Chrétien and grown aboard Salyut 7 in 1982.....	80
Fig. 3.1: Mechanism of action of $\alpha$ -toxin.....	88
Fig. 3.2: Colour appearance of NG and LSMMG-grown cultures of <i>S. aureus</i> .....	92
Fig. 3.3: Absorption spectrum of pigments extracted from <i>S. aureus</i> .....	93
Fig. 3.4: Production of staphyloxanthin by <i>S. aureus</i> grown under NG and LSMMG.....	94
Fig. 3.5: Secretion of sheep and rabbit erythrocytes haemolysins by <i>S. aureus</i> grown under NG and LSMMG.....	96
Fig. 4.1: Two-dimensional gel electrophoresis. ....	104
Fig. 4.2: SDS-PAGE of soluble proteins in the culture supernatant of <i>S. aureus</i> grown under NG and LSMMG.....	114
Fig. 4.3: 2D electrophoresis of soluble proteins in the culture supernatant of <i>S. aureus</i> isolate RF6 grown under NG and LSMMG.....	115
Fig. 4.4: Quantification of soluble proteins in the culture supernatants of <i>S. aureus</i> .....	116
Fig. 4.5: 2D electrophoresis of soluble proteins in the culture supernatants of <i>S. aureus</i> grown under NG and LSMMG. ....	118
Fig. 4.6: 2D electrophoresis of intracellular proteins of <i>S. aureus</i> grown under NG and LSMMG. ....	120
Fig. 4.7: Proteins identified by LC/MS-MS. ....	121
Fig. 4.8: Examples of poor gel resolution due to prior sample freezing before IEF. ....	135
Fig. 4.9: Examples of poor gel resolution due to non-optimal IEF conditions. ....	136
Fig. 4.10: Examples of poor gel resolution due to gel polymerisation.....	137
Fig. 5.1: Typical sequence of procedures used for microarray analysis.....	141
Fig. 5.2: Sequential steps during qRT-PCR quantification with SYBR green dye. ....	143
Fig. 5.3: Thermal profile used for qRT-PCR. ....	148
Fig. 5.4: Condition tree of <i>S. aureus</i> grown under NG and LSMMG. ....	150

Fig. 5.5: Comparative gene expression of <i>S. aureus</i> isolate RF11 under NG and LSMMG. ....	152
Fig. 5.6: RNA quality assessment by RNA native agarose gel electrophoresis of <i>S. aureus</i> grown under NG and LSMMG. ....	154
Fig. 5.7: Ct values obtained as qRT-PCR data. ....	156
Fig. 5.8: qRT-PCR amplification plots obtained with specific primers for 16s rRNA. ....	157
Fig. 5.9: Amplification plots obtained from comparison of <i>hla</i> expression in NG and LSMMG. ....	158
Fig. 5.10: Dissociation curve (T <sub>m</sub> ) for determination of the quality of PCR products produced during the qRT-PCR reaction. ....	161

Fig. 6.1: From DNA to protein. ....	174
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Table 1.1: Some of the facilities now available that provide microgravitational conditions for various periods of time. ....	33
Table 1.2: Example of ground-based models which reduce the effects of the gravitational vector on subjects or samples under study. ....	34
Table 1.3: Summary of in-flight and ground-based studies performed on different bacterial species. ....	43

Table 2.1: Characteristics of the 20 <i>S. aureus</i> isolates screened when plated on sheep blood agar. ....	70
Table 2.2: Susceptibility of <i>S. aureus</i> to antibiotics under NG and LSMMG. ....	73
Table 2.3: Summary of the effects of LSMMG on <i>S. aureus</i> growth kinetics, antibiotic susceptibility and cell morphology. ....	81

Table 3.1: Summary of the effect of LSMMG on <i>S. aureus</i> production of virulence determinants. ....	101
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Table 4.1: The most abundant proteins found in the supernatant proteome of <i>S. aureus</i> . ...	122
Table 4.2: Summary of <i>S. aureus</i> global regulatory mechanisms which may be involved in response to LSMMG environment. ....	131
Table 4.3: Summary of the effect of LSMMG on <i>S. aureus</i> proteome. ....	138

Table 5.1: Oligonucleotide primers designed for qRT-PCR. ....	147
Table 5.2: <i>S. aureus</i> genes up- and down-regulated following growth under LSMMG, compared to NG. ....	153
Table 5.3: Comparison of up- and down-regulated genes of <i>S. aureus</i> grown under NG and LSMMG. ....	160
Table 5.4: Summary of <i>S. aureus</i> global regulatory mechanisms which may be involved in response to LSMMG environment. ....	168
Table 5.5: Summary of the effect of LSMMG on <i>S. aureus</i> transcriptome. ....	170

Table 6.1: Effect of LSMMG on <i>S. aureus</i> and bacterial targets with potential application for development of vaccines and antibacterial agents. ....	178
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## ABBREVIATIONS

1D	one dimension
2D	two dimensions
3D	three dimensions
$\mu\text{A}$	microampere
$\mu\text{g}$	microgram
$\mu\text{l}$	microlitre
$\mu\text{m}$	micrometre
$^{\circ}\text{C}$	degree Celsius
BCPR	Bioastronautics Critical Path Roadmap
BHI	brain-heart-infusion
BSA	bovine serum albumin
cDNA	complementary DNA
CFU	colony forming unit
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CLSI	Clinical and Laboratory Standard Institute
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ESA	European Space Agency
<i>g</i>	gravity
g	gram
h	hour
HARV	High Aspect Ratio Vessel
HCl	hydrochloric acid
HUMEX	human exploratory mission
IEF	isoelectric focusing
IPG	immobilised pH gradient
ISS	International Space Station
kDa	kiloDalton
kg	kilogram



kV	kilovolt
LB	Luria-Bertani
LC	liquid chromatography
LSMMG	low-shear modelled microgravity
m	metre
M	molar
mA	milliampere
mg	milligram
MHB	Mueller-Hinton broth
MIC	minimum inhibitory concentration
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MS	mass spectrometry
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MSSA	methicillin-susceptible <i>Staphylococcus aureus</i>
MW	molecular weight
MWCO	molecular weight cut-off
NASA	National Aeronautics and Space Administration
NG	normal gravity
nl	nanolitre
nm	nanometre
PBP	penicillin binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
psi	pound per square inch
RCCS	Rotary Cell Culture System
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
qRT-PCR	quantitative reverse transcriptase-PCR
s	second

SAg	superantigen
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	scanning electron microscope
STS	space transportation system
TAE	tris-acetate-EDTA
TCA	tricarboxylic acid
TEM	transmission electron microscopy
TGS	tris-glycine-SDS
Tris	trishydroxymethylaminomethane
TSB	tryptic soya broth
TSST	toxic shock syndrome toxin
UK	United Kingdom
USA	United States of America
V	volt

***Imagination is more important than knowledge...***  
*Albert Einstein*

# **CHAPTER ONE**

## **Introduction**

## **1.1 The exploration of space**

Curiosity and willingness to learn, the desire to investigate and the need to explore are, in all probability, the main motivations for both scientists and broader humanity to constantly seek answers to fundamental questions regarding the physical world. Driven by this innate human thirst for knowledge and progress, space exploration has become a reality and should be considered as one of mankind's greatest achievements to date.

Half a century after the first manned mission beyond the confines of Planet Earth, the prospect of living and working in space continues to stimulate broad public interest, due to both the potential economic and political impact on our societies. Space exploration is increasingly a symbol of global multinational co-operation and of improvement in the quality of our daily lives. The use of space technologies has been extended beyond the Earth's orbit, with applications in medicine, communications, Earth observation and many other fundamental and applied areas of research and exploration (Mindell *et al.*, 2008).

Although very sophisticated equipment has been extensively employed in space, the presence of humans beyond Earth's boundaries has become essential in order to undertake tasks that robots cannot perform. The first manned space flight took place in 1961, when the Russian cosmonaut Yuri Gagarin orbited the Earth in 108 minutes aboard Vostok 1. During the same decade, the USA also developed similar space programmes, such as the Mercury and Gemini missions. The knowledge acquired during these first successful manned missions facilitated the design and assembly of more sophisticated spacecraft for the later Apollo programmes and culminated in the arrival of humankind at the Moon in 1969. More than just a national achievement, these manned missions essentially confirmed that humans could survive in space.

The 1970s were marked by the realisation of the first space stations, the Soviet Salyut (Bluth and Helppie, 1987) and the American Skylab (Belew, 1977). Being temporarily manned, both the Soviet and American programmes demonstrated that it was possible for humans to live and work in space for extended periods of time, up to

several months. In addition, these two programmes furthered knowledge of the effects of space flight on the human body, materials and fluids, and allowed a more in-depth study of the Earth and Universe. Also, during this decade, the first joint flight between the USA and the Soviet Union, the Apollo-Soyuz test programme, marked the beginning of international co-operation in space with its attendant positive political impact.

The Space Shuttle era began during the 1980s. A number of space transportation systems (STS), the American shuttles *Challenger*, *Discovery*, *Columbia*, *Atlantis* and *Endeavour*, were designed to provide frequent and reliable access to the space environment. As a component of the ongoing partnership between the USA National Aeronautics and Space Administration (NASA) and the European Space Agency (ESA), these Shuttle craft functioned as international short-stay orbital laboratories, the Spacelab, where several scientific experiments, mainly in the fields of biomedicine and physics, took place (Halstead and Dufour, 1986). Shuttle flights are still a reality and they continue to provide essential information in several research areas and to support other space missions.

A further significant achievement in terms of the space sciences was the construction of more complex and sophisticated space stations that allowed humans to remain in space for extended periods of time. As a consequence, a large number of investigations have taken place over the last twenty to thirty years that have substantially increased our knowledge of the effects of space flight on the human body as well as on a variety of materials and fluids. Mir became the first permanently manned space station, was designed by the Russians and orbited the Earth between 1986 and 2001.

For many years, only the Americans and Russians contributed actively to the space industry. Nowadays, it is a global co-operation, with more than 20 countries involved. In this context, an agreement achieved in 1985 enabled an international partnership to construct the International Space Station (ISS), which was occupied by its initial crew in 2000. This space station is scheduled to be completed in 2010 and will play a major role in space research.

In January 2004, President George W. Bush announced the new *Vision for Space Exploration*. The return to the moon, with the possibility to work and live on our natural satellite for extended periods of time was one of the key aims of this initiative and is likely to be pursued by the current USA Administration and its successors over the next twenty to thirty years. The experience and knowledge gained will be applied to future exploration with the aim of reaching other destinations, in particular the planet Mars.

An ambitious mission to the Red Planet will involve travel in space for great distances over long durations. Outbound and inbound flights will take up to 5-6 months and it will be necessary for astronauts to stay on the surface of the planet for up to 570 days, until the alignment of the planets allows their return to Earth (Ball and Evans, 2001). During these 2-3 year missions, crew members will be exposed to microgravity approaching  $10^{-4}$ - $10^{-6}$  g in Earth orbit, 0.17 g on the Moon and 0.38 g on the Martian surface. It is therefore fundamental to focus ground and space research on the understanding and prevention of the adverse effects on the human body of prolonged exposure to space and planetary environments (Ball and Evans, 2001; Mindell *et al.*, 2008).

## **1.2 The effects of space flight on the human body**

The detrimental effects on the well being of astronauts arising from long-term space flight, with its attendant extended exposure to microgravity and intense radiation (ionising and non-ionising), are well documented (Ball and Evans, 2001; Sonnenfeld and Shearer, 2002). The risk factors are recognised and form key elements of the NASA's Bioastronautics Critical Path Roadmap (BCPR) (Osborn *et al.*, 2005), a framework which guides NASA's strategy to assess, understand, mitigate and manage the risks associated with long-term exposure of astronauts to the space environment. Similarly, ESA has conducted a study on human exploratory missions (HUMEX), which critically assesses human responses, limits and needs with regard to planetary and interplanetary environments (Horneck *et al.*, 2003).

As the distance and duration of missions progressively increase, careful planning of biomedical support provided to crew members is essential for the success of upcoming missions and for the maintenance of the physiological and psychological integrity of the crew. A risk reduction strategy for human space exploration is thus a priority. In this context, particular interest has been centred on the effects of microgravity on the human body; issues may include decreases in bone mineral density with high probability of bone fracture, renal stone formation due to elevated urine calcium resulting from bone re-absorption, muscular atrophy, anaemia and decreased cardiovascular performance. In addition, isolation for long periods of time, noise, confinement and restricted working environment can lead to physical and psychological stresses, sleep and nutritional disturbance (Ball and Evans, 2001).

*In vivo* and *in vitro* studies on the effects of space flight on the immune system have demonstrated a significant reduction in the efficacy of cellular immune function and have been extensively reviewed (Cogoli, 1981; Cogoli, 1993a; Cogoli, 1993b; Cogoli, 1993c; Cogoli, 1994; Cogoli, 1996; Meshkov, 2001; Borchers *et al.*, 2002; Sonnenfeld and Shearer, 2002; Sonnenfeld *et al.*, 2003). A potent inhibition of lymphocyte activation has been repeatedly demonstrated (Cogoli *et al.*, 1979; Cogoli *et al.*, 1980; Cogoli *et al.*, 1984; Cogoli, 1993b) and is associated with monocyte malfunction and reduced phagocytic index (Bechler *et al.*, 1992; Cogoli, 1993b; Kaur *et al.*, 2005). Alterations in cytokine production have also been reported (Talas *et al.*, 1984; Sonnenfeld *et al.*, 1990; Sonnenfeld, 1994; Batkai *et al.*, 1999; Felix *et al.*, 2004), as has inhibition of natural killer cell activity (Talas *et al.*, 1984; Rykova *et al.*, 1992; Tuschl *et al.*, 1993; Konstantinova *et al.*, 1993; Stowe *et al.*, 2001) and changes in leukocyte sub-populations in the bone marrow (Sonnenfeld *et al.*, 1992; Ortega *et al.*, 2009) and spleen (Pecaut *et al.*, 2000).

Several haematological changes have also been noted and include reduction of the red blood cell count and haemoglobin mass accompanied by loss of plasma volume (Cogoli, 1981). It has been hypothesised that the effect of space flight on the immune system may be attributed in the main to stress factors. This is consistent with the findings that the microgravity environment increases the production of stress hormones such as cortisol (Leach *et al.*, 1996; Stowe *et al.*, 2001) and catecholamines (Padgett and Glaser, 2003; Aviles *et al.*, 2005).



These alterations in immune competence are likely to compromise the health of astronauts, particularly if they are maintained for the duration of a long mission. The cellular components of the immune system play a central role in the control of bacterial and viral pathogens, limiting their ability to colonise, invade and spread within the body. Decreased immune function could lead to an increased risk of microbial infection. The reactivation of latent herpes virus during space flight (Stowe *et al.*, 2001) is a clear manifestation of changes in the immune status of astronauts and an indication that extended flight may challenge the immune defences of even the fittest astronaut.

There are a range of opportunistic pathogens, including bacteria, fungi and viruses that depend on reduced immune function to cause serious infectious disease, and some of these will inevitably accompany the crew into deep space. Although there is no documented evidence that an onboard infection has resulted in the abortion of a space flight, microbial infections have caused significant problems during orbital missions. Analysis of medical events among cosmonauts during several Russian and American missions indicated that a significant number of episodes of infection occurred, including conjunctivitis and acute respiratory and dental infections. A Soviet cosmonaut, Yuri Romanenko, experienced debilitating toothache for over two weeks during a 96-day flight aboard Salyut 6. The Soviet space programme had no contingency plan in place to deal with dental emergencies and the problem could only be remedied after scheduled touchdown. Similarly, 26 instances of infection were reported for American astronauts during the Space Shuttle programme STS-1 to STS-89 (Ball and Evans, 2001). These medical episodes may be somewhat indicative of some decline in immune function as well as changes in saprophytic flora.

There is a growing body of evidence suggesting that the risk of infection during extended flight may be compounded as a result of the influence of microgravity and the deep space environment on the ecology and behaviour of microorganisms, particularly bacteria. In addition to its negative impact on the immune response, space flight leads to profound changes in the composition of the bacterial microflora of the intestinal (Nefedov *et al.*, 1971; Shilov *et al.*, 1971; Ilyin, 2005) and nasal tracts (Shilov *et al.*, 1971; Decelle and Taylor, 1976). It has been reported that as early as two weeks into the confinement imposed by space flight there is a significant

reduction in the number of bacterial species that can be isolated from the intestinal tract as well as an interchange of bacteria between crew members (Nefedov *et al.*, 1971; Shilov *et al.*, 1971; Ilyin, 2005). For example, a simplification of the anaerobic intestinal bacterial flora was noted in the Apollo and Skylab crews, favouring the emergence of robust Gram-negative aerobic species such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and various Enterobacteriaceae (Taylor, 1974a).

Alterations in the immune competence and microflora of crew members are likely to compromise the health of astronauts, particularly in terms of development of infectious diseases. It is therefore essential to quantitatively and qualitatively control and manage the presence of important bacterial opportunistic pathogens in enclosed environments.

### **1.3 Opportunistic pathogens in space**

The optimal functioning of the spacecraft and the well-being of astronauts are two major factors, which will determine the success of a space mission. Rather than relying on in-flight solutions, potential hazards must be identified early and preventative measures taken. One of the potential risks for astronauts is microbial contamination of the space environment. Characterisation of the microbiological population onboard is thus essential and has been a major concern since the early days of space exploration (Spizizen, 1971; Taylor, 1974b). Throughout the intervening years, a number of procedures have been developed and optimised for monitoring these microbial populations, including collection of surface swabs, air and water samples before, during and/or after space flight. The concentration of microorganisms is determined and microbial identification is carried out using techniques such as polymerase chain reaction (PCR) (Guarnieri *et al.*, 1997; Novikova *et al.*, 2006).

Although astronauts are quarantined prior to flight, their resident intestinal, oral and nasal flora will make a large contribution to the microbial reservoir onboard an extended mission. Around  $10^{14}$  microorganisms, the overwhelming majority of

which are bacteria, are known to reside in the intestinal tract and they constitute 50-60% of the faecal mass (Guarner and Malagelada, 2003; Kraehenbuhl and Corbett, 2004). This population is comprised of about 500 bacterial species, although 30-40 species account for 99% of the total; most are anaerobes and the colon contains more bacterial mass than other regions of the intestinal tract (Guarner and Malagelada, 2003; Pai and Kang, 2008). Similarly, the microbiota of the skin, the oral and the nasal passages warrant attention. Transient or persistent carriage of streptococci and staphylococci in the nasal and upper airways is ubiquitous in the general population. For example, in a healthy human population, approximately 60% of individuals carry *Staphylococcus aureus* at any one time (Kluytmans *et al.*, 1997).

Spacecraft and orbiting space stations are restricted working environments with constant rotation of crew members and working materials. In spite of all precautions, a variety of microorganisms maintain a presence inside the spacecraft; they can, with implementation of the correct procedures, be reduced but not eliminated (Pierson, 2001; Novikova *et al.*, 2006). Microbial contamination of the spacecraft and accumulation of potential pathogens is, therefore, likely to occur and can negatively impact the health, safety, and performance of the crew as well as affect the integrity of the spacecraft.

For these reasons, measures are constantly and rigorously implemented in an attempt to attain an environment as germ-free as possible. Space vehicles are equipped with efficient air filtration systems and surfaces are regularly cleaned and disinfected. The environmental conditions are strictly controlled and microbiological monitoring of air, water and surfaces is regularly performed (Pierson, 2001). Extensive environmental microbiological studies have been performed aboard Apollo (Ferguson *et al.*, 1975), Skylab (Taylor *et al.*, 1977) and various Space Shuttles (Pierson *et al.*, 1993) missions. Mir (Kawamura *et al.*, 2001; Novikova, 2004; Ott *et al.*, 2004) and ISS (Castro *et al.*, 2004; Novikova *et al.*, 2006) have provided the opportunity to improve the knowledge of the dynamics of microbial population and long-term habitability aboard spacecraft.

While fungal levels in the spacecraft are usually low and remain low throughout the mission, bacterial levels tend to increase during flight and the bacterial species

identified are commonly human commensal microorganisms (Pierson, 2001). *Staphylococcus*, *Bacillus*, *Micrococcus* and *Corynebacterium* are the most common bacterial genera recovered from air and surface samples. Interestingly, bacteria of the genus *Staphylococcus* have been consistently identified as the most predominant contaminants of spacecraft and space stations, particularly in air and surface samples (Pierson, 2001). *Staphylococcus* sp. were identified in 55.5% of the surface samples and 53.2% of the air samples recovered from Mir (Novikova, 2004). Similarly, this genus was identified in 84% of the surface samples and 38.8% of the air samples recovered from ISS (Novikova *et al.*, 2006). *S. aureus* has been identified in a significant proportion of these isolates, reaching values higher than 3% in both surface and air samples collected (Novikova, 2004; Novikova *et al.*, 2006).

In addition, identification of several species of bacteria (Kawamura *et al.*, 2001; Castro *et al.*, 2004; Novikova, 2004; Ott *et al.*, 2004; Novikova *et al.*, 2006), fungi (Kawamura *et al.*, 2001; Castro *et al.*, 2004; Novikova, 2004; Ott *et al.*, 2004; Novikova *et al.*, 2006), and reactivated latent viruses (Stowe *et al.*, 2001; Pierson *et al.*, 2007), particularly from long-term manned missions, suggests that these organisms are able to survive and proliferate in zero gravity. An increased presence of opportunistic pathogens and a decrease in saprophyte microorganisms have been recorded in samples taken from astronauts during space flight (Ilyin, 2005). Given the depressed immune status of astronauts (Sonnenfeld and Shearer, 2002) and recent increases in the frequency and duration of space missions, there is a high risk of onboard infections. Opportunistic pathogens such as *S. aureus* are, therefore, a major concern.

Currently available data support the notion that significant transfer of *S. aureus* occurred between crew members during several space missions, from the early Apollo (Ferguson *et al.*, 1975; Decelle and Taylor, 1976) and Skylab (Taylor *et al.*, 1977) series to more recent Shuttle (Pierson, 2001), Mir (Kawamura *et al.*, 2001; Novikova, 2004; Ilyin, 2005) and ISS (Castro *et al.*, 2004; Novikova *et al.*, 2006) missions. This process may have been accelerated in a microgravity environment by the generation of larger respiratory droplets, with a longer settle time, compared to those found on Earth.

*S. aureus* may cause serious, potentially life-threatening infections in individuals with even moderately compromised immune systems (Gillaspy *et al.*, 2006) and the infections they cause are often difficult to treat in terrestrial healthcare institutions, let alone within the confines of a space vessel.

## **1.4 *Staphylococcus aureus*: an opportunistic pathogen**

Staphylococci (from the Greek *staphyle* = bunch of grapes + *coccus* = granule) are round-shaped Gram-positive bacteria, often characterised by grape-like cluster formations. The genus *Staphylococcus* was first identified by Ogston (1881), after recovery of the bacterium from pus of surgical abscesses in patients suffering from acute inflammatory disease. As a result of their ubiquity and adaptability, staphylococci are frequently found as part of the normal human microflora, in the main colonizing skin and mucous membranes (Roberts, 1948; Noble *et al.*, 1967). Their characterisation is usually based on a range of biochemical, physiological and morphological parameters, such as catalase, coagulase and toxin production, as well as colony pigmentation and cell morphology (Götz *et al.*, 2006).

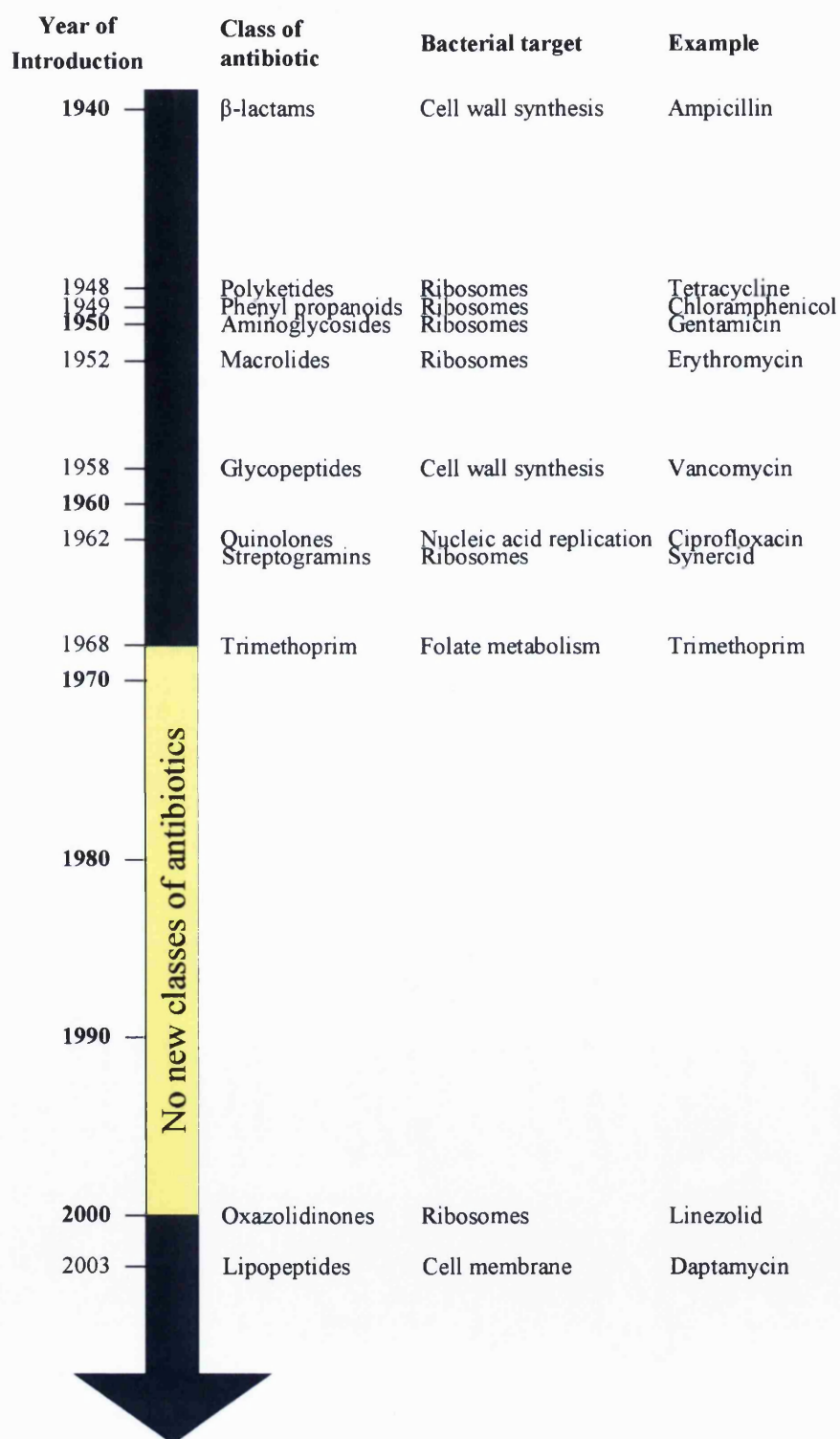
The species designation *S. aureus* (from the Latin *aureus* = golden) was proposed by Rosenbach (1884) highlighting the typical yellow colony pigmentation of this bacterium. These spherical cocci are approximately 1  $\mu\text{m}$  in diameter and are part of the normal human microflora, primarily colonizing the upper respiratory tract (Williams, 1963; Peacock *et al.*, 2001). In a healthy human population, approximately 20% of individuals are persistent *S. aureus* carriers; 60% are intermittent carriers and 20% are persistent non-carriers (Kluytmans *et al.*, 1997). Nasal carriage of *S. aureus* has been considered as an important risk factor for staphylococcal disease, particularly in immunocompromised individuals (Peacock *et al.*, 2001).

### 1.4.1 Treatment of staphylococcal infections

Together with other opportunistic pathogens, *S. aureus* is one of the main causes of community and hospital-acquired infectious diseases and is a major cause of mortality. It is responsible for a variety of infectious diseases, particularly in immunocompromised individuals; these range from local skin infections to more severe systemic infections such as endocarditis and pneumonia. Despite the availability of antimicrobials, some staphylococcal infections produce mortality rates of approximately 20–40% (Mylotte *et al.*, 1987) and this reaches around 90% if severe staphylococcal infections are left untreated (Smith and Vickers, 1960).

With the introduction of penicillin in 1944, the incidence of morbidity and mortality due to infection, including those due to staphylococci, was significantly reduced. However, penicillin-resistant staphylococci were isolated from infectious lesions within a few months of the introduction of benzylpenicillin (Barber and Rozwadowska-Dowzenko, 1948). By the late 1960s, nearly 80% of *S. aureus* clinical isolates were resistant to penicillin and this number has now increased to over 90% (Livermore, 2000; Lowy, 2003). The primary resistance mechanism to first generation penicillins is the production of  $\beta$ -lactamases, enzymes encoded by acquisition of the *blaZ* resistance gene (Lowy, 2003).

Methicillin was introduced in 1960. Like other  $\beta$ -lactams, this antibiotic inhibits the synthesis of the bacterial cell wall; however, it is effective against benzylpenicillin-resistant *S. aureus* isolates as it is insensitive to the action of  $\beta$ -lactamases. Nevertheless, within a year of methicillin introduction, the first methicillin-resistant *S. aureus* (MRSA) isolate was identified in the United Kingdom (Barber, 1961; Jevons, 1961). In contrast to the mechanism of resistance to the earlier penicillins, the primary resistance mechanism of methicillin is not due to the production of  $\beta$ -lactamases but to the expression of the *mecA* resistance gene by MRSA, with consequent production of penicillin binding protein PBP2a (Lowy, 2003).



**Fig. 1.1:** Key time points in the development and introduction of new classes of antibiotics into clinical practice. Between 1968 and 2000 there were no discoveries of new classes of antibiotics (Walsh, 2003). Note: example drugs were not necessarily introduced on the dates shown.

In response to the emergence of resistance, a variety of antibiotics with distinct mechanisms of action have been developed (Fig. 1.1). These include drugs such as tetracycline (polyketide), chloramphenicol (phenyl propanoid), streptomycin (aminoglycoside) and erythromycin (macrolide). All were initially active against *S. aureus* but extensive acquired resistance to these antibiotics now restricts their therapeutic utility.

The use of vancomycin (glycopeptide) is considered as the last resource for the treatment of MRSA infections; however, reduced susceptibility to this and other glycopeptide antibiotics has been already reported. The first cases were identified in Japan (Hiramatsu *et al.*, 1997a; Hiramatsu *et al.*, 1997b; Hiramatsu, 1998) but since these initial reports, other countries have identified MRSA clinical isolates with reduced susceptibility to vancomycin (Ploy *et al.*, 1998; Kim *et al.*, 2000; Oliveira *et al.*, 2001; Trakulsomboon *et al.*, 2001; Song *et al.*, 2004; Wang *et al.*, 2004; Torun *et al.*, 2005). The first vancomycin resistant isolate was reported in Michigan (MMWR, 2002). Fortunately, high vancomycin resistance levels are not common and this antibiotic remains effective against many MRSA strains.

After the introduction of Trimethoprim in the late 1960s, for many years there were no discoveries of antibiotics with novel mechanism of action. During these decades, chemical modification of the existing antibiotics was utilised to enhance their bacterial activity against resistant strains. For that reason, new generations of cephalosporins (Thornsberry, 1985) and quinolones (Martin *et al.*, 1998) are more effective and potent against bacteria which were resistant to the previous generations. Only recently, new classes of antibiotics such as linezolid (oxazolidinone) and daptomycin (lipopeptide) have been licensed; however, cases of reduced susceptibility and resistance to these antibiotics have been already reported (Tsiodras *et al.*, 2001; Pillai *et al.*, 2002; Wilson *et al.*, 2003; Baysallar *et al.*, 2004; Mangili *et al.*, 2005; Roberts *et al.*, 2006b)

Resistance has emerged rapidly and frequently to a variety of antibiotics (Lacey, 1984a; Lacey, 1984b) and the acquisition of multiple resistance genes by many pathogens is leading to the appearance of difficult-to-treat multi-drug resistant pathogens in the hospital and community environments (Levy, 2005). A study of



over 3,000 *S. aureus* clinical isolates established that more than 80% of the MRSA strains were multi-drug resistant, displaying resistance to at least five different antibiotics (Fluit *et al.*, 2001). Resistance is quickly becoming more frequent and complex, developing faster than introduction of new antibiotics in the market. The discovery of new classes of antibiotics requires the identification of potential novel bacterial targets, a process which is usually both lengthy and expensive and, for that reason, does not attract many pharmaceutical industries to invest in this area. The prospects for the identification of novel antibacterial targets and, consequently, the development of new drugs are currently very limited, highlighting the urgent need to find novel ways to treat bacterial infections (Projan and Youngman, 2002; Projan, 2002; Lowy, 2003; Projan and Shlaes, 2004).

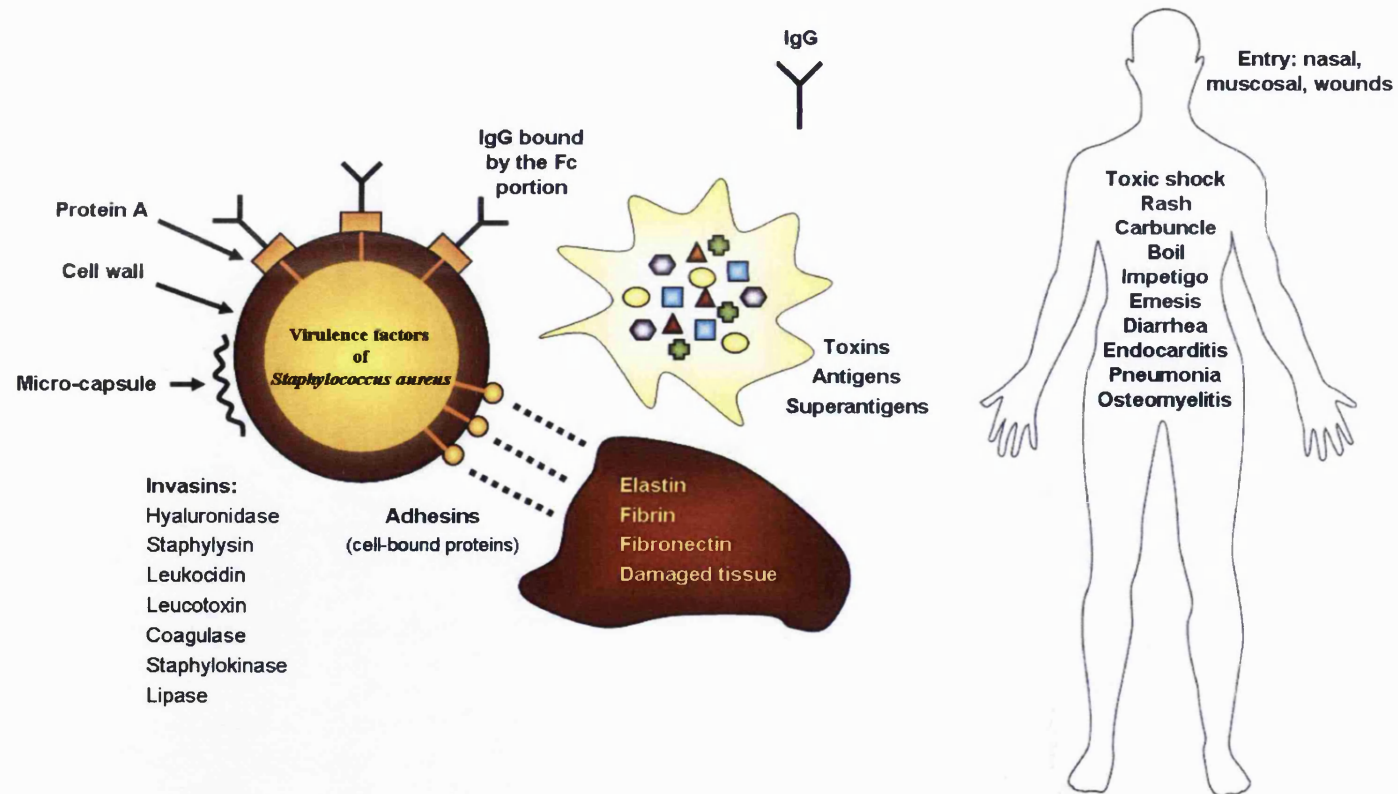
### **1.4.2 Pathogenicity and virulence**

In addition to its capacity to acquire and disseminate antibiotic resistance genes, *S. aureus* is able to produce a wide range of virulence factors, including a variety of cell associated and extracellular proteins (Fig. 1.2). Rather than causing a disease, the primary purpose of staphylococcal virulence factors is to promote adaptation and survival of the bacterium to adverse environments (Honeyman *et al.*, 2001). Its extreme adaptive capacity has enabled this bacterium to become a very successful opportunistic pathogen (Lindsay, 2008).

The staphylococcal cell wall is a thick layer composed of peptidoglycan, teichoic acids and proteins. The peptidoglycan component confers rigidity and structural integrity on the cell and the wall plays a central role in the adaptation and survival of the pathogen. It is involved in mechanisms of resistance to  $\beta$ -lactam and glycopeptides antibiotics that act on the cell wall, in evasion of the host immune system and in attachment to the surface of host cells, often through interactions with host proteins. Most clinical isolates also express a polysaccharide capsule that confers anti-phagocytic function (Götz *et al.*, 2006; Fischetti *et al.*, 2006).

## *Staphylococcus aureus*

## Effects on host (local and systemic)



**Fig.1.2:** Virulence factors of *S. aureus* and possible local and systemic effects on the host.

Bacterial surface-associated proteins play a variety of roles in staphylococcal pathogenesis. Some are able to bind to host tissues or molecules, enabling evasion of host defence mechanisms and promoting colonisation. The first cell surface protein identified in *S. aureus* was protein A (Sjodahl, 1977a), which is present on most isolates. It binds to the Fc terminal of IgG molecules and interferes with the phagocytosis of opsonised bacteria (Wright *et al.*, 1977; Sjodahl, 1977b; Potter *et al.*, 1997). Other cell surface proteins such as fibronectin (Flock *et al.*, 1987; Froman *et al.*, 1987), fibrinogen (Boden and Flock, 1989; Ni *et al.*, 1998), collagen (Holderbaum *et al.*, 1987; Switalski *et al.*, 1993; Hienz *et al.*, 1996) and elastin (Park *et al.*, 1991) bind selectively to host matrix and plasma proteins, thereby promoting colonisation of host tissues (Foster and Hook, 1998; Götz *et al.*, 2006; Fischetti *et al.*, 2006).

Many *S. aureus* isolates secrete toxins with cytolytic or superantigen (SAg) activity that damage host tissues and have a decisive role in pathogenesis. The membrane active proteins can include  $\alpha$ -,  $\beta$ -,  $\delta$ -,  $\lambda$ -toxins and leucocidin; these will be described in more detail in chapter three. *S. aureus* can produce one or more enterotoxins that are frequently responsible for food poisoning manifestations. Toxic shock syndrome is a potentially fatal acute systemic disease caused by a staphylococcal toxin which takes the same name, toxic shock syndrome toxin (TSST). Exfoliative toxin is responsible for staphylococcal scalded skin syndrome, in which the outer layer of the epidermis separates from the underlying tissues (Rogolsky, 1979; Fischetti *et al.*, 2006).

*S. aureus* may also produce several extracellular enzymes, such as coagulase, clumping factor, staphylokinase, hyaluronidase and lipase (Fischetti *et al.*, 2006). These invasins are capable of degrading host tissues, disrupting physical barriers, increasing nutrient availability and thereby promoting colonisation (Honeyman *et al.*, 2001).

The mechanisms of regulation of the production of virulence factors by *S. aureus* is a complex and coordinated process and is dependent of the physiological status of the cell and the nature of its immediate environment. Key staphylococcal global virulence regulatory systems have been described previously and extensive studies

have been undertaken in the attempt to understand how these systems interact with each other (Novick, 2003; Lindsay, 2008). Probably the best characterised loci are the accessory gene regulator, *agr* (Recsei *et al.*, 1986), the staphylococcal accessory regulator, *sar* (Cheung *et al.*, 1992), the *S. aureus* exoprotein expression gene, *sae* (Giraud *et al.*, 1994) and the alternative sigma factor, *sigB* (Wu *et al.*, 1996). These regulatory systems are fundamental to the expression of *S. aureus* virulence genes such as  $\alpha$ -toxin, coagulase and protein A (Novick, 2003; Lindsay, 2008). Further details will be discussed throughout this thesis.

## **1.5 Simulating microgravity on Earth**

Experiments performed during space flight provide a unique opportunity to study the simultaneous effects of the various physical stresses associated with the environment beyond Earth's gravitational field. It is possible, for example, to determine how microgravity, radiation and acceleration simultaneously affect the properties of different materials, fluids and organisms. The knowledge acquired onboard can then be applied in areas such as physics, medicine and biology.

Although space research can be a rewarding and exciting experience, there are clear technical difficulties. The access to such resources is severely limited as there are infrequent flight opportunities and insufficient funds to cover all but a few in-flight experiments. For this reason, the nature and the number of experiments approved by the various space agencies is restricted for each, relatively infrequent, flight opportunity. Careful planning is clearly essential in order to reduce the probability of experimental failure as it is unlikely that repeat opportunities will present themselves (Pierson *et al.*, 2007).

Sample preparation and analysis is a key issue and a frequent limitation of in-flight experiments. Frequently, samples must be prepared well in advance of lift-off and experimental material must withstand storage onboard the space vehicle after the experiment has been performed, during the period after landing, before processing in the terrestrial laboratory after recovery and prior to analysis. Prolonged storage of

samples is usually not ideal, as it may interfere with the outcome of experimental procedures (Clément and Slenzka, 2006; Pierson *et al.*, 2007).

During a mission, the limited working environment and conditions of microgravity restrict the manipulations and procedures that can be performed onboard and even relatively simple experiments require substantial crew time and expertise. The equipment must be very light, compact, simple and be able to adapt to the physical space and the nature of the experiments planned for any particular mission (Pierson *et al.*, 2007).

In addition to these predictable limitations, many unforeseen difficulties may arise during a mission. Instrument failure and break-down, operator errors, failure to correctly perform a task or sample contamination may cause complete loss of a carefully planned investigation. Moreover, since crew rotation is a frequent occurrence during a space mission, reproducibility is a technically challenging issue (Clément and Slenzka, 2006).

For the reasons stated, experiments performed during space flight have a high probability of failure and data obtained in this way often cannot be validated by repetition in order to obtain statistically significant data sets. Moreover, it is not possible to determine, for example, the effects of microgravity as a single parameter on a given sample due to omnipresent external factors such as acceleration, radiation and vibration.

As a result of the limitations associated with in-flight experimentation, ground-based experimental procedures have been developed to replicate some, but not all, of the characteristic features of the space environment and these models form an essential component of much research in the microgravity field. Models are a quick and reliable way of simulating space flight conditions and allow an approximation of single or multiple physical stresses associated with space flight. Because they provide more accessible working conditions, ground-based studies can be performed prior to space flight and the data obtained used for planning or correlation studies (Häder *et al.*, 2005; Clément and Slenzka, 2006). Tables 1.1 and 1.2 summarise some

of the various procedures that have been developed in order to provide microgravitational conditions for various periods of time.

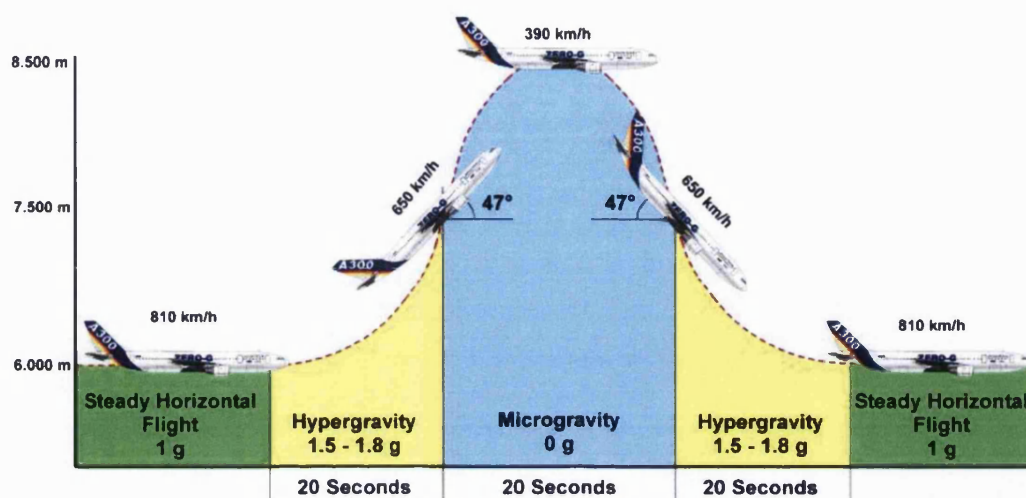
**Table 1.1:** Some of the facilities now available that provide microgravitational conditions for various periods of time (Clément and Slenzka, 2006).

Microgravity facility	Gravity ( $g$ )	Duration
Drop Tower	$10^{-6}$	2-5 s
Parabolic Flight	$10^{-2}$	20-25 s
Sounding Rocket	$10^{-6}$	5-15 min
Space Shuttle	$10^{-6}$	12-16 days
Space Station	$10^{-6}$	45 days or more

The choice of the microgravity facility or the ground-based model adequate for a particular study may depend on several parameters and is frequently based on a cost-effective perspective. Facilities such as the drop tower and parabolic flights provide short periods of microgravity which are suitable for studies involving the effect of microgravity on the properties of fluids and materials (Clément and Slenzka, 2006). Parabolic flights (Fig. 1.3) have become very popular in the last few years and have been utilised by various research groups to experience short periods of  $10^{-2}$   $g$  weightlessness. Experiments are performed aboard an aircraft which follows a specific trajectory: a gradual ascent at an angle of approximately  $47^\circ$  for 20 s produces an acceleration of 1.8  $g$ , followed by a 20 s parabolic manoeuvre of reduced gravity and a final  $47^\circ$  descent during a 20 s period. In general, thirty parabolas are flown in one parabolic flight campaign and the flight is repeated during three consecutive days. A significant amount of data can thus be obtained (Häder *et al.*, 2005; Clément and Slenzka, 2006).

**Table 1.2:** Example of ground-based models which reduce the effects of the gravitational vector on subjects or samples under study (Klaus, 2001; Clément and Slenzka, 2006; Hemmersbach et al., 2006).

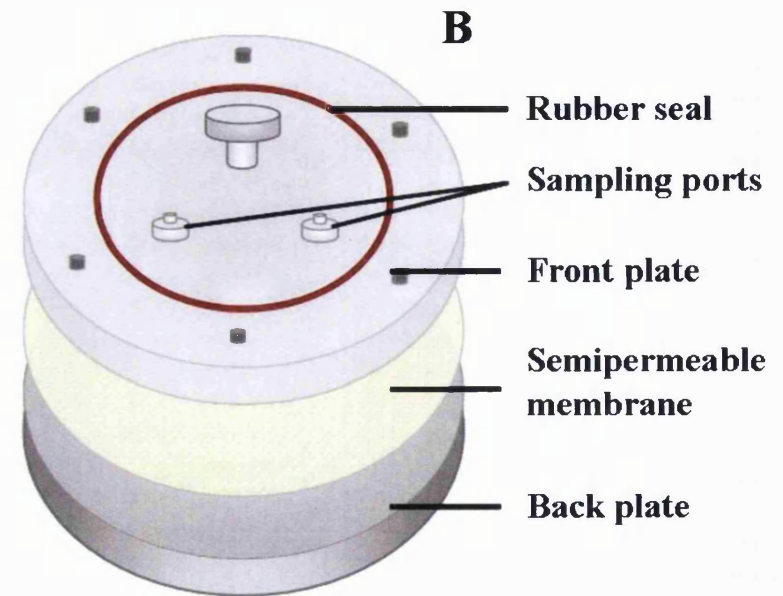
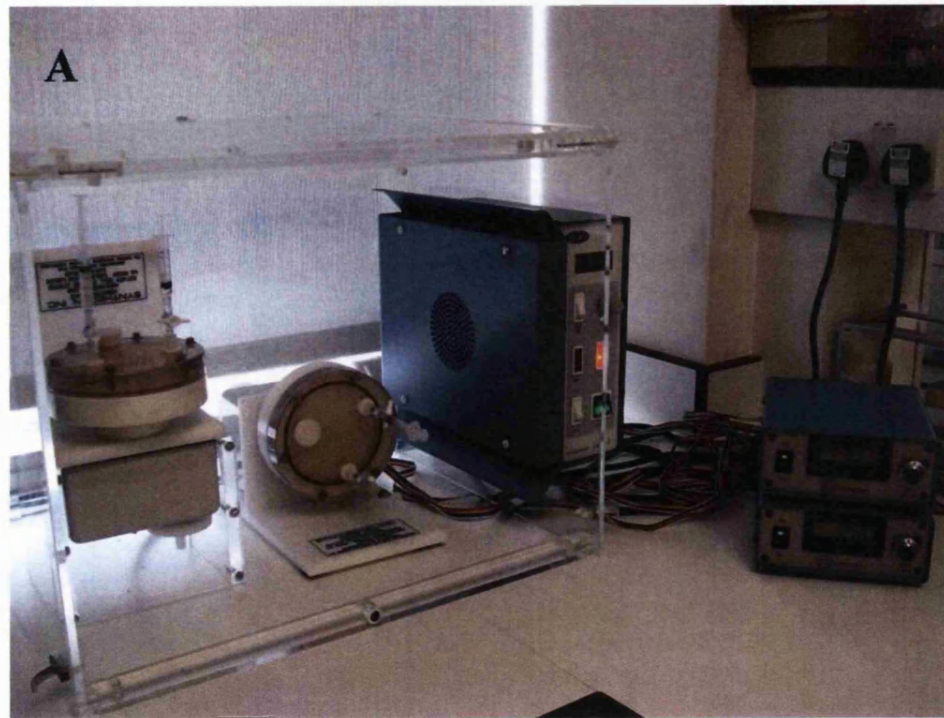
Ground-based model	Description	References
<b>Rotary Cell Culture System and clinostat</b>	Continuous rotation about a horizontal axis reduces effects of gravity on cells or plants	(Klaus, 2001; Nickerson <i>et al.</i> , 2003; Wang <i>et al.</i> , 2006)
<b>Diamagnetic levitation</b>	Levitation reduces gravitational vector on diamagnetic materials	(Valles, Jr. and Guevorkian, 2002; Coleman <i>et al.</i> , 2007; Dijkstra, 2008)
<b>Head-out water immersion model</b>	Water immersion reduces gravitational effects on a neutrally buoyant human subject	(Bonde-Petersen <i>et al.</i> , 1983; Egawa <i>et al.</i> , 2000)
<b>Hindlimb suspension model</b>	Rats are suspended by their tails eliminating the gravitational load to the hind limbs	(Templeton <i>et al.</i> , 1988; Nash <i>et al.</i> , 1991; Morey-Holton and Globus, 1998)



**Fig. 1.3:** Parabolic flight illustrating the typical flight parabola of an aircraft (A300) providing alternating periods of hyper- and hypo-gravity (image source: [www.esa.int](http://www.esa.int)).

The short periods of weightlessness provided by parabolic flights and other facilities can be useful for some studies, particularly in the field of physics; however, some biology experiments may require longer periods of exposure to the microgravity “trigger” and would have thus to be performed aboard a spacecraft or a space station (Clément and Slenzka, 2006). This option is usually extremely expensive and of difficult access and appeals for the use of ground-based models, whenever possible, as more cost-effective options. Moreover, cell culture requires sterile environments and must thus be performed using systems able to provide such experimental conditions. For these reason, novel procedures to examine the effects of simulated microgravity at the cellular level have been developed in recent years. The NASA biotechnology group developed the Rotary Cell Culture System (RCCS), a patented and licensed bioreactor which has been subsequently commercialised by Synthecon, Inc., Houston, Texas. The RCCS provides an optimised suspension culture environment and has been commonly used to study the effects of low-shear modelled microgravity (LSMMG) at the cellular level (Fig. 1.4).



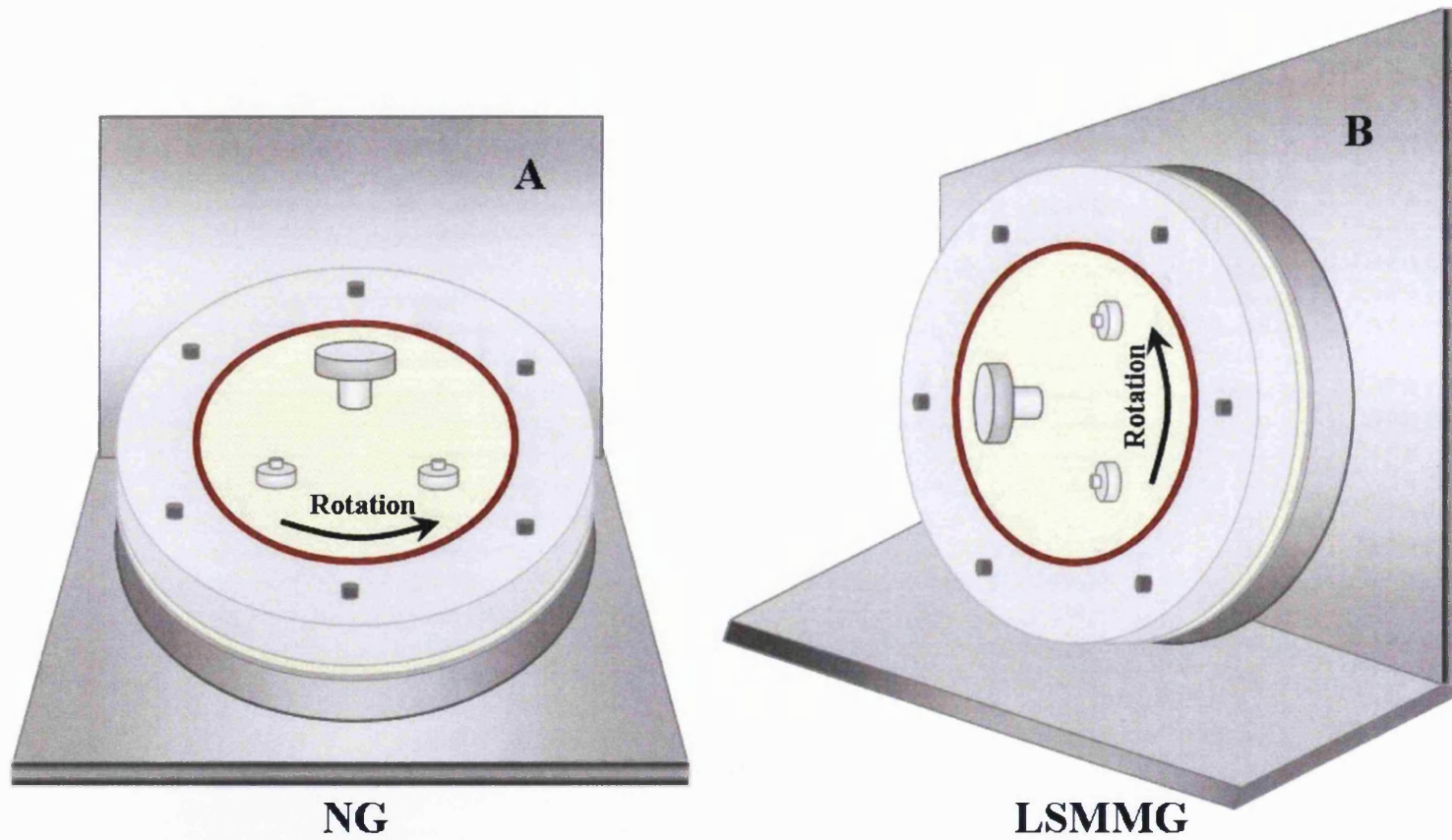


**Fig. 1.4:** The Rotary Cell Culture System (RCCS). (A) Laboratory experiment setup. (B) Elements of the High Aspect Ratio Vessel (HARV).

This equipment consists of a cylindrical High Aspect Ratio Vessel (HARV) connected to a motor and a platform on which the vessel rotates. The HARV is a rotating bioreactor which separates into two pieces: on the front face are located a fill port and two sampling ports; the back unit contains a semi-permeable membrane that allows oxygenation by diffusion. Once assembled, the vessel can be completely filled with broth and inoculum. The HARV is then attached to the platform, which can be positioned to grow cells in normal gravity (NG) or LSMMG by altering the physical position of the culture vessel (Fig. 1.5).

As the acceleration due to gravity on Earth is  $9.8 \text{ m.s}^{-2}$  (1 g), it is physically impossible for the RCCS to completely reduce or eliminate the gravitational field acting on the sample. However, the effects of this physical force on cells in culture can be minimised. When the HARV is rotated about the horizontal axis (Fig. 1.5 B), the direction of the gravity vector is accordingly re-oriented: under this mode of growth all elements within the vessel rotate together as a single entity and cells are maintained in suspension in the culture medium and do not sediment.

Because the vessel and the liquid within it are constantly and continuously rotated, the cells are subject to hydrodynamic forces, such as centrifugal and Coriolis, that are proportional to the rotation speed. The rate of rotation of the HARV should thus be optimised and should be sufficient to maintain cells in constant “free-fall” but slow enough to minimise the Coriolis and centrifugal vectors, preventing sedimentation of the suspended cells (Hammond and Hammond, 2001; Klaus, 2001). The centrifugal force explains mathematically the outward movement that an object experiences in a rotating device; in its turn, the Coriolis pseudo force defines the apparent curved path of the object when observed from inside the rotation frame of reference when, in fact, the particle is travelling in a straight path (Klaus, 2001).



**Fig. 1.5:** Operating orientations of the Rotary Cell Culture System (RCCS). Cell can be cultured under NG (A) or LSMMG (B) conditions by changing the orientation of the HARV culture vessel.

Fluid mechanics within the HARV can be estimated using calculations based on the Navier-Stokes law for flow around spherical objects (Gao *et al.*, 1997; Klaus *et al.*, 1998; Hammond and Hammond, 2001; Klaus, 2001):

$$V_{\text{sed}} = 2/9 r^2 (\rho_{\text{particle}} - \rho_{\text{fluid}}) (g / \mu)$$

The terminal velocity ( $V_{\text{sed}}$ ) of a spherical particle with a given radius ( $r$  in cm) through a fluid under the influence of gravity ( $g$ ) depends on the particle and fluid density ( $\rho$  in  $\text{g.cm}^{-3}$ ) and the fluid viscosity ( $\mu$  in  $\text{kg.h}^{-1}.\text{m}^{-1}$ ) (Klaus *et al.*, 1998; Hammond and Hammond, 2001):  $g$  is the acceleration due to gravity, which on Earth is  $9.8 \text{ m.s}^{-2}$ ;  $r$  is defined as

$$r = (3V/4\pi)^{1/3}$$

where  $V$  is the particle or cell volume in  $\text{cm}^3$  (Klaus *et al.*, 1998). When filling the vessel with medium, all air bubbles should be removed to eliminate turbulence and to ensure a sustained low-shear ( $< 1 \text{ dyn/cm}^2$ ) environment. The maximum shear stress ( $\tau_{\text{max}}$ ) is a function of terminal velocity and can also be determined using the following equation (Hammond and Hammond, 2001):

$$\tau_{\text{max}} = 3 \mu V_{\text{sed}} / (2 r)$$

In this model, bacteria are therefore considered as spherical particles. Several parameters such as the size and density of the bacteria, the density and viscosity of the medium and vessel geometry must be considered in order to determine the gravitational force that cells sense in the HARV (Klaus, 2001). Using this mathematical model, it has been confirmed that the existence of a LSMMG environment is about  $10^{-2} g$  for bacterial cells when the HARV is at a constant speed of 25 rpm (Unsworth and Lelkes, 1998; Nickerson *et al.*, 2000; Lynch and Matin, 2005).

Bacteria can be cultured in this unique continuous free-fall environment, which is generally accepted to simulate conditions of microgravity during cell growth and usually referred to as low-shear modelled microgravity (LSMMG) (Wilson *et al.*, 2002a; Nickerson *et al.*, 2003). Cells will still experience unit gravity but the constant rotation of the vessel prevents sedimentation. The gravitational vector is randomised over the cell surface and is offset by hydrodynamic forces, being time-averaged to near-zero (Unsworth and Lelkes, 1998; Hammond and Hammond, 2001; Klaus, 2001). In contrast, in a typical static terrestrial bacterial culture, cells tend to sediment to the bottom of the flask during the growth period. Agitation is often used to promote cell suspension and growth. Similar growth conditions are experienced when the HARV is rotated about the vertical axis (Fig. 1.5 A) since cells are subject to normal gravitational forces, thus serving as a control (1 g) environment.

Although originally designed to create a low-shear low-turbulence environment for cell growth and to simulate the microgravity conditions experienced during space flight, modelled microgravity systems have been widely used over a range of areas of research. The RCCS can be used to grow and study both eukaryotic and prokaryotic cells with applications in cancer research (Clejan *et al.*, 1996; Ingram *et al.*, 1997; Grimm *et al.*, 2002; Nakamura *et al.*, 2002), tissue regeneration and transplantation (Plett *et al.*, 2001; Montufar-Solis *et al.*, 2004; Navran, 2008), drug delivery (Nickerson *et al.*, 2007; Vamvakidou *et al.*, 2007) as well as in microbiology and infectious disease (Nickerson *et al.*, 2001; Carterson *et al.*, 2005; Nickerson *et al.*, 2007). A number of cell types have been grown in the HARV including bone (Klement and Spooner, 1993), cartilage (Freed and Vunjak-Novakovic, 1997; Montufar-Solis *et al.*, 2004), liver (Mitteregger *et al.*, 1999), neural (Ma *et al.*, 2008), renal (Kaysen *et al.*, 1999) and tumour (Rhee *et al.*, 2001) cells.

In contrast to traditional monolayer eukaryotic cell culture, cultivation of various mammalian cell types under a LSMMG environment produces highly differentiated and organised three-dimensional (3D) cell aggregates, structurally and functionally resembling tissues that have developed *in vivo*. A similar effect has been observed in space (Hymer *et al.*, 1996; Unsworth and Lelkes, 1998) highlighting the similarity between both environments. Such observations have important biological and medical implications as the LSMMG-generated cell phenotype and its attendant

physiology are not generally observed when conventional cell culture methods are adopted. These microgravity-generated 3D tissues could thus represent an alternative approach to donor organs for transplantation and be useful for the production of antibodies and antigens (Unsworth and Lelkes, 1998; Navran, 2008).

Growth in the RCCS also mimics the low-shear environment of certain areas of the human body such as the uterus and intestinal epithelium and may provide information regarding the adaptation of commensal and pathogenic bacteria to these niche environments (Beeson *et al.*, 2000; Cai *et al.*, 2000; Guo *et al.*, 2000; Nickerson *et al.*, 2001).

In addition, modelled microgravity systems have been frequently used to simulate the gravitational effects of space flight and to study the effect of simulated microgravity on the structural characteristics and physiology of bacterial cells. Studies of microbes in this unique environment may additionally provide important information regarding the behaviour of microorganisms in unusual and extreme environments (Fang *et al.*, 1997b; Nickerson *et al.*, 2000; Lynch *et al.*, 2004).

## **1.6 Effects of microgravity on microorganisms**

The harmful effects of prolonged exposure to a weightless environment to the human immune system are well documented (Sonnenfeld and Shearer, 2002). As a result, risk assessments of infectious disease during space missions are in the main based on the capacity of the immune system of the astronaut to resist infection (Osborn *et al.*, 2005). However, changes in key microbial characteristics as a result of exposure to microgravity will also impact astronauts' health and performance during a mission.

It is well established that microorganisms have a remarkable ability to adapt to different environments and to respond to stress (Hecker and Volker, 2001; Gasch and Werner-Washburne, 2002; Hengge-Aronis, 2002; Lindsay, 2008). Accordingly, changes in physical forces, such as shear and gravity, are very likely to impact on microbial physiology. I believe that opportunities exist to establish the impact of

microgravity on the bacterial phenotype, with respect to physiology, structure and behaviour, not only as an indicator of the potential for infection of those exposed to low-gravity forces, but also as an environmental signalling issue, in particular in relation to the important opportunistic invader of spacecraft *S. aureus*, in its own right.

The effects of microgravity on microorganisms have been previously described and, in general, reports are variable and controversial. Experiments performed as early as 1957 aboard unmanned satellites and later aboard manned spacecraft and space stations have been described and reviewed by several authors (Taylor, 1974b; Dickson, 1991; Mishra and Pierson, 1992; Nickerson *et al.*, 2003; Leys *et al.*, 2004; Nickerson *et al.*, 2004). A variety of microorganisms have been studied both during space flight (Taylor, 1974b; Dickson, 1991; Mishra and Pierson, 1992; Leys *et al.*, 2004) and, more recently, under modelled microgravity (Nickerson *et al.*, 2003; Nickerson *et al.*, 2004). The majority of the studies indicate significant differences between bacteria grown under 1 g and reduced gravity. However, various technologies and experimental approaches have been adopted in these investigations, which may challenge the interpretation and comparison of the data obtained and may also explain some of the differences that were found between studies.

Microorganisms appear to respond in different ways to variations in gravitational forces. A number of explanations have been suggested for this fact and the majority are associated with physical factors, such as decreased mass diffusion or shear levels as well as the development of microenvironments with consequent changes in the distribution of nutrient and waste products (Klaus *et al.*, 1997; Nickerson *et al.*, 2004); however, the specific mechanism(s) involved are at present unknown. Nevertheless, the knowledge gain with such experimentations can provide important and unique information on microbial physiology, pathogenicity and virulence. Table 1.3 summarises the various in-flight and ground-based studies performed on different bacteria.

**Table 1.3:** Summary of in-flight and ground-based studies performed on different bacterial species. References are indicated. ND: not described.

Bacterial species	Space flight	RCCS
<b>Growth kinetics</b>		
<i>Bacillus subtilis</i>	(Mennigmann and Lange, 1986)	ND
<i>Escherichia coli</i>	(Mattoni, 1968)	(Fang <i>et al.</i> , 1997a)
	(Bouloc and D'Ari, 1991)	(Kacena <i>et al.</i> , 1997)
	(Gasset <i>et al.</i> , 1994)	(Kacena and Todd, 1997)
	(Tixador <i>et al.</i> , 1994)	(Brown <i>et al.</i> , 2002)
	(Thevenet <i>et al.</i> , 1996)	
	(Kacena <i>et al.</i> , 1997)	
	(Kacena and Todd, 1997)	
	(Klaus <i>et al.</i> , 1997)	
	(Kacena <i>et al.</i> , 1999a)	
	(Kacena <i>et al.</i> , 1999b)	
	(Brown <i>et al.</i> , 2002)	
<i>Salmonella enterica</i>	(Mattoni, 1968)	(Wilson <i>et al.</i> , 2002a)
		(Wilson <i>et al.</i> , 2002b)
<b>Antibiotic susceptibility</b>		
<i>Escherichia coli</i>	(Tixador <i>et al.</i> , 1985a)	ND
	(Tixador <i>et al.</i> , 1985b)	
	(Lapchine <i>et al.</i> , 1986)	
	(Tixador <i>et al.</i> , 1994)	
	(Kacena and Todd, 1999)	
<i>Staphylococcus aureus</i>	(Tixador <i>et al.</i> , 1985a)	ND
	(Tixador <i>et al.</i> , 1985b)	
<b>Cell morphology</b>		
<i>Escherichia coli</i>	(Tixador <i>et al.</i> , 1985a)	ND
	(Tixador <i>et al.</i> , 1994)	
<i>Staphylococcus aureus</i>	(Tixador <i>et al.</i> , 1985a)	



Bacterial species	Space flight	RCCS
<b>Stress resistance</b>		
<i>Escherichia coli</i>	ND	(Lynch <i>et al.</i> , 2004)
<i>Salmonella enterica</i>	ND	(Nickerson <i>et al.</i> , 2000) (Wilson <i>et al.</i> , 2002a)
<b>Secondary metabolism</b>		
<i>Escherichia coli</i>	ND	(Fang <i>et al.</i> , 1997a) (Fang <i>et al.</i> , 2000b) (Gao <i>et al.</i> , 2001)
<i>Streptomyces clavuligerus</i>	ND	(Fang <i>et al.</i> , 1997c)
<i>Streptomyces hygroscopicus</i>	ND	(Fang <i>et al.</i> , 2000a)
<i>Bacillus brevis</i>	ND	(Fang <i>et al.</i> , 1997b)
<b>Virulence</b>		
<i>Escherichia coli</i>	ND	(Chopra <i>et al.</i> , 2006)
<i>Salmonella enterica</i>	(Wilson <i>et al.</i> , 2007)	(Nickerson <i>et al.</i> , 2000) (Chopra <i>et al.</i> , 2006)
<b>Protein production</b>		
<i>Pseudomonas aeruginosa</i>	ND	(Guadarrama, 2006)
<i>Salmonella enterica</i>	(Wilson <i>et al.</i> , 2007)	(Nickerson <i>et al.</i> , 2000) (Chopra <i>et al.</i> , 2006)
<i>Streptococcus pneumoniae</i>	ND	(Orihuela, 2001)
<b>Gene expression</b>		
<i>Escherichia coli</i>	ND	(Tucker <i>et al.</i> , 2007)
<i>Salmonella enterica</i>	(Wilson <i>et al.</i> , 2007)	(Wilson <i>et al.</i> , 2002b) (Chopra <i>et al.</i> , 2006)
<i>Streptococcus pneumoniae</i>	ND	(Orihuela, 2001)

### 1.6.1 Growth kinetics

The first investigations on the effects of space flight on microorganisms focused on growth kinetics, antibiotic susceptibility and cell morphology of *Escherichia coli*, *Bacillus subtilis* and *Salmonella enterica* (Taylor, 1974b). Studies on the effects of space flight on bacterial growth generally indicate shortened lag phase duration (Thevenet *et al.*, 1996; Klaus *et al.*, 1997; Kacena *et al.*, 1999a; Kacena *et al.*, 1999b), extended exponential growth phase (Klaus *et al.*, 1997; Kacena *et al.*, 1999a; Kacena *et al.*, 1999b) and higher final cell density (Mennigmann and Lange, 1986; Kacena *et al.*, 1999a; Kacena *et al.*, 1999b). Nevertheless, some authors contradict these findings, suggesting no differences between in-flight and ground-grown cells (Boulloc and D'Ari, 1991).

Similar studies performed using the RCCS are also conflicting. When *S. enterica* was grown in minimal medium under conditions of LSMMG, a shortened generation time was noted compared to NG (Wilson *et al.*, 2002a). However, such effects could not be reproduced when the growth was undertaken in rich, nutrient-replete medium (Wilson *et al.*, 2002b). Similarly, the growth of *E. coli* in minimal medium appeared to be altered under LSMMG, as increased cell dry weight was obtained compared to the 1 g control (Fang *et al.*, 1997a).

It has been hypothesised that these differences may be due to nutrient availability and are to a large extent dependent on the growth medium utilised (Klaus *et al.*, 1997). Sedimentation forces are reduced in microgravity and consequently the microenvironment in which the cells are growing is altered; there is a more homogeneous cell distribution within the culture medium, favouring nutrient availability and dissemination of waste products (Klaus *et al.*, 1997; Kacena *et al.*, 1999a). Support for this hypothesis has been obtained by Kacena and Todd (1997); *E. coli* and *B. subtilis* were cultured on solid medium during space flight in order to minimise indirect physical effects. No differences were found between in-flight and ground cultures, suggesting that changes in fluid dynamics and extracellular transport (rather than cellular dynamics) are responsible for the differences previously reported for liquid culture (Kacena and Todd, 1997; Kacena *et al.*, 1997).

The same research group also examined the effect of agitation during cell growth in 1 *g* (Kacena *et al.*, 1999a). Static cultures of *E. coli* and *B. subtilis* were compared with shaker-bath samples of the same bacterial species. The final cell concentration obtained for static *E. coli* cultures was 13% lower than the simultaneous agitated culture. This observation provides evidence that agitation may influence bacterial growth, probably by increasing nutrient availability and dissemination of waste products, a similar effect to the one described in weightless conditions. No significant differences were found comparing static and agitated *B. subtilis* cultures, which can be explained by the motility of this bacterium (Kacena *et al.*, 1999a).

The variable and somewhat conflicting data so far available in this field clearly indicates that further work is needed both in space flight and under simulated microgravity conditions to better elucidate the effect of an altered gravitational field on bacterial growth kinetics.

### **1.6.2 Antibiotic susceptibility and cell morphology**

Microgravity appears to affect the susceptibility of bacteria to antibiotics and cell morphology (Tixador *et al.*, 1985a). In-flight studies of antibiotic susceptibility appeared to increase the resistance of staphylococci and *E. coli* to a range of antibiotics (Tixador *et al.*, 1985a; Lapchine *et al.*, 1986). The MICs of oxacillin, chloramphenicol and erythromycin for *S. aureus* and of colistin and kanamycin for *E. coli* were increased under space flight conditions compared to ground controls (Tixador *et al.*, 1985b). Similar results were obtained during experiments performed in subsequent flights (Lapchine *et al.*, 1986; Mishra and Pierson, 1992).

The mechanism for this increased resistance is still unclear; however, altered mass diffusion and cellular transport mechanisms proposed by Klaus and co-workers (1997) could be a possible explanation since antibiotic diffusion in the media and cellular penetration may be consequently altered. Kacena and Todd (1999) provided some evidence that support this hypothesis. When MIC testing against gentamicin

was performed in *E. coli* grown in agar instead of liquid culture, no changes in antibiotic susceptibility were observed comparing in-flight and ground cultures.

Although the reported increased antibiotic resistance tends to be reversible upon return to Earth (Tixador *et al.*, 1985a; Lapchine *et al.*, 1986), it is an occurrence that may potentially compromise the treatment of onboard infections and consequently impact health and performance of crew members. Studies in this area should therefore be extended using different experimental conditions, such as different classes of antibiotics as well as various bacterial species. In addition, the use of ground-based models to study the effect of simulated microgravity on bacterial antibiotic susceptibility would also be of value.

### 1.6.3 Stress resistance

It has been suggested that simulated microgravity may activate the general bacterial stress response, inducing resistance to a variety of stresses (Nickerson *et al.*, 2000; Wilson *et al.*, 2002a). When *S. enterica* was grown under LSMMG conditions the bacterium became more resistant to acid, thermal and osmotic stress in comparison to NG (Nickerson *et al.*, 2000; Wilson *et al.*, 2002a). Resistance to macrophage killing was also increased under the same conditions (Wilson *et al.*, 2002a). However, LSMMG-grown cells were more sensitive to oxidative stress than NG grown bacteria (Wilson *et al.*, 2002a). Such observations are consistent with microarray data that indicate down-regulation of peroxide resistance genes under LSMMG (Wilson *et al.*, 2002b). A similar study was performed in *E. coli* demonstrating that LSMMG-grown cells were more resistant to hyperosmosis and low pH (Lynch *et al.*, 2004). These findings suggest that LSMMG differentially affects bacterial resistance to several environmental stresses (Nickerson *et al.*, 2004).

Stress responses in bacteria are known to be regulated by sigma factors (Yildiz and Schoolnik, 1998; Hecker and Volker, 2001; Hengge-Aronis, 2002; Rollenhagen *et al.*, 2003); particularly in *E. coli* and *Salmonella*, RpoS ( $\sigma^S$ ) is the primary regulatory locus which is responsible for expression of genes associated with resistance to

various environmental stresses such as acid and osmotic shock (Fang *et al.*, 1992; Lee *et al.*, 1994; Hengge-Aronis, 2002). However, general stress resistance under LSMMG was shown to be independent on this major regulator of environmental stress (Wilson *et al.*, 2002a; Lynch *et al.*, 2004). Nevertheless, resistance to environmental stress has been frequently associated with bacterial virulence potential (Yildiz and Schoolnik, 1998; Horsburgh *et al.*, 2002) and should thus be considered as valuable information, particularly in the study of the effect of gravitational forces on the virulence properties of opportunistic pathogens.

#### **1.6.4 Production of secondary metabolites**

The production of secondary metabolites under LSMMG conditions has been previously studied and the data indicate that bacteria may alter metabolic activity in this environment (Demain and Fang, 2001). The production of antibiotics was shown to be reduced, in particular the production of microcin B17 by *E. coli* (Fang *et al.*, 1997a; Fang *et al.*, 2000b),  $\beta$ -lactams by *Streptomyces clavuligerus* (Fang *et al.*, 1997c) and rapamycin by *Streptomyces hygroscopicus* (Fang *et al.*, 2000a). In contrast, the production of gramicidin S by *Bacillus brevis* (Fang *et al.*, 1997b) appeared to be unaffected by LSMMG. These findings suggest that growth under LSMMG does not consistently reduce the production of secondary metabolites and that microbes may respond in specific ways to alterations in the gravitational field (Nickerson *et al.*, 2004).

Interesting findings were also obtained comparing RCCS-production of secondary metabolites with shaking flasks. While under standard laboratory growth conditions the presence of glycerol in the growth media usually inhibits production of gramicin by *B. brevis*, such a repressive effect was not observed during growth in the HARV, both under NG and LSMMG conditions (Fang *et al.*, 1997b); similarly, the presence of glucose did not reduce the production of microcin B17 by *E. coli* when cells were grown in the RCCS (Fang *et al.*, 2000b). In addition, the accumulation site of secondary metabolites was found to be altered in HARV-cell growth compared to shaking flasks (Fang *et al.*, 1997a); while microcin B17 accumulation is usually

intracellular under standard laboratory growth conditions, growth under low shear-stress environment increased the secretion of this antibiotic by *E. coli* and its consequent accumulation in the extracellular environment (Fang *et al.*, 1997a). Since the effects reported were comparable both under NG and LSMMG, it has been hypothesised that growth in the HARV affects the production and cellular localization of secondary metabolites due to the low-shear stress conditions (Gao *et al.*, 2001; Nickerson *et al.*, 2004). This hypothesis is sustained by the observation of increased production of microcin B17 by *E. coli* when a single Teflon bead was added to the HARV, which may have enhanced shear-stress in the growth vessel (Gao *et al.*, 2001). The underlying mechanisms of these observations are still unclear.

These studies have provided important information on the environmental effects on production of secondary metabolites by microorganisms, with potential applications in biotechnology and pharmaceutical research. Although it is still unclear how growth in the HARV or under LSMMG influences the production of secondary metabolites by microorganisms, both in terms of concentration and cellular localization, it has been proposed that the low-shear characteristics of the HARV environment could be optimised for microbial growth and exploited to increase secretion and extracellular accumulation of cellular products with interest in bioprocessing and biotechnological industries (Fang *et al.*, 1997a; Nickerson *et al.*, 2004).

### 1.6.5 Virulence

Previous reports suggest that LSMMG enhances virulence of *S. enterica* (Nickerson *et al.*, 2000; Chopra *et al.*, 2006; Wilson *et al.*, 2007). As reported by Nickerson and co-workers, *S. enterica* grown under LSMMG and tested in a murine model showed a decreased average host time to death and LD<sub>50</sub> (the oral lethal dose of bacteria required to kill 50% of the tested mice) when compared to bacteria grown under NG. LSMMG-bacteria more readily colonised murine liver and spleen and showed enhanced invasive potential compared to NG-grown cells (Nickerson *et al.*, 2000).

The production of tumour necrosis-factor alpha in LSMMG-infected epithelial cells was also shown to be increased (Chopra *et al.*, 2006). Using the antiorthostatic tail suspension mouse model, reduced LD<sub>50</sub> was also observed for mice infected with *S. enterica* under simulated microgravity conditions compared to mice infected under normal gravitational conditions (Chopra *et al.*, 2006). In recent virulence studies performed using *S. enterica* grown during space flight, increased virulence of this bacterium compared to ground-cultured cells was found in a murine model. This conclusion was based on the observation of decreased time to death, increased percent mortality and decreased LD<sub>50</sub> after mice infection with space flight-cultures (Wilson *et al.*, 2007). These data are consistent with previous ground-based studies (Nickerson *et al.*, 2000).

The molecular mechanisms involved in the increased virulence induced by LSMMG found in *S. enterica* were later studied by DNA microarray analysis; however, no virulence genes were shown to be induced during growth in simulated microgravity; in fact, two major *Salmonella* pathogenicity islands were found to be down-regulated (Wilson *et al.*, 2002b). These findings raised the possibility that LSMMG may be enhancing *Salmonella* virulence by a novel mechanism which has not been yet described (Wilson *et al.*, 2002b; Wilson *et al.*, 2007).

LSMMG appears to markedly affect the production of the enterotoxigenic *E. coli* (ETEC) heat-labile enterotoxin and enhance the production of tumour necrosis factor-alpha in murine macrophages infected with enteropathogenic *E. coli* (EPEC) (Chopra *et al.*, 2006). These findings suggest that microgravity may act as an environmental signal enhancing bacterial virulence (Nickerson *et al.*, 2000). However, toxin production by *Pseudomonas aeruginosa* did not seem to be affected by microgravity since no significant differences were found in production of exotoxin A under LSMMG compared to NG (Guadarrama *et al.*, 2005).

It is important to indicate that the study of the effect of space flight and LSMMG on bacterial virulence, as well as other studies which require bacterial subculture, may be limited to some extent. It has been reported that gravitational effects are transitory and terrestrial subculture of microgravity-grown cells revert to the bacterial phenotype previously displayed under normal gravitational force (Tixador *et al.*,

1985b). If this statement is proven to be true, virulence studies may be somewhat affected, since the bacterial phenotype of the infecting bacteria may not be the same as for microgravity-grown cells. Careful interpretation of the available data should be thus implemented.

### 1.6.6 Protein production

There are limited published studies on the effects of microgravity on bacterial protein production and secretion. Proteomic analyses have demonstrated significantly decreased protein production and several differences in whole cell protein profiles of *S. enterica* under LSMMG compared to NG (Nickerson *et al.*, 2000; Chopra *et al.*, 2006). Thirty-eight proteins were found reduced threefold or more under LSMMG conditions compared to NG; however, the nature of these proteins has not been yet identified (Nickerson *et al.*, 2000). In recent studies performed by Nickerson and co-workers (2007), the proteomic profile of *S. enterica* grown during space flight was compared to NG and LSMMG conditions. In similar fashion to ground-based experiments, the production level of 73 proteins of various functions was found to be reduced during flight compared to ground controls (Wilson *et al.*, 2007).

Preliminary studies performed in *Streptococcus pneumoniae* indicate multiple quantitative and qualitative differences in protein production under NG and LSMMG, although no further information has been so far provided (Orihuela, 2001). In contrast, no differences in protein production were noted for *P. aeruginosa* when grown in the HARV for 12 or 24 h comparing NG and LSMMG cultures (Guadarrama, 2006). However, these later studies were performed using a HARV rotation speed of 15 rpm when the conditions usually adopted are 25 rpm (Nickerson *et al.*, 2000).

The lack of data in terms of the effect of space flight and LSMMG on bacterial proteomic profile clearly indicates that further research is needed in this field. Studies on the effects of space flight and LSMMG on the proteomic profiles of different bacteria may provide important information on adaptational mechanisms of



bacteria to this unique environment as well as allow the identification of new targets with potential use for the development of novel vaccines and therapeutics.

### 1.6.7 Gene expression

There is some evidence that growth under conditions of microgravity may alter gene transcription (Wilson *et al.*, 2002b; Chopra *et al.*, 2006; Wilson *et al.*, 2007). Microarray analysis revealed LSMMG-induced alterations in the expression of 22 (Chopra *et al.*, 2006) and 163 genes in *S. enterica* (Wilson *et al.*, 2002b); however, it has not been possible to identify any core cellular function affected by LSMMG in *S. enterica* since identification of these genes showed them to be spread throughout the entire chromosome. Nevertheless, this microarray data showed that LSMMG may regulate genes involved in iron metabolism, suggesting a role of the protein Fur in responses to LSMMG (Wilson *et al.*, 2002b).

Recently, *S. enterica* was grown during the space shuttle *Atlantis* mission STS-115 and its gene expression profile was analysed and compared with NG- and LSMMG-grown cells. Differential expression of 167 genes with variable function was found for in-flight cultures compared to the ground controls; in particular, 69 genes were found to be up-regulated and 98 down-regulated. The analysis of these microarray studies strongly suggests the involvement of the RNA-binding regulatory protein Hfq in the response of *S. enterica* to microgravity (Wilson *et al.*, 2007). This protein is known to be involved in the *S. enterica* stress response and in expression of certain virulence mechanisms (Figueroa-Bossi *et al.*, 2006; Sittka *et al.*, 2007; Ansong *et al.*, 2009). In contrast, no differences were found in a study of gene expression of *S. pneumoniae* grown under LSMMG compared to NG (Orihuela, 2001).

The effect of LSMMG and nutrient availability has also been studied in *E. coli*. The bacterium was grown in minimal and rich media, under both NG and LSMMG and the gene expression profiles were compared. Although reproducible transcriptional alterations were observed, no specific LSMMG responsive genes were identified. Growth in minimal medium induced changes in loci associated with the cell

envelope while growth in rich medium altered the expression of genes involved in translation (Tucker *et al.*, 2007).

Further studies on the effects of space flight and LSMMG on the transcriptomic profiles of different bacteria are clearly necessary and these should provide important information on the adaptational mechanisms of bacteria to this unique environment. By itself or together with proteomic studies, transcriptomics may allow the identification of new bacterial targets for the development of novel ways to prevent or treat infectious disease.

## **1.7 Aims**

There is a growing body of evidence suggesting that the risk of infection during extended flight may be compounded as a result of the influence of microgravity and the deep space environment on the ecology and behaviour of microorganisms, particularly bacteria. In addition to its negative impact on the immune response (Sonnenfeld and Shearer, 2002), space flight leads to profound changes in the composition of the bacterial microflora of the intestinal and nasal tracts (Nefedov *et al.*, 1971; Ilyin, 2005), to changes in bacterial growth kinetics (Klaus *et al.*, 1997; Kacena *et al.*, 1999a), and to reduction of the susceptibility of bacteria to commonly used antibiotics (Tixador *et al.*, 1985b). There are indications that some bacteria become more virulent when cultured under conditions of modelled microgravity (Nickerson *et al.*, 2000; Wilson *et al.*, 2007). However, the reasons for these findings remain unknown.

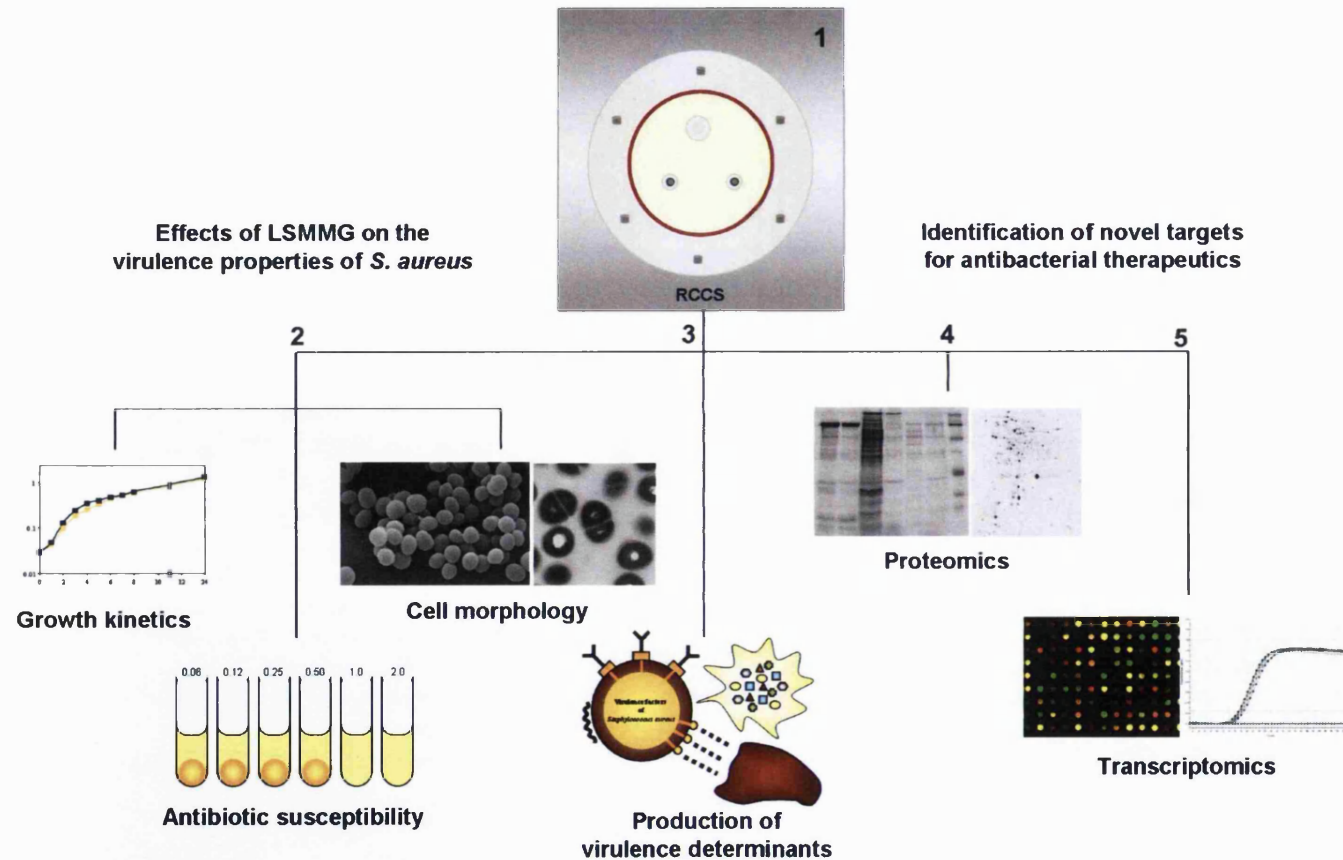
Microbiological monitoring of air and surfaces within the ISS indicates that bacteria of the genus *Staphylococcus* are found in high frequency (Novikova *et al.*, 2006). *S. aureus*, an opportunistic pathogen with the capacity to cause severe debilitating infection, constitutes a significant proportion of these isolates and is thus likely to pose a risk of infection on extended missions. Few experiments have been performed with staphylococci aboard space shuttles (Tixador *et al.*, 1985a); similarly, previous ground-based studies in the main investigated the effects of simulated microgravity

on Gram-negative bacteria such as *E. coli* and *S. enterica* (Nickerson *et al.*, 2004) and not on key Gram-positive pathogens such as *S. aureus*.

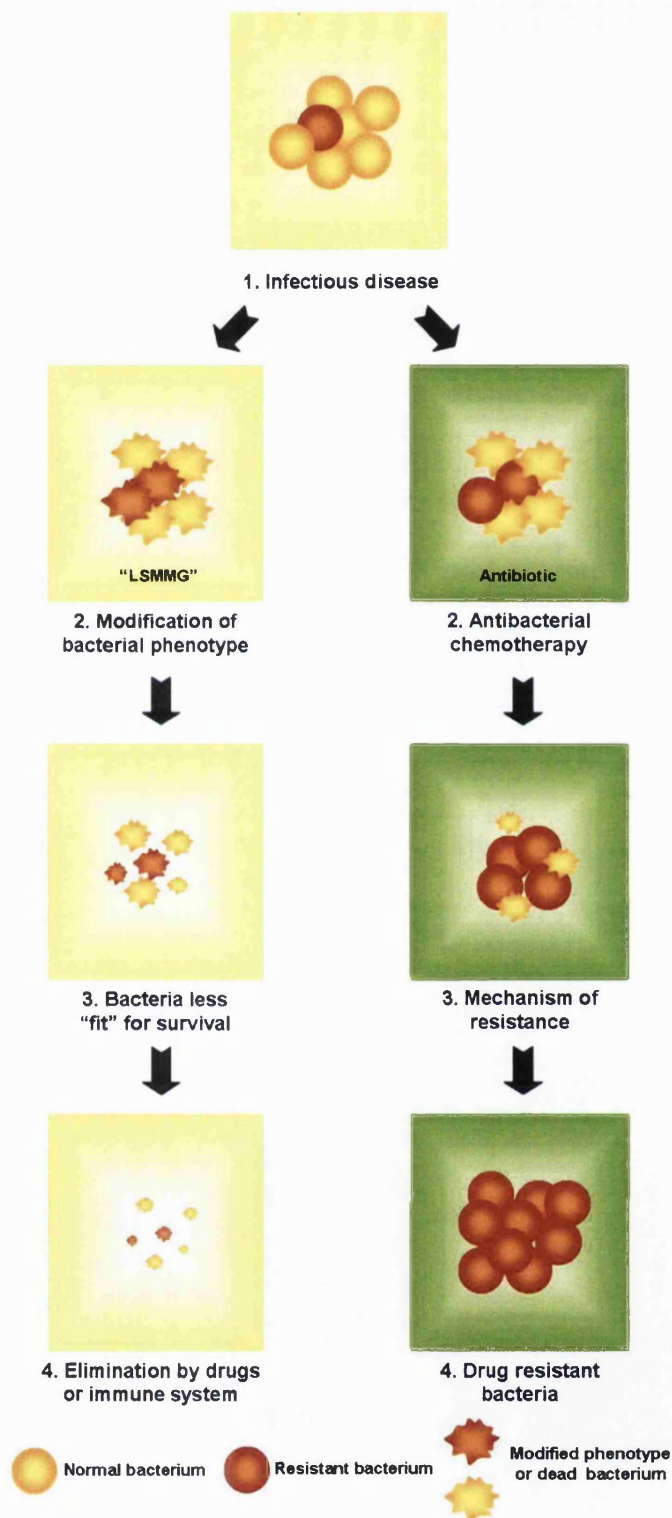
This study will evaluate the effects of simulated microgravity on the biology of *S. aureus* and attempt to identify the mechanisms involved in mechanical sensing of this environmental factor (Fig. 1.6). Several parameters such as growth kinetics, antibiotic susceptibility, production of virulence determinants and gene expression will be determined and discussed in terms of the potential impact of microgravity-induced phenotypic modifications on extended missions into deep space. Investigation of the effects of different gravitational forces on microorganisms may have potential future applications in both academic and economic terms.

*S. aureus* shows a strong tendency to accumulate antibiotic resistance genes and has become increasingly resistant to a variety of antimicrobial agents. The limited prospects for the identification of novel antibacterial targets and, consequently, the development of new drugs have led to an overreliance on modification of the existing antibiotic structures in order to increase their efficacy and potency against MRSA strains. However, there is an urgent need for the discovery of new classes of antibiotics with new targets for chemotherapeutic attack (Projan and Youngman, 2002; Projan, 2002; Lowy, 2003; Projan and Shlaes, 2004).

Although recent work has provided important information on the complex regulatory networks that control gene expression in staphylococci (Goerke *et al.*, 2001; Xiong *et al.*, 2006), the environmental cues that stimulate the synthesis and transport of virulence and antibiotic resistance determinants are poorly understood (Novick, 2003). The study of *S. aureus* in low-shear low-turbulence conditions may provide a better understanding of the pathogenicity and virulence mechanisms of this bacterium, as well as reveal novel regulatory components that lead to down-regulation of virulence determinants. The use of LSMMG provides the opportunity to identify regulators of the pathogenic phenotype which would represent attractive targets for antibacterial therapeutics.



**Fig. 1.6:** Aims and strategies of the study of the effect of simulated microgravity on the virulence properties of the opportunistic bacterial pathogen *S. aureus*. After growth in the Rotary Cell Culture System (RCCS) (1) *S. aureus* parameters such as antibiotic susceptibility and cell morphology (2), production of virulence determinants (3), proteomic (4) and transcriptomic (5) profile will be analysed.



**Fig. 1.7:** An alternative approach to antibacterial chemotherapy based on inhibition of the expression of bacterial virulence and antibiotic resistance genes, allows host defences to overwhelm the invader or rejuvenate existing clinical antibiotics whose activity is compromised by resistance.

Conventional antibiotics impose intense selective pressure on bacterial populations that gradually leads to the emergence of drug and multi-drug resistance. For this reason, there is an urgent need for the development of novel therapeutic options that suppress or abrogate the emergence of drug resistant strains. An alternative approach to antibacterial chemotherapy is based on inhibition of the expression of virulence and antibiotic resistance genes, allowing host defences to overwhelm the invader or rejuvenating existing clinical antibiotics whose activity is compromised by resistance. Such a mechanism is less likely to rapidly select the drug-resistant genotype (Alksne and Projan, 2000; Taylor *et al.*, 2002; Cegelski *et al.*, 2008). In this study, simulated microgravity is utilised as an environmental trigger for a potential modification of the *S. aureus* phenotype so that bacteria become less “fit” with respect to survival in the human body and more easily eliminated by antibacterial drugs or by the immune system (Fig. 1.7).

Figure 1.6 and 1.7 summarise the aims and strategies of the current study of the effect of simulated microgravity on the virulence properties of the opportunistic bacterial pathogen *S. aureus*.

## **CHAPTER TWO**

**Effect of LSMMG on the growth kinetics, antibiotic susceptibility and cell morphology of *S. aureus***

## 2.1 Introduction

Attempts have been made since the early 1970s to determine the effects of microgravity on a variety of microorganisms. Many of these studies were undertaken as internal investigations for space agencies and were not reported in the open literature but a significant proportion of this body of data has subsequently been reviewed and included are data obtained during space flight (Taylor, 1974b; Dickson, 1991; Mishra and Pierson, 1992; Leys *et al.*, 2004) and using ground based models (Nickerson *et al.*, 2003; Nickerson *et al.*, 2004). *E. coli*, *B. subtilis* and *S. enterica* are the most well-characterised bacteria under microgravity conditions. So far, only one study of the effects of space flight on *S. aureus* has been published, suggesting alterations in the bacterial phenotype in terms of antibiotic susceptibility and cell morphology (Tixador *et al.*, 1985b).

Reports on the effects of microgravity on bacterial growth, viability, morphology and antibiotic susceptibility are variable and controversial. For this reason, the available data must be carefully interpreted, as reported changes in bacterial phenotype in response to variations in the gravitational field may not be attributable to changes in gravity *per se* but may have resulted due to the method or the test conditions employed. Moreover, the use of different bacterial species and strains as well as experimental conditions complicates comparison of results obtained by the various research groups.

Also important to note is that space biology is constantly affected by unforeseen difficulties. Limitations associated with prolonged or inadequate sample storage, lack of sophisticated equipment, insufficient number of controls or experiment repetitions, may cause a total loss of an investigation planned for several years that may not be able to be repeated in the future (Clément and Slenzka, 2006). Consequently, in some published studies, the interpretation of data may be handicapped by lack of evidence of biological reproducibility and statistical insufficiencies such that the conclusions drawn may be open to doubt. These issues have been described in chapter one and examples will emerge during the course of this thesis.



Changes in the properties of bacteria as a result of exposure to microgravity may have a negative impact on the health of astronauts in-flight and may therefore affect their performance during a mission. Some of these effects have been described in general terms in chapter one. This chapter focuses on bacterial growth kinetics, antibiotic susceptibility and cell morphology under simulated reduced gravity.

### **2.1.1 Effect of microgravity on bacterial growth kinetics**

One of the first studies on the effects of microgravity on bacterial growth kinetics was conducted in 1967 aboard Biosatellite II. *S. enterica* and *E. coli* were cultivated during Earth-orbital flight and their final cell density was increased compared to ground controls (Mattoni, 1968; Tixador *et al.*, 1985a). Similarly, *B. subtilis* was grown under space flight conditions during Spacelab D1 shuttle mission *Challenger* STS-61-A in 1985 and an increased bacterial growth rate and higher final cell population in microgravity were demonstrated (Mennigmann and Lange, 1986).

A set of experiments performed aboard seven American space shuttle missions between April 1991 and March 1994 (STS-37, -43, -50, -54, -57, -60, -62) revealed differences in the growth characteristics of *E. coli* (Klaus *et al.*, 1997). Only limited sample numbers were recovered during these experiments, which considerably affected statistical interpretation, but a trend to shortened lag phase and a significantly higher final cell density in stationary phase was noted. Growth rate was apparently unaffected during the increased duration of the exponential phase.

Later, Thévenet and co-workers (1996) investigated two *E. coli* strains, a motile strain and a non-motile mutant, aboard the American shuttle mission *Columbia* STS-65. Two in-flight cultures, one on a static rack (0 g) and a control sample on a centrifuge running at 1 g, were compared with two other Earth controls, one on a static rack (1 g) and another one on a centrifuge running at 1.4 g. No differences were found in the growth kinetics between the flight and ground samples for the motile strain. In contrast, for the non-motile mutant, the lag phase was considerably shorter in 0 g when compared with the flight and ground controls. These results

suggest alterations in cell growth under microgravity but the data could not be statistically interpreted since a considerable fraction of the mutant inoculum was unable to form colonies after recovery of the samples.

Subsequent investigations performed during STS-63 shuttle mission *Discovery* in 1995 included extensive control culture conditions and cell-growth data taken several times during in-flight growth of *E. coli* and *B. subtilis* strains (Kacena *et al.*, 1999a). Analysis of the data obtained indicated that cultures grown during space flight displayed a shorter lag period, an increased exponential growth rate and a higher final cell concentration compared to ground controls. Similar results were found later by the same group of researchers aboard space station Mir (Kacena *et al.*, 1999b).

The majority of the studies of the effects of space flight on the growth kinetics of bacteria indicate differences between in-flight and ground cultures. Reports on the effects of microgravity on bacterial growth, viability, morphology and antibiotic susceptibility are variable and controversial, including a shortened lag phase duration and higher final cell density. Nonetheless, some authors have contradicted these findings, particularly when examining *E. coli* during space flight conditions, and it has been suggested that microgravity has no effect on bacterial growth kinetics (Bouloc and D'Ari, 1991; Tixador *et al.*, 1994; Gasset *et al.*, 1994).

More recently, ground-based experiments have been performed to determine the effects of microgravity on the growth kinetics of bacteria; such experiments will not be influenced by non-gravity-related conditions found during space flight, such as increased solar and cosmic radiation and vibration. In the main, bacterial growth experiments conducted under simulated microgravity underpin the conclusions drawn for in-flight experiments (Klaus *et al.*, 1998) but there is again some conflicting evidence. It is clear that the conditions generated under simulated microgravity may affect nutrient flux and changes in the availability of essential nutrients must be taken into account when interpreting the data from such work (Baker *et al.*, 2004).

When *S. enterica* was grown in minimal salts medium under LSMMG, a shortened generation time was found compared to NG cultures (Wilson *et al.*, 2002a). Such

effects could not be reproduced when the same bacterium was grown in complex media (Wilson *et al.*, 2002b). Similarly, LSMMG-grown *E. coli* reached higher final cell density compared to NG-grown bacteria when minimal medium was used (Fang *et al.*, 1997a). Interestingly, many of the studies performed in space that reported changes in bacterial growth kinetics (Klaus *et al.*, 1997; Kacena *et al.*, 1999a; Kacena *et al.*, 1999b; Brown *et al.*, 2002) also used minimal growth medium.

Effects of space flight, simulated microgravity and hypergravity on *E. coli* have been simultaneously determined by Brown and co-workers (Brown *et al.*, 2002). The bacteria were grown aboard STS-95 shuttle mission *Discovery* and were compared with ground controls. Cultures grown in space reached higher final cell populations than ground controls, suggesting stimulated bacterial growth. To a lesser extent, this trend was also found for cultures grown under simulated microgravity. In contrast, and as expected, hypergravity decreased the size of the final bacterial population.

### **2.1.2 Effect of microgravity on bacterial antibiotic susceptibility and morphology**

Treatment of infections among crew members during space flight could be compromised by temporary or permanent changes in bacterial antibiotic susceptibility as a result of exposure to the space environment. Interestingly, changes in antibiotic susceptibility have been associated with modifications in the ultrastructure of the staphylococcal cell wall. Increased levels of resistance to vancomycin were found to be accompanied by reduced rates of cell wall turnover and autolysis, increased thickness of cell wall and reduced cell division (Sieradzki and Tomasz, 2003). Similarly, changes in the staphylococcal susceptibility to  $\beta$ -lactam antibiotics and an increased cell wall thickness were observed during growth in the presence of polyphenols (Hamilton-Miller and Shah, 1999; Stapleton *et al.*, 2007).

There is some evidence that bacterial isolates recovered in-flight from astronauts were more resistant to antibiotics than isolates collected pre- or post- flight from the

same individuals (Tixador *et al.*, 1985b). Other early studies performed during Skylab missions 2 and 4 revealed changes in antibiotic susceptibility and cell morphology of *B. subtilis* (Tixador *et al.*, 1985b) compared to these parameters determined on Earth.

These observations provoked a more detailed examination that was carried out in 1982, as part of the Cytos 2 experiment aboard the Soviet space station Salyut 7 (Tixador *et al.*, 1985a; Tixador *et al.*, 1985b). *E. coli* and *S. aureus* isolates from the commensal flora of the French astronaut Jean-Loup Chrétien were examined for their antibiotic susceptibility and morphology during orbital flight and compared to ground controls. Two antibiotics were tested against the *E. coli* isolate, colistin and kanamycin; the *S. aureus* isolate was tested against oxacillin, chloramphenicol and erythromycin. MICs were determined for in-flight cultures and were compared to ground controls; the data indicated a fourfold decrease in antibiotic susceptibility for the *E. coli* strain and a twofold decrease for the *S. aureus* isolate. These differences were considered by the authors to be significant, particularly for *E. coli*. The reduced antibiotic susceptibility was transitory, as terrestrial subculture of the flown bacteria following return to Earth showed that both bacteria reverted to the phenotype displayed prior to the flight.

The effect of space flight on the bacterial ultrastructure, in the absence of antibiotics, was also recorded during this mission (Tixador *et al.*, 1985a). Cells were cultivated without antibiotics during space flight and after recovery they were fixed and analysed in standard laboratory conditions. No differences were observed for *E. coli* but a thickening of the staphylococcal cell wall was noted during in-flight growth.

Later in 1985, as part of the European Biorack 58F programme Antibio (D1 mission), a similar experiment was performed aboard the American space shuttle *Challenger* (Lapchine *et al.*, 1986). The activity of colistin was tested against *E. coli* under different gravitational conditions. This experiment differed from the one carried out by Tixador and co-workers mainly because it included several controls: two in-flight cultures, one on a static rack (0 g) and another one in centrifugally-simulated 1 g, were compared with two Earth controls, one on a static rack (1 g) and another on a centrifuge running at 1.4 g. A twofold decrease in susceptibility to

colistin was found for the in-flight cultures compared with the Earth controls and was considered by the authors to be significant.

Changes in *E. coli* antibiotic susceptibility were also studied as part of the Antibio 23F experiment during the STS-42 mission aboard the US space shuttle *Discovery* in 1992 (Tixador *et al.*, 1994). Bacteria were grown in the presence of different concentrations of dihydrostreptomycin and the MIC determined. Results obtained were somewhat inconclusive, as only small differences were found between the flight cells and the ground experiments. In similar fashion to the Cytos 2 experiment, *E. coli* ultrastructure was examined by electron microscopy of fixed cells and it was concluded that bacterial cell morphology was not affected by flight conditions.

These studies are in the main inconclusive and clearly indicate that further work is needed to clarify the effects of microgravity on the growth kinetics, antibiotic susceptibility and cell morphology of bacteria. This chapter is a contribution to the knowledge in this area and focuses on the Gram-positive bacterium *S. aureus*. The effect of LSMMG on *S. aureus* growth kinetics antibiotic susceptibility and cell morphology was determined.

## **2.2 Materials and Methods**

### **2.2.1 Bacterial strains**

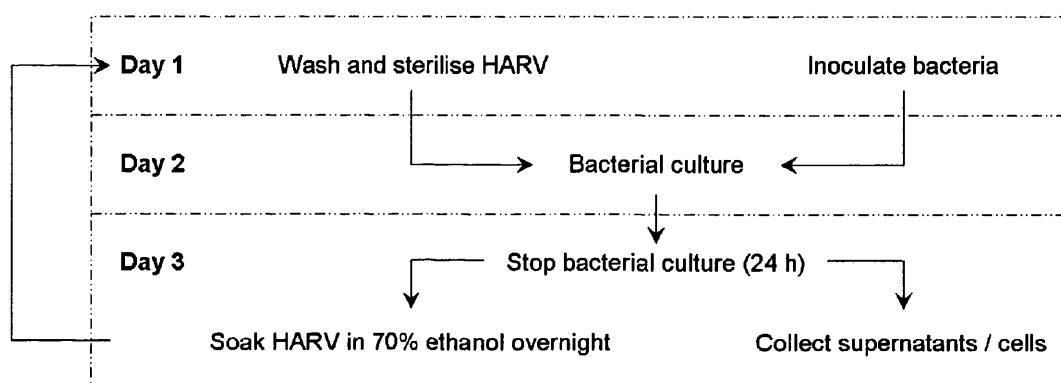
All studies were performed using methicillin susceptible *S. aureus* (MSSA) isolates RF1, RF6 and RF11. These bacteria are clinical isolates from the Royal Free Hospital, London and were a gift from J.M.T. Hamilton-Miller.

These isolates were selected for study on the basis of differences in their growth, pigmentation and haemolysin secretion following culture on sheep blood agar. Initially, 20 *S. aureus* isolates were screened for these phenotypic parameters. One single colony of each *S. aureus* isolate was diluted in 3 ml of Müller-Hinton broth

(MHB) (Oxoid, Basingstoke, UK) and 10 µl of the suspension plated on sheep blood agar. After overnight incubation at 37°C, plates were examined in the usual manner.

### 2.2.2 HARV preparation and sterilisation

The following diagram explains the experimental procedures for bacterial growth in the RCCS:



**Fig. 2.1:** Experimental procedures for bacterial growth in the RCCS.

The RCCS can be provided with autoclavable or disposable HARVs. Only two autoclavable vessels were available during the course of this project, limiting the final number of samples that could be collected. On average, two experimental procedures were performed per week, as three days were required for each bacterial growth experiment. The HARV is very susceptible to physical damage. Continuous autoclaving and usage may lead to leakage and breakage. Replacement of damaged pieces was required during the course of this project, delaying sample collection and significantly increasing the cost of the project.

The HARV contains a semi-permeable membrane that allows oxygenation by diffusion. This membrane is particularly susceptible to damage and may easily become impregnated with dirt residues which may block oxygen diffusion through the membrane or cause the vessel to be toxic and thus unsuitable for cell growth. For this reason, before each experiment, the two halves of the HARV were separated and all the pieces were washed; special attention was given to the oxygenator membrane.

Membrane aeration may be limited for specific bacterial species and can be improved using an air pump to gently aerate the culture whilst keeping the shear forces low. Since *S. aureus* is a facultative aerobe, membrane aeration is sufficient for growth and was the adopted method throughout this project. However, low oxygenation may affect cell growth and yield and this should be considered when interpreting data.

Before autoclaving, the vessels were assembled and soaked in 70% ethanol. The plastic valves were removed and autoclaved separately from the vessel. The peripheral screws were loosened one turn, the cell ports covered with aluminium foil and all the components were then autoclaved for 30 min at 110°C. Sterility conditions were verified by incubation of sterile MHB in the HARV at 37°C for 18 h and confirmation of absence of growth detected as turbidity. Contamination was sometimes observed in the vessel. To reduce this occurrence, the vessels were assembled within a class II microbiological safety cabinet and all materials required for bacterial growth were manipulated with extreme care to avoid contamination.

As indicated in the RCCS specifications, to obtain good representative samples the rotation of the vessel should not be stopped during sampling. The growth media should be withdrawn whilst the vessel is rotating by manipulating the syringes attached to the sampling ports. However, cell sedimentation may occur within the control vessel due to its horizontal position, which may interfere with the sampling procedure and the reproducibility of samples collected. In order to quantify the effects of cell sedimentation in the HARV, samples were removed using two different sampling procedures: during continuous rotation of the HARV and following discontinuation of vessel rotation.

### **2.2.3 Bacterial culture**

Bacteria were grown in MHB using Synthecon RCCS-1 HARVs (obtained from Cellon S.A., Bereldange, Luxembourg) housed in a 37°C incubator. Bacterial cultures were incubated in MHB for 18 h at 37°C. The overnight culture was then

inoculated at a dilution of 1:50 into 50 ml of fresh MHB and subsequently introduced into the HARV. Care was taken to ensure that the reactor was completely filled with medium, with complete absence of air bubbles. The bioreactors were then orientated to grow cells under conditions of LSMMG (rotation about the horizontal axis) or NG (rotation about the vertical axis). In all experiments, LSMMG and NG cultures were grown simultaneously with a rotation rate of 25 rpm for up to 24 h at 37°C. At these rotation speed LSMMG-cells experience simulated microgravity conditions equivalent to  $10^{-2}$  g (Nickerson *et al.*, 2000). Cells were harvested by centrifugation at 3,000 g for 10 min and the culture supernatants were retained for further analysis.

#### **2.2.4 Determination of growth kinetics and final cell density**

The growth of the cultures in the HARV was monitored over a period of 8 h by measuring the cell density at  $\lambda_{600\text{nm}}$  using a Perkin Elmer Lambda 25 spectrophotometre (Buckinghamshire, UK). A final measurement was taken after 24 h of growth. Numbers of viable bacteria were determined by serial dilution and plating on to nutrient agar. The samples were removed *via* the HARV sampling port using two different sampling procedures: during continuous rotation of the HARV and following discontinuation of vessel rotation. During the sampling procedure, care was taken to ensure that bubbles were not introduced into the vessel. The experiment was repeated twice for each *S. aureus* isolate.

After 24 h of growth in the HARV, cells were harvested by centrifugation at 3,000 g for 10 min at 4°C, pellets were frozen at -80°C for at least 2 h and then freeze-dried using a VirTis Advantage freeze dryer (VirTis, Hampshire, UK). The thermal stage was set at -20°C for 2 h and the primary drying was carried for 14 h at 200 psi. The secondary drying was set at 10°C for 8 h at a reduced pressure of 50 psi. Final cell density was determined as cell dry weight (mg). For statistical analysis, the experiment was repeated six times for each *S. aureus* isolate (n=6).



### **2.2.5 Determination of Minimum Inhibitory Concentration (MIC)**

The antibiotics used in this study were kindly provided by the following companies: GlaxoSmithKline (flucloxacillin), Eli Lilly (vancomycin hydrochloride) and Sanofi Aventis (erythromycin).

The antibiotic susceptibility of the isolates was compared by MIC determinations using the standardised broth dilution method, according to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI). The assays were performed in 96-well microtitre trays using serial twofold dilutions of the antibiotics flucloxacillin, vancomycin and erythromycin and a bacterial inoculum of  $10^4$  CFU. The dilutions of the different antibiotics and bacteria were prepared in MHB. Bacterial cultures grown for 24 h under NG and LSMMG were diluted to obtain a final bacterial count of approximately  $10^4$  CFU/ml; 100  $\mu$ l of this suspension were dispensed into the microtitre plate wells. The final volume per well was 200  $\mu$ l. *S. aureus* ATCC29213 was used as an indicator strain. Presence or absence of growth was read after 24 h of incubation at 37°C. Bacterial growth was detected in the form of clumps of bacteria at the bottom of the well or as turbidity; a clear well indicated inhibition of growth. MIC values were determined to be the lowest antibiotic concentration at which no visible bacterial growth occurred. Viable counts from the diluted bacterial suspensions were performed to confirm that the correct bacterial inoculum was achieved in each well. Each *S. aureus* isolate was grown in three independent HARV cultures. Three MIC determinations per isolate (one for each HARV culture) were performed (n=3). Duplicates were included for all MIC determinations.

### **2.2.6 Morphologic analysis by electron microscopy - scanning electron microscopy (SEM) and transmission electron microscopy (TEM)**

SEM was performed by Mr. David McCarthy at The School of Pharmacy, University of London. TEM was performed by Mr. Innes Clatworthy at The Royal Free Hospital, London.

After 24 h growth in the HARV, cells were recovered by centrifugation at 3,000 g for 10 min, washed twice with 50 ml of phosphate buffered saline (PBS) and fixed in 1 ml of 1.5% w/v glutaraldehyde for at least 2 h at room temperature.

For scanning electron microscopy (SEM), the pellet was washed once with 1 ml of 70% ethanol and twice with the same volume of 100% ethanol. Air-dried, gold-coated preparations were examined using a FEI XL30 scanning electron microscope.

For transmission electron microscopy (TEM), the cells were treated with osmium tetroxide and embedded in epoxy resin. Sectioning and staining with uranyl acetate was followed by Reynolds' lead citrate. Ultra-thin sections were viewed and photographed using a Philips 201 transmission electron microscope.

Each *S. aureus* isolate was grown in two independent HARV cultures. SEM and TEM experiments were repeated twice for each isolate (one for each HARV culture).

## **2.3 Results**

### **2.3.1 Selection of *S. aureus* isolates**

The selection of *S. aureus* isolates for further investigation was based on colony size, pigmentation and haemolysin secretion following growth on sheep blood agar. A collection of 20 different methicillin susceptible *S. aureus* (MSSA) isolates was screened based on these phenotypic parameters. The results are summarised in table 2.1. Isolates RF1, RF6 and RF11 were selected on the basis of differences in their phenotypic characteristics. By analysing colony size when cultures were plated on agar, isolate RF6 was found to be the slowest growing isolate while RF11 grew more rapidly than the other two isolates. In terms of colony pigmentation, RF1 produced light yellow colonies while RF6 colonies were more intense yellow than the others. Haemolysin secretion was detected by the appearance of zones of haemolysis, visualised as a halo around the bacterial colonies. All three isolates were found to

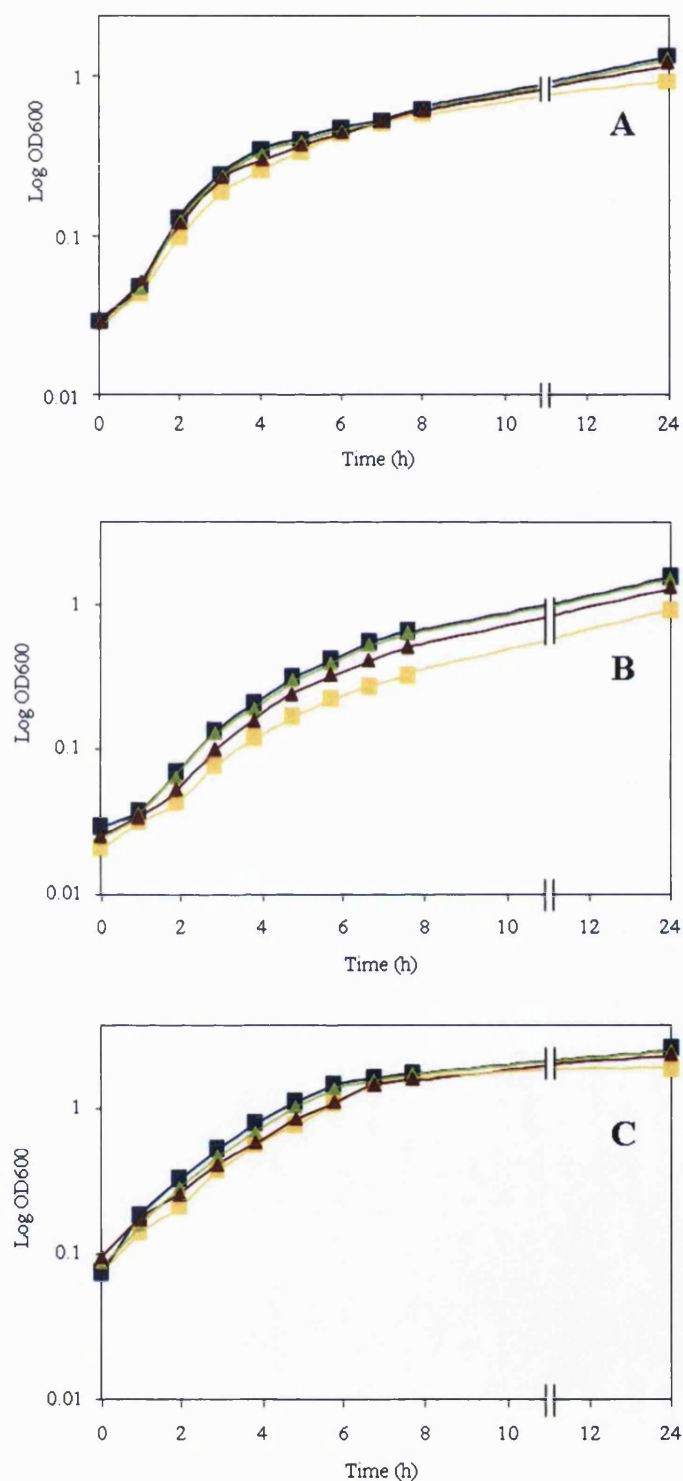
lyse sheep erythrocytes but RF1 appeared to secrete more toxins, followed by isolate RF6 and finally RF11, which secreted less total haemolysin.

**Table 2.1:** Characteristics of the 20 *S. aureus* isolates screened when plated on sheep blood agar. Colony size was compared according to the approximate colony diameter and haemolysin secretion was compared according to the approximate diameter of the halo formed around the bacterial colonies. + < 0.5 mm, 1 mm < ++ > 0.5 mm, +++ > 1 mm.

MSSA Isolate	Colony Size	Colony pigmentation	Haemolysin secretion
RF1	++	Light yellow	+++
RF2	+++	Light yellow	+
RF3	++	Yellow	+
RF4	+++	Bright yellow	+++
RF5	+++	Bright yellow	++
RF6	+	Bright yellow	++
RF7	+++	Light yellow	+++
RF8	+	Light yellow	+
RF9	++	Light yellow	+
RF10	++	Light yellow	+
RF11	+++	Yellow	+
RF12	++	Light yellow	+
RF13	++	Light yellow	++
RF14	++	Light yellow	+
RF15	+++	Light yellow	+
RF16	+++	Yellow	+
RF17	+++	Yellow	++
RF18	+	Light yellow	+
RF19	++	Light yellow	+
RF20	+	Light yellow	+

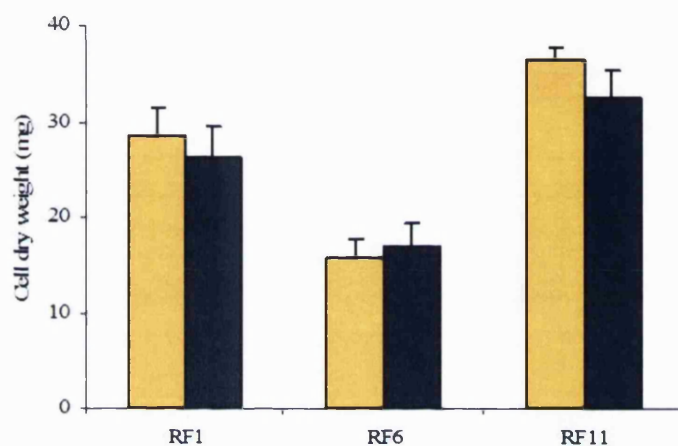
### 2.3.2 Effect LSMMG on *S. aureus* growth kinetics

*S. aureus* isolates RF1, RF6 and RF11 were grown in the HARV under conditions of NG and LSMMG over a 24 h period. OD measurements performed during the growth period are shown in figure 2.2. As indicated in the RCCS specifications, to obtain good representative samples, the rotation of the vessel should not be stopped during sampling. The growth media should be withdrawn whilst the vessel is rotating by manipulating the syringes attached to the sampling ports. Samples were removed using two different sampling procedures: during continuous rotation of the HARV and following discontinuation of vessel rotation. The growth curves obtained for each sampling method are shown in figure 2.2.



**Fig. 2.2:** Growth kinetics of *S. aureus* isolates RF1 (A), RF6 (B) and RF11 (C). NG vessel in continuous (■) or discontinuous (▲) rotation and LSMMG vessel in continuous (■) or discontinuous rotation (▲).

Under LSMMG, the absence of sedimentation may result in a more homogeneous cell distribution within the vessel and therefore no differences were noted between the two sampling methods. Cells within the control vessel (horizontal position) may sediment on the bottom of the HARV and a difference between the two sampling methods was particularly evident at the 24 h time point.



**Fig. 2.3:** Final cell density determined as cell dry weight of *S. aureus* isolates RF1, RF6 and RF11 grown under NG (■) and LSMMG (■) after 24 h. Error bars represent 1 SD; n=6;  $p > 0.05$  in all three isolates.

No major differences were found with regard to the shape of the growth curve, rate of growth or final density of the *S. aureus* isolates RF1, RF6 and RF11 when growth under NG conditions was compared to LSMMG conditions over a 24 h period. A slower growth rate was noted in cells grown under NG compared to LSMMG-grown bacteria; however, no significant changes in the final cell density ( $p > 0.05$ ), determined as cell dry weight after 24 h of growth, were noted (Fig. 2.3).

### 2.3.3 Effect of LSMMG on susceptibility of *S. aureus* to antibiotics

The effect of growth under LSMMG conditions on susceptibility to the antibiotics erythromycin, flucloxacillin and vancomycin were determined using *S. aureus* isolates RF1, RF6 and RF11. The MIC values obtained for three individual experiments are shown in table 2.2.

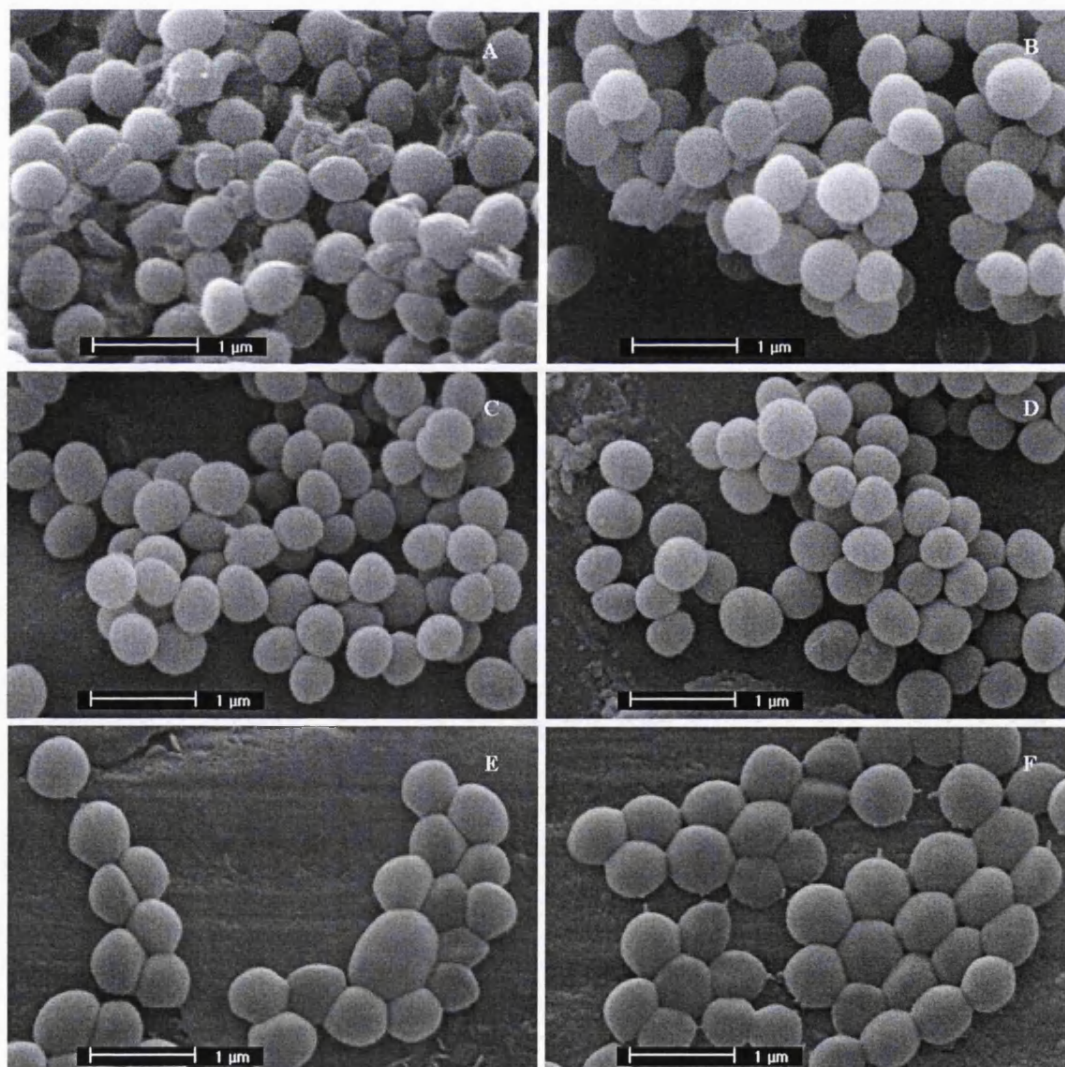
**Table 2.2:** Susceptibility of *S. aureus* isolates RF1, RF6 and RF11 to antibiotics under NG and LSMMG. The MIC values indicated are from three individual experiments (n=3).

Antimicrobial Agent	Minimum inhibitory concentration (mg/L)																	
	RF1						RF6						RF11					
	NG			LSMMG			NG			LSMMG			NG			LSMMG		
Erythromycin	1	1	0.5	1	1	1	0.25	0.25	0.12	0.12	0.12	0.12	>128	>128	>128	>128	>128	>128
Flucloxacillin	0.12	0.25	0.25	0.12	0.12	0.25	0.25	0.25	0.25	0.25	0.25	0.12	0.12	0.25	0.12	0.12	0.12	0.12
Vancomycin	0.5	1	1	0.5	0.5	1	0.5	1	0.5	0.5	0.5	0.5	0.5	1	1	0.5	1	1

No major differences were found in the antibiotic susceptibility of the three *S. aureus* isolates to the three antibiotics studied. The MICs determined for NG cultures and the ATCC29213 control were comparable.

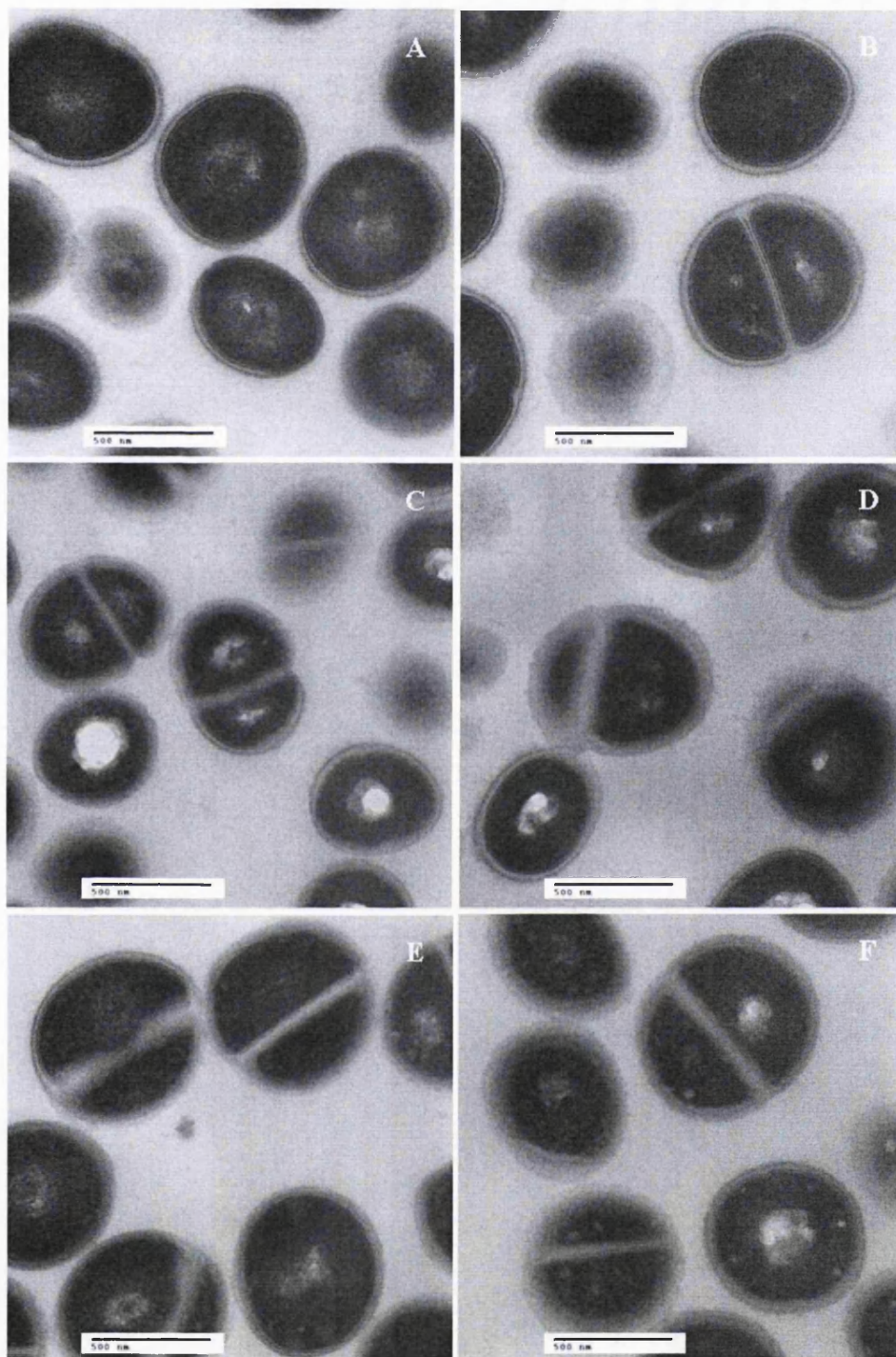
#### 2.3.4 Effect of LSMMG on *S. aureus* morphology

The effects of simulated microgravity on the morphology of *S. aureus* were studied by SEM and TEM. Some of the images obtained are shown in figures 2.4 and 2.5.



**Fig. 2.4:** Scanning electron microscopy images of *S. aureus*. RF1 grown under NG (A). RF1 grown under LSMMG (B). RF6 grown under NG (C). RF6 grown under LSMMG (D). RF11 grown under NG (E). RF11 grown under LSMMG (F).





**Fig. 2.5:** Transmission electron microscopy images of sections of *S. aureus*. RF1 grown under NG (A). RF1 grown under LSMMG (B). RF6 grown under NG (C). RF6 grown under LSMMG (D). RF11 grown under NG (E). RF11 grown under LSMMG (F).

No differences in *S. aureus* morphology attributable to changes in gravity were evident.



## 2.4 Discussion

Many bacteria, including *S. aureus*, display intra-species variation with regard to both the genotype and the phenotype. Deriving the complete genome sequences of strains from the same species has highlighted the extent of this variation and illustrated the degree of intra-species diversity (Lan and Reeves, 2000; Chan, 2003). In terms of space biology, the importance of intra-specific differences in response to simulated microgravity has been emphasised. *Acinetobacter radioresistens* ATCC49000, *Stenotrophomonas paucimobilis* ATCC10829 and one isolate of each of the species, originally recovered from the water supply aboard the ISS, were compared under conditions of NG and LSMMG. Although no differences were found for the ISS isolates and *A. radioresistens* ATCC49000, simulated microgravity reduced the rate of growth of *S. paucimobilis* ATCC10829 compared to NG (Baker and Leff, 2005).

In order to ensure that appropriate strains were selected for this study of the effect of microgravity on the staphylococcal phenotype, I first examined 20 randomly selected *S. aureus* isolates from clinical samples processed at a nearby general hospital. The characterisation of clinical isolates, particularly under different environmental conditions such as microgravity, is important since they represent real pathogens which may express virulence factors that might not be expressed by laboratory strains. Moreover, as there is evidence that microgravity may increase bacterial antibiotic resistance (Tixador *et al.*, 1985b), all isolates examined in the present study were methicillin susceptible to allow further studies on the effect of microgravity on *S. aureus* antibiotic susceptibility.

The isolates were plated on to sheep blood agar and selected on the basis of their phenotypic characteristics. These included differences in bacterial growth kinetics, pigmentation and haemolysin production: the latter was detected by examining the zones of haemolysis surrounding bacterial colonies grown on blood agar plates. Isolate RF1 was selected for its light yellow pigmentation and RF6 for its bright yellow colour, indicating copious pigment production. Haemolysin secretion was observed for all three isolates but was found to be more pronounced with isolates

RF1 and RF6; RF11 showed reduced haemolysin secretion compared to the other two isolates. The study of these parameters is discussed further in chapter three.

Bacterial metabolic activity is growth-phase dependent and is tightly controlled by a complex regulatory network. Alterations in growth kinetics induced by LSMMG may indicate that cells are able to respond differently to environmental changes and may also impact the study of other parameters of interest. Therefore, the selected *S. aureus* isolates were grown in the HARV and the effects of LSMMG on the growth kinetics studied.

To better understand any microgravity-induced changes in bacterial growth kinetics, bacterial growth was monitored throughout the lag, logarithmic and stationary phases of the batch growth cycle. The lag phase commences immediately after inoculation into fresh medium. During this phase, the population remains temporarily unchanged since bacteria need to adapt to the nutrient composition. Cells are not able to divide, but they may grow in volume or mass, synthesising RNA and proteins, and they increase their metabolic activity. The length of the lag phase depends on a range of factors, such as the age and size of the inoculum and the time needed for cells to adapt to the fresh medium. Consequently, the growth medium used will have a major impact on the metabolic state and composition of bacteria at this stage.

The exponential or logarithmic (log) phase is characterised by cell division and intense metabolic activity. Again, growth rate will depend on the culture medium used, temperature, nutrient consumption and excretion of waste products by bacteria. During the stationary phase the growth rate slows as a result of nutrient depletion and accumulation of toxic products. It is during this phase that the bulk of staphylococcal virulence factors are secreted and the maximum cell population density is achieved.

No major differences were found with regard to the growth kinetics of the *S. aureus* isolates RF1, RF6 and RF11 when grown under NG and LSMMG. However, a slower growth rate was found for cells grown under NG compared to LSMMG-grown bacteria. A possible explanation may be related to the slow rotation of the vessels and the effects of gravity. Cells within the control vessel (horizontal position) may sediment on the bottom of the HARV; under LSMMG, the absence of

sedimentation may result in a more homogeneous cell distribution within the vessel. These differences may have impacted on the sampling procedure and could explain the small differences in growth rate between NG- and LSMMG grown cells depicted in Fig. 2.2.

As described in chapter one, the HARV contains a semi-permeable membrane that allows oxygenation by diffusion. Although aeration may be limited in the growth vessels, *S. aureus* are facultative aerobes and are thus able to grow under restricted oxygen conditions (Fischetti *et al.*, 2006; Somerville and Proctor, 2009). The final culture OD and cell mass are however reduced compared to the OD normally determined for this bacterium after a 24 h growth in a shake flask culture, reaching a maximum of OD 3 at  $\lambda_{600\text{nm}}$ .

It has been suggested that microgravity can act as an environmental signal affecting bacterial growth (Klaus *et al.*, 1997; Kacena *et al.*, 1999a; Kacena *et al.*, 1999b), although it has also been proposed that differences between microgravity-grown and normal gravity-grown cells may be due to differences in nutrient availability resulting from altered diffusion characteristics (Klaus *et al.*, 1997; Kacena *et al.*, 1999a). As bacteria readily respond to changes in nutrient flux, this could, in part, explain these contradictions. Moreover, changes in growth rate, shape of the growth curve and final biomass appear to manifest more readily in simple salts media rather than in more complex growth environments (Wilson *et al.*, 2002a; Wilson *et al.*, 2002b). As staphylococci have complex nutritional requirements, the experiments in this project could not be undertaken using chemically defined minimal salts media. Thus, the data obtained using nutritionally complex MHB lend some support to the view that it is a combination of the use of minimal medium and LSMMG-induced increased metabolic activity that drives growth in the microgravity environment (Nickerson *et al.*, 2004). It is important to note that differences between NG- and LSMMG-grown cells may also be due to changes in other parameters such as hydrodynamics, oxygen tension and shear stress.

Previous studies suggest that the susceptibility of bacteria to antibiotics, although transitory, is altered under conditions of microgravity (Tixador *et al.*, 1985b; Lapchine *et al.*, 1986). These authors determined the MICs for oxacillin,

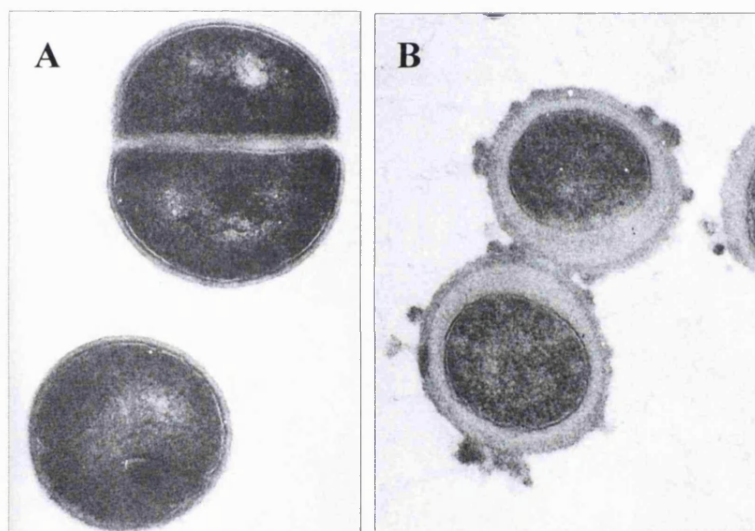
chloramphenicol and erythromycin against *S. aureus* and a twofold increase was found in-flight compared to ground controls (Tixador *et al.*, 1985b). However, care must be taken when interpreting such small differences in MICs. It is generally accepted that a twofold difference in MIC values is not significant given the technical limitations of the bioassay (Andrews, 2001). Moreover, the method used by Tixador *et al.* (1985b) for the measurement of the MIC was based on visual observation of changes in colour induced by acidification of the culture medium following glucose utilisation during bacterial growth. The choice of a suitable method to use for MIC determination in space flight conditions is challenged by the limitations which may affect space research. For this reason, Tixador and co-workers most certainly selected the most appropriate conditions for the described study. Nevertheless, it is important to refer that the method used contrasts with the recommended turbidity endpoint and could lead to inaccuracies in in-flight determinations.

Within this context, the effect of modelled microgravity on *S. aureus* antibiotic susceptibility was determined. Three different antimicrobial agents were selected based on their differing mechanisms of action: the macrolide erythromycin inhibits proteins synthesis by binding to the 50S ribosome; the  $\beta$ -lactam, flucloxacillin, and the glycopeptide, vancomycin, interfere with different stages of the cell wall peptidoglycan synthesis. LSMMG elicited no effects on the susceptibility of the *S. aureus* isolates RF1, RF6 and RF11 against the three antibiotics studied. These results are similar to those obtained by Orihuela *et al.* (2001) who found that, when *Streptococcus pneumoniae* was grown in the HARV, there were no differences in antibiotic susceptibility between NG- and LSMMG-cultures.

It is important to note that for MIC determination of bacteria under LSMMG, cultures were removed from the HARV, diluted and transferred to static 96-well plates. Once removed from the vessel, cells were no longer under the influence of simulated microgravity for the duration of the assay. Moreover, it was reported that during the Cytos 2 (Tixador *et al.*, 1985b) and Biorack 58F (Lapchine *et al.*, 1986) experiments, antibiotic susceptibility patterns reverted to pre-flight values when in-flight cultures were sub-cultured in a terrestrial environment. If microgravity-induced changes are phenotypic in nature, as seems most likely, it will be difficult to detect

small variations in MIC values using this method due to the technical issues described above.

Resistance to certain antibiotics is related to the increased thickness of the staphylococcal cell wall; these changes alter the state of penetration of antibiotic into the cell (Hanaki *et al.*, 1998; Sieradzki and Tomasz, 2003). In contrast to the images obtained from the *S. aureus* cultured aboard Salyut 7 (Fig. 2.6), no discernable differences were found in staphylococcal cell morphology as revealed by SEM (Fig. 2.4) and TEM (Fig. 2.5).






**Fig. 2.6:** Transmission electron microscopy images of sections of *S. aureus* isolated from J. L. Chrétien and grown aboard Salyut 7 in 1982. Control (A) and in-flight cultures (B) (Tixador *et al.*, 1985a).

Examination of the images obtained during the Cytos 2 experiment (Tixador *et al.*, 1985a) showed the ground controls to have a typical staphylococcal cell ultrastructure, with clearly differentiated peptidoglycan cell walls adjacent to the cytoplasmic membrane and septum formation in the plane of cell division. In-flight cultures clearly show a different morphology, which has been interpreted as a very large increase in the thickness of the staphylococcal cell wall (Tixador *et al.*, 1985a; Lapchine *et al.*, 1986). However, the in-flight image does not resemble that of staphylococcal cells, even accounting for the fact that the bacteria may have

undergone extensive phenotypic modification: the layers external to the cytoplasmic membrane appear much less dense than those associated with staphylococci with thickened cell walls (Hamilton-Miller and Shah, 1999) and the presence of vesicles associated with an outer membrane appears very similar to Gram-negative bacterial cells (Khandelwal and Banerjee-Bhatnagar, 2003). These doubts raise the possibility that figure 2.6 may represent an image of a Gram-negative contaminant and not that of *S. aureus* cells.

In summary (Table 2.3), growth in LSMMG does not appear to have an effect on *S. aureus* growth kinetics, antibiotic susceptibility or gross cell morphology. It would be of value to repeat these experiments using different ground-based models and aboard the ISS to better understand how changes in gravity, as well as other parameters associated with the space environment, may affect bacterial cells.

**Table 2.3:** Summary of the effects of LSMMG on *S. aureus* growth kinetics, antibiotic susceptibility and cell morphology described in the present chapter.

Effect of LSMMG on <i>S. aureus</i>	
	No effect on growth kinetics
	No effect on antibiotic susceptibility
	No effect on cell morphology

## **CHAPTER THREE**

### **Effect of LSMMG on the production of virulence determinants by *S. aureus***

## **3.1 Introduction**

*S. aureus* produces a wide range of virulence factors that are essential for the pathogenesis of the bacterium and have been described in general terms in chapter one. This section focuses on the effects of LSMMG on some of the virulence properties of *S. aureus*, such as pigment production and toxin secretion, providing a better understanding of the virulence mechanisms of this opportunistic pathogen as well as contributing to the risk assessment for crew members during space flight.

### **3.1.1 Production of carotenoid pigments by *S. aureus***

One readily-observed characteristic of *S. aureus* is its yellow to orange colour, particularly when grown to stationary phase. Pigmentation is usually more pronounced after growth for more than 24 hours. Ogston (1881) examined patients suffering from acute inflammatory disease and observed that purulent lesions were often yellow in colour. The colour of pus from abscesses was already known to be due to the presence of infecting “staphylococci”. In recognition of the yellow colour of these bacteria, Rosenbach (1884) soon thereafter proposed the species name: *S. aureus* (from the Latin *aureus* = golden).

The most ubiquitous group of bacterial pigments belongs to the class of carotenoid pigments and these were first identified in bacteria by Zopf (1889). A large amount of effort has been made to identify the carotenoids present in bacteria and, in particular, in *S. aureus*. Different methods have been developed for extraction, isolation and purification of bacterial pigments; many of them were adapted from methods previously optimised for the extraction of pigments from plants and other species (Sobin and Stahly, 1942). Identification of bacterial pigments is usually based on their spectral properties and the determination of their absorption maxima.

The first attempt to identify the carotenoids present in *S. aureus* was published by Chargaff (1933), who found the pigment zeaxanthin. In later studies, it was reported



that *S. aureus* produces diversified carotenoid pigments and zeaxanthin may not always be present (Sobin and Stahly, 1942; Douglas and Parisi, 1966; Hammond and White, 1970). Furthermore, it has been reported that pigment production may vary from strain to strain (Sobin and Stahly, 1942).

In 1981 a novel carotenoid, staphyloxanthin, was described in *S. aureus* (Marshall and Wilmoth, 1981a). This newly identified structure was proposed to be the major carotenoid produced by the bacterium after prolonged cultivation. The identification of staphyloxanthin as a staphylococcal carotenoid had probably been delayed because the compound is unstable under conditions of extreme pH and it almost certainly would have been degraded during extraction in most, if not all, previously published studies (Marshall and Wilmoth, 1981a). Using more modern methodologies, Marshall and Wilmoth (1981a; 1981b) isolated, purified and determined the chemical structure of 17 different staphylococcal carotenoids and proposed a pathway for carotenoid biosynthesis in *S. aureus*.

The chemical structure of staphyloxanthin and the elucidation of the staphyloxanthin biosynthetic pathway were first determined using a chemical approach in 1981 (Marshall and Wilmoth, 1981b). These structural and biosynthetic elements were later confirmed by NMR and DNA sequencing techniques, which also provided information on the organisation of the operon encoding the enzymes involved in the staphyloxanthin pathway (Wieland *et al.*, 1994; Pelz *et al.*, 2005).

The complete staphyloxanthin biosynthesis operon *crtOPQMN* is composed of five genes. All these genes and their corresponding enzymes were shown to be necessary for the synthesis of staphyloxanthin (Pelz *et al.*, 2005). The first stage of carotenoid pigment production in *S. aureus* results from the action of two enzymes, dehydrosqualene synthase and dehydrosqualene desaturase, which are products of the *crtMN* operon (Wieland *et al.*, 1994). The reaction product of these enzymes is 4,4'-diaponeurosporene, the first detectable major carotenoid, produced after approximately 12 h cultivation (Wieland *et al.*, 1994). After prolonged culture (24-36 h), this yellow intermediate is converted, through a series of enzymatic reactions

catalysed by *crtOPQ*-encoded enzymes, to an orange product, staphyloxanthin, the major carotenoid of *S. aureus* (Marshall and Wilmoth, 1981b; Pelz *et al.*, 2005).

Upstream of *crtO* there is a sigma B ( $\sigma^B$ ) dependent promoter. This alternative sigma factor is necessary for the synthesis of staphyloxanthin and is thought to directly and positively regulate expression of the *crtMN* operon (Kullik *et al.*, 1998). When  $\sigma^B$  deletion mutants were analysed, reduced colony pigmentation was observed (Kullik *et al.*, 1998; Morikawa *et al.*, 2001). It has been reported that the cold shock protein CspA modulates pigment production and is required for maximal production of pigment: loss of CspA resulted in decreased expression of genes in operon *crtMN*, probably by expression modulation of  $\sigma^B$  (Katzif *et al.*, 2005). Further details of the gene regulation in *S. aureus* will be described in chapter five.

One of the fundamental goals of research in this area is the determination of the function of pigments in bacteria. Carotenoids, especially those from fruit and vegetables, have long been recognised as potent antioxidants that facilitate a variety of essential biological functions (Krinsky, 1989; Edge *et al.*, 1997; El-Agamey *et al.*, 2004). Similarly, it has been suggested that carotenoids may protect bacteria from oxidants released by neutrophils and macrophages (Rosen and Klebanoff, 1979; Dahl *et al.*, 1989; Liu *et al.*, 2004).

The use of *S. aureus* mutants with disrupted carotenoid biosynthesis has been useful for the clarification of the pigment function of this bacterium. By a comparison of wild-type *S. aureus* with a mutant lacking pigment production, the mutant was found to be more susceptible to oxygen species and phagocyte-mediated killing than the wild-type (Liu *et al.*, 2005). These data were later confirmed by Clauditz and co-workers (2006). Pigment production has also shown to contribute to the virulence of *S. aureus* in a murine subcutaneous model: lesions generated by a pigmented strain were significantly larger than non-pigmented mutants (Liu *et al.*, 2005). As a result, staphylococcal carotenoids have been considered to have potential as novel targets for antibacterial therapy (Liu *et al.*, 2005).

### 3.1.2 Secretion of toxins by *S. aureus*

*S. aureus* secretes several extracellular proteins, many of which are toxins with damaging effects on host cells; they make a significant contribution to the pathogenesis of the organism. Among these toxins are several haemolysins, including  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ - toxins, and their molecular biology, biological properties and synthesis have been reviewed by a number of authors (Wiseman, 1975; Rogolsky, 1979; Dinges *et al.*, 2000; Fischetti *et al.*, 2006). Particular interest has been given to  $\alpha$ -toxin and, therefore, details of this protein will be emphasised throughout this chapter.

The sensitivity of cells to staphylococcal toxins may vary amongst different animal species and is dependent on the type and quantity of toxin secreted. All major exotoxins have the capacity to lyse sheep and rabbit erythrocytes, with the exception of  $\beta$ -haemolysin, which is unable to lyse rabbit erythrocytes (Fischetti *et al.*, 2006). Whilst red cells from these species are highly susceptible to  $\alpha$ -toxin, much higher toxin concentrations are required to damage human erythrocytes (Bhakdi *et al.*, 1984).

The traditional assay employed for the identification of staphylococcal toxins, a method still in use, involves the determination of the susceptibility of erythrocytes from different animal species and, for this reason, they are frequently referred to as haemolysins. However, most of these secreted staphylococcal toxins are also able to lyse nucleated cells, such as monocytes, lymphocytes, erythrocytes, platelets, and endothelial cells (Bhakdi and Tranum-Jensen, 1991).

The genes that encode  $\alpha$ -,  $\beta$ -,  $\delta$ - and  $\gamma$ - toxins are respectively *hla*, *hlb*, *hld* and *hlg* and are under the control of the accessory gene regulator, *agr*. Gene expression for toxin secretion occurs mainly during late exponential and stationary phases of bacterial growth. Further details regarding expression of these genes will be presented in chapter five.

The mechanism of action of staphylococcal exotoxins continues to be the subject of much research. It is known that they damage the target cell membrane by formation

of a transmembrane pore that disrupts the phospholipid bilayer, leading to osmotic imbalance and, frequently, cell death (Iacovache *et al.*, 2008). The precise role of these toxins in human disease remains, however, unknown.

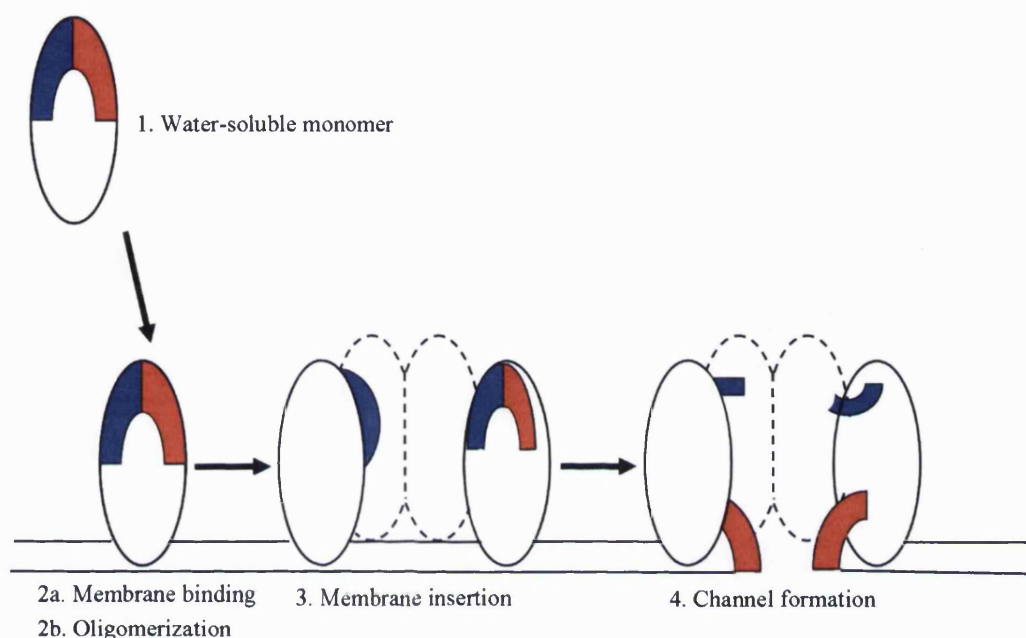
### 3.1.2.1 Alpha-toxin

$\alpha$ -toxin was first identified by Morgan and Graydon (1936) and is the most potent and well characterised membrane-damaging toxin produced by pathogenic staphylococci. It has been suggested that  $\alpha$ -toxin, among other factors, is required for *S. aureus* virulence and makes a major contribution to morbidity and mortality in human infections (Fischetti *et al.*, 2006). However, some key laboratory observations raise issues concerning the contribution of the toxin to the pathogenesis of staphylococcal disease and to its precise role in human pathology. For example, 1000fold higher concentrations of  $\alpha$ -toxin are required to lyse human erythrocytes in comparison to rabbit erythrocytes, which are highly susceptible to  $\alpha$ -toxin (Bhakdi *et al.*, 1984; Fischetti *et al.*, 2006). In humans, platelets and monocytes are the most susceptible cell types to  $\alpha$ -toxin while lymphocytes and granulocytes are naturally resistant (Bhakdi *et al.*, 1988; Bhakdi *et al.*, 1989).

The mechanism of action of  $\alpha$ -toxin has been described in detail (Fussle *et al.*, 1981; Bhakdi *et al.*, 1981; Bhakdi *et al.*, 1984; Bhakdi and Tranum-Jensen, 1987; Hildebrand *et al.*, 1991; Valeva *et al.*, 1997; Montoya and Gouaux, 2003) and follows a series of sequential events (Valeva *et al.*, 1997) (Fig. 3.1). The protein is expressed and secreted by *S. aureus* as a water-soluble monomer of approximately 33 kDa (Bhakdi *et al.*, 1981; Fairweather *et al.*, 1983). After secretion, the soluble toxin diffuses away from the bacterium and binds to the plasma membrane of target host cells.

There are indications that binding of  $\alpha$ -toxin to the target membrane can occur via specific host cell receptors (Cassidy and Harshman, 1976; Hildebrand *et al.*, 1991). The high susceptibility of certain target cells, even when the toxin is released in low concentrations, is caused by the presence of these specific binding sites (Hildebrand

*et al.*, 1991). In fact, specific binding may be essential for pathogenicity since staphylococcal toxins are usually released in low concentrations, in environments where they can be easily and rapidly cleared. However, this specificity has not been yet confirmed nor have the receptors been identified (Menestrina *et al.*, 2001).



**Fig. 3.1:** Mechanism of action of  $\alpha$ -toxin. The protein is expressed and secreted by *S. aureus* as a water-soluble monomer (1). The toxin diffuses away from the bacterium, binds to the plasma membrane of target host cells (2a) and oligomerises into a circular non-transmembrane pre-pore (2b). Oligomerisation triggers a series of conformational changes and membrane insertion of a portion of each protomer (3). Finally, formation of a cylindrical pore occurs (4).

The membrane-bound monomer oligomerises into a circular non-transmembrane pre-pore (Fang *et al.*, 1997d). Oligomerisation triggers a series of conformational changes and membrane insertion of a portion of each protomer by a mechanism that is not well understood. Finally, formation of a cylindrical pore occurs (Belmonte *et al.*, 1987; Song *et al.*, 1996; Gouaux, 1998).

Whilst the structure of the soluble form of  $\alpha$ -toxin is still unknown, the structural conformation of the oligomeric pore has been determined (Song *et al.*, 1996). For

many years the transmembrane pore was thought to be a hexameric complex (Fussle *et al.*, 1981; Bhakdi *et al.*, 1981; Ikigai and Nakae, 1985; Tobkes *et al.*, 1985; Olofsson *et al.*, 1988). Recent studies using X-ray crystallography analysis of the detergent-solubilised  $\alpha$ -toxin oligomer, which represents the oligomeric pore, have revealed a heptameric structure with a mushroom-like shape (Song *et al.*, 1996). High-resolution images of the pore structure confirm previous findings (Gouaux *et al.*, 1994). Interestingly, later studies using atomic force microscopy have once again suggested the possibility of a hexameric structure of the pore (Czajkowsky *et al.*, 1998).

### 3.1.2.2 Other staphylococcal toxins

$\beta$ -toxin is a sphingomyelinase first described by Glenn and Stevens (1935), which damages membranes rich in sphingomyelin (Doery *et al.*, 1963; Doery *et al.*, 1965). The classical test for  $\beta$ -toxin is lysis of sheep erythrocytes since this toxin does not lyse rabbit cells (Fischetti *et al.*, 2006). The majority of human isolates of *S. aureus* do not express  $\beta$ -toxin.

$\delta$ -toxin (Williams and Harper, 1947) was discovered after the other staphylococcal toxins but has subsequently been well characterised and has been the subject of much attention due to its widespread distribution amongst *S. aureus* strains (Fischetti *et al.*, 2006).  $\delta$ -toxin is usually co-expressed with  $\alpha$ -toxin since the gene encoding for  $\delta$ -toxin, *hld*, overlaps a major regulatory system *agr*, which regulates *hla* transcription (Janzon *et al.*, 1989). It is a very small peptide of 26 aminoacids produced by 97% of *S. aureus* strains but its exact role in disease is still unknown (Dinges *et al.*, 2000).

$\gamma$ -toxin (Smith and Price, 1938), along with leucocidin, belongs to the group of bi-component leucotoxins. The cytotoxic activity of this toxin results from the synergistic action of two proteins, the S and the F components (for slow- and fast-eluting proteins in an ion-exchange column) (Woodin, 1959; Woodin, 1960). Similarly to  $\alpha$ -toxin, the proteins are secreted by *S. aureus* as water-soluble

monomers, bind to the membrane of susceptible cells and form hetero-oligomeric transmembrane pores (Colin *et al.*, 1994; Meunier *et al.*, 1997; Roblin *et al.*, 2008).

As described in chapter one, the production of virulence factors by *S. aureus* is an essential condition for the adaptation and survival of the bacterium to adverse environments. I set out to investigate if changes in gravity conditions are likely to alter *S. aureus* phenotype and virulence properties, such as pigment production and toxin secretion, parameters evaluated in this section.

## **3.2 Materials and Methods**

### **3.2.1 Extraction and quantification of carotenoid pigments**

Carotenoids were isolated from *S. aureus* essentially as previously described (Marshall and Wilmoth, 1981a; Wieland *et al.*, 1994). NG- and LSMMG-grown cells were harvested by centrifugation at 3,000 g for 10 min at 4°C, washed twice with PBS and freeze-dried. Pellets were frozen at -80°C for at least 2 h and then freeze-dried using a VirTis Advantage freeze dryer (VirTis, Hampshire, UK). The thermal stage was set at -20°C for 2 h and the primary drying was carried for 14 h at 200 psi. The secondary drying was set at 10°C for 8 h at a reduced pressure of 50 psi. The cells (10 mg dry weight) were suspended in 500 µl of methanol, heated at 55°C in a water bath for 5 minutes, cooled and removed by centrifugation. The extraction was repeated twice, until no further pigment could be extracted. Exposure to bright light was avoided to avoid photodegradation of carotenoids. The combined concentrations of staphyloxanthin and intermediate carotenoids in the methanol layer were quantified by measuring the absorbance of solutions at  $\lambda_{462\text{ nm}}$ , the absorbance maximum of the primary carotenoid pigment staphyloxanthin, using a Perkin Elmer Lambda 25 spectrophotometer.

### 3.2.2 Assays for staphylococcal haemolytic activity

The haemolytic activity of supernatants from NG or LSMMG bacterial cultures grown for 24 h was determined using sheep and rabbit erythrocytes. Defibrinated blood (10 ml; E&O Laboratories, Bonnybridge, UK) was centrifuged at 1,000 g for 15 min at 4°C, erythrocytes were washed three times with cold sterile PBS and suspended in the same buffer to a final concentration of 1% v/v. The supernatants collected from NG- and LSMMG-grown cultures were normalised using values based on cell dry weight and the normalised samples were added to aliquots of the erythrocyte suspension to a final volume of 1.5 ml. The mixtures were incubated at 37°C for 30 min followed by 25 min at 4°C. An erythrocyte suspension in PBS or ammonia solution (10% w/w NH<sub>3</sub>) was included in each assay as 0% and 100% haemolysis controls respectively. After centrifugation to remove intact cells, the degree of erythrocyte lysis was determined spectrophotometrically by measuring the absorbance of the supernatants at  $\lambda_{540\text{nm}}$  (Perkin Elmer Lambda 25 spectrophotometer).

### 3.2.3 Statistical analysis

For the preparation of carotenoids, as relatively small quantities of dried bacteria were recovered from HARV cultures (15-20 mg), each *S. aureus* isolate was grown in six independent cultures. Six staphyloxanthin determinations per isolate (one for each HARV culture) were performed (n=6). Replicate data was combined to calculate mean values.

To assay the staphylococcal haemolytic activity, each *S. aureus* isolate was grown in two independent HARV cultures. Six haemolysin assays for each HARV culture (twelve per isolate) were performed (n=6). Duplicates were included in experiment. Replicate data was combined to calculate mean values.

The values obtained for each individual experiment were compared using paired Student's *t*-test ( $p < 0.01$ ).



### 3.3 Results

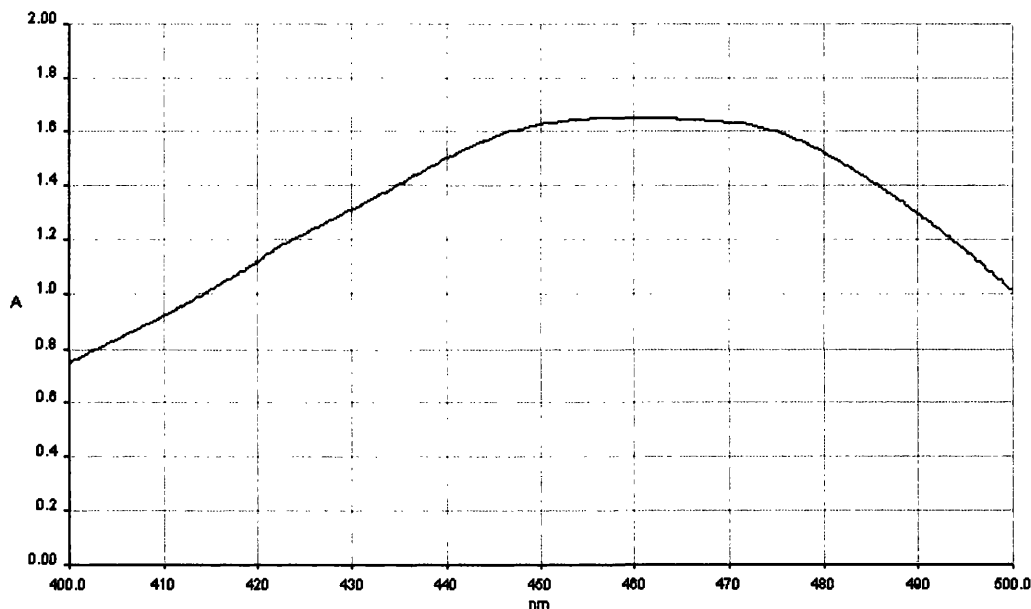
#### 3.3.1 Effect of LSMMG on *S. aureus* carotenoid pigment production

During growth of the *S. aureus* isolates in the HARV, clear colour differences were noted between NG- and LSMMG-cells when the bacteria were allowed to grow to stationary phase. With all three isolates studied, the suspension within the vessel grown under NG consistently produced a brighter yellow pigmentation than LSMMG-grown cells. It was noted that the distribution of NG-grown bacteria within the vessel was less homogeneous than that found with LSMMG-grown cells; the latter appeared more homogeneous and displayed less yellow pigmentation (Fig. 3.2). This relationship was found throughout the course of the study. For this reason, carotenoids were extracted from the isolates following growth in the HARV to study the effects of LSMMG on *S. aureus* pigment production.



**Fig. 3.2:** Colour appearance of NG and LSMMG-grown cultures of *S. aureus* RF6. Cells grown under NG produce brighter yellow pigmentation compared to LSMMG-grown cells. Similar results were observed for *S. aureus* isolates RF1 and RF11.

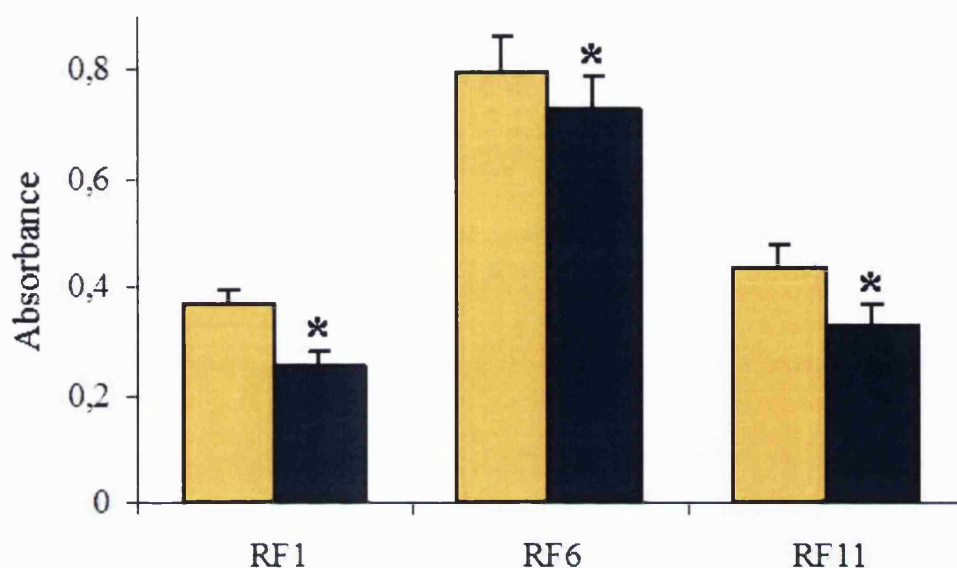
It has been reported that staphyloxanthin, the major pigment produced by *S. aureus*, shows an absorption maximum of 462 nm (Wieland *et al.*, 1994). To confirm this observation, the absorption profiles of pigments extracted from RF6 were determined between  $\lambda_{400\text{nm}}$  and  $\lambda_{500\text{nm}}$ ; this isolate was selected as it appeared to produce larger quantities of pigment when examined following growth on agar plates (Fig. 2.1). A typical absorption spectrum obtained from RF6 extracts is shown in figure 3.3.



**Fig. 3.3:** Absorption spectrum of pigments extracted from *S. aureus* isolate RF6 using the procedure of Wieland *et al* (1994).

In agreement with published work (Wieland *et al.*, 1994), the absorption maximum was found to be at 462 nm, characteristic of the major staphylococcal carotenoid, staphyloxanthin.

In order to determine whether LSMMG influenced the production of carotenoid by *S. aureus*, pigments were extracted with warm methanol as described, followed by quantification of staphyloxanthin at  $\lambda_{462\text{nm}}$  (Fig. 3.4).



**Fig. 3.4:** Production of staphyloxanthin by *S. aureus* isolates RF1, RF6 and RF11 grown under NG (■) and LSMMG (■). Error bars represent 1 SD; n=6; \*p<0.005.

As shown in figure 3.4, the production of pigment is clearly different among the three *S. aureus* isolates studied, with isolate RF6 showing higher absorbance values at  $\lambda_{462\text{nm}}$ , thus suggesting higher production of staphyloxanthin per unit cell volume than either RF1 or RF11. These results complement the relative pigment production observed when isolates were plated on blood agar (Fig. 2.1).

Under LSMMG, all three isolates produced significantly lower amounts of staphyloxanthin compared to NG-grown bacteria ( $p < 0.005$ ), suggesting that simulated microgravity can alter pigment production by *S. aureus*.

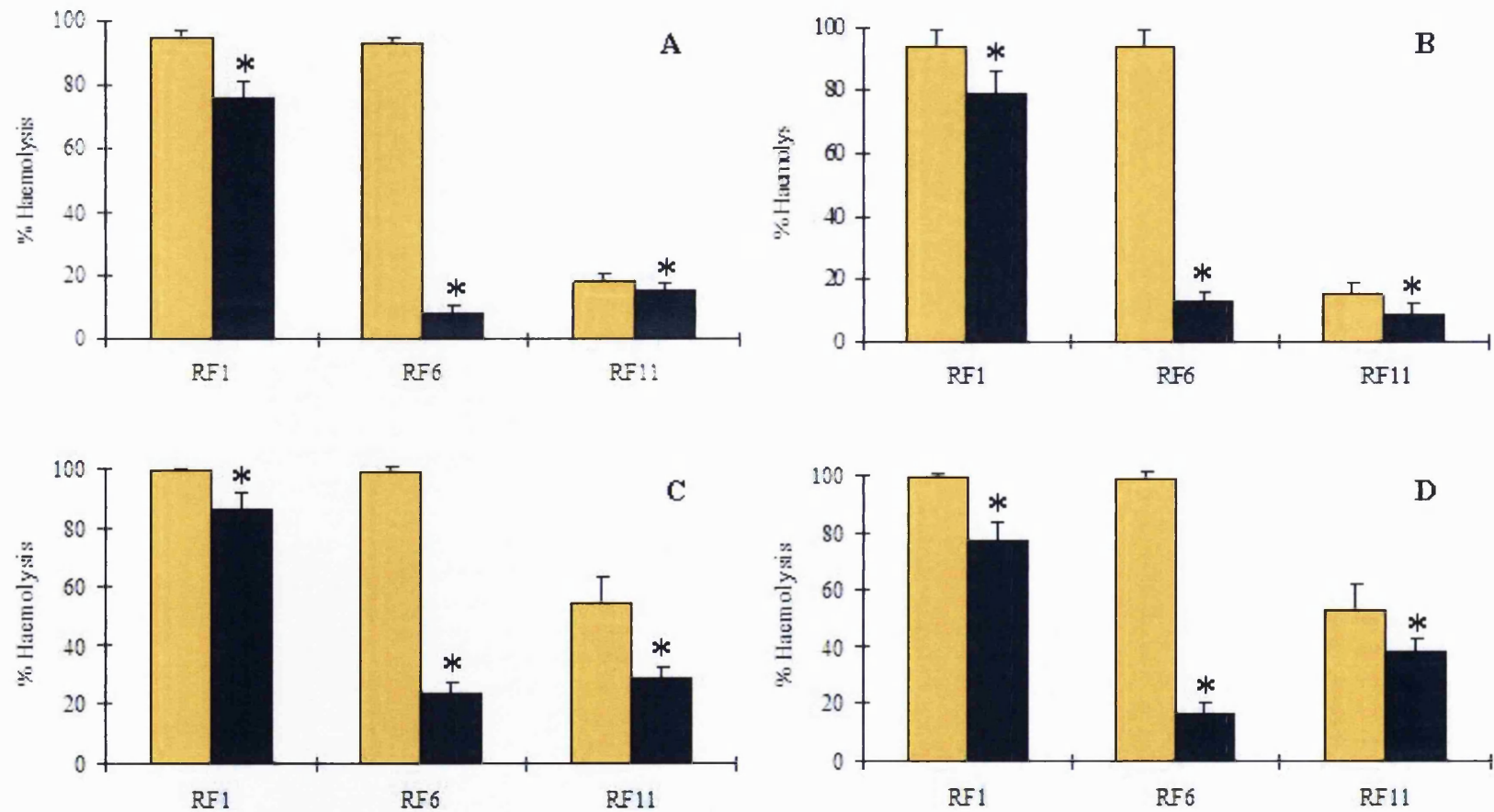
### 3.3.2 Effect of LSMMG on *S. aureus* haemolysin secretion

The identification of zones of haemolysis surrounding bacterial colonies grown on blood agar plates is a reliable, cost-effective and convenient procedure for

establishing toxin secretion by staphylococci. As described in chapter two, this method was used for the selection of the *S. aureus* isolates RF1, RF6 and RF11, based on semi-quantitative determination of haemolysin secretion. All three isolates secreted haemolysins when plated on sheep blood agar (Fig. 2.1). The presence of secreted haemolysins in culture supernatants was quantified following growth of *S. aureus* isolates RF1, RF6 and RF11 in the HARV. An assay for staphylococcal haemolytic activity was performed using both sheep and rabbit blood and the results are shown in figure 3.5.

As expected, all three isolates were able to lyse both rabbit and sheep erythrocytes. However, clear quantitative differences were found in haemolysin secretion among isolates and between NG and LSMMG samples. RF11 was found to produce lower amounts of haemolysins compared with RF1 and RF6. When the gravity conditions were altered, all three isolates significantly reduced the production of haemolysins under LSMMG compared to NG ( $p < 0.01$ ). In particular, a marked reduction was noted in isolate RF6, where toxin secretion was almost completely absent under LSMMG.

As described in the introduction of this section,  $\beta$ -haemolysin, in contrast to the  $\alpha$ - and  $\delta$ -forms, is unable to lyse rabbit erythrocytes. Moreover, the sensitivity of erythrocytes to the different staphylococcal haemolysins varies amongst animal species. The capacity of the culture supernatants to lyse rabbit cells was thus compared with their ability to lyse sheep erythrocytes. Comparison of figure 3.5 A and B with C and D indicates that  $\beta$ -toxin activity may have contributed significantly to the overall lytic profile, although the increased haemolysis of rabbit erythrocytes is likely to be due to increased sensitivity of these cells to  $\alpha$ -toxin compared to sheep erythrocytes. This observation was particularly interesting in the case of isolates RF6 and RF11, where the percentage of lysis of rabbit erythrocytes was higher compared to sheep erythrocytes.



**Fig. 3.5:** Secretion of sheep (A and B) and rabbit (C and D) erythrocytes haemolysins by *S. aureus* isolates grown under NG (■) and LSMMG (■). Two supernatants, supernatant 1 (A and C) and supernatant 2 (B and D) were tested for each *S. aureus* isolate. Error bars represent 1 SD; n=6; \*p<0.01.

### 3.4 Discussion

The production of virulence factors is a key feature of *S. aureus* pathogenicity and is essential for the adaptation of this bacterium to different environmental conditions. Changes in gravity conditions are thus likely to alter *S. aureus* phenotype and virulence properties. In this section I evaluated the impact of LSMMG on pigment production and haemolysin secretion by *S. aureus*.

The clear pigmentation differences noted between NG- and LSMMG-cells when the bacteria were allowed to grow to stationary phase may be related to the impact of the physical orientation of the vessel on gravity-induced cell sedimentation. Under conditions of LSMMG, the vessel is vertically orientated and the cell suspension is rotated at constant velocity with a gravity vector near zero. In this simulated weightless environment, absence of sedimentation produces better cell distribution and thus the cell suspension is more homogenous and lighter in colour. In contrast, the control vessel is horizontally orientated, the suspended cells experience unit gravity and they tend to sediment slightly faster than in LSMMG conditions, leading to a brighter yellow pigmentation, particularly at the bottom of the vessel. NG-grown cells harvested by centrifugation displayed more yellow pigmentation when examined by eye than LSMMG-grown cells, emphasising the fact that differences observed within the HARV were not related to alterations in cell sedimentation.

Thus, differences in pigmentation are attributable to a reduction in carotenoid pigment production when the bacteria are grown under conditions of simulated microgravity. For quantification of carotenoid pigments, several factors were taken into account. It is known that pigment production can vary among species and from strain to strain and can also be dependent on growth conditions. *S. aureus* is no exception (Sobin and Stahly, 1942). For example, Douglas and Parisi (1966) have shown that pigmentation differences between staphylococcal strains could be explained by quantitative or qualitative alterations in the production of carotenoids.

Carotenoid pigments can be extracted from the cells and readily identified by spectrometric analysis. It is important to note that absorption spectra can vary not

only with the chemical structure of the pigments present in a given sample but also with the solvent in which they are dissolved after extraction. The chemical structure of pigments can be quite variable and depends, for example, on the number of carbons, the oxygen functionalities (hydroxyl, methoxy, oxo, epoxy, carboxy or aldehydic groups) and presence or absence of cyclic structures. A very important characteristic of carotenoids is the presence of double bonds in a conjugated system, which has a large influence on their spectral properties; they typically absorb light between 400 and 500 nm (Armstrong and Hearst, 1996; Gruszecki and Strzalka, 2005).

It has been previously shown that staphyloxanthin is the major carotenoid pigment produced by *S. aureus* by cells that are allowed to grow to stationary phase, especially after 24 h of growth (Marshall and Wilmoth, 1981a). This carotenoid has a characteristic absorption spectrum with a single broad peak at approximately 462 nm (Wieland *et al.*, 1994), which has been confirmed spectrophotometrically in the present study.

Interestingly, pigment production has been linked with virulence of *S. aureus*. Liu and co-workers (2005) have suggested that staphylococcal carotenoids can promote oxidant resistance and phagocyte survival, representing a potential novel target for antimicrobial therapy. Staphyloxanthin, as the major pigment produced by *S. aureus*, may enable the bacterium to combat the immune system and survive in the infected host (Pelz *et al.*, 2005). The finding that, under LSMMG, *S. aureus* produces lower quantities of carotenoid pigments could indicate that the virulence of this bacterium is altered when grown under these conditions.

It has been suggested that alterations in pigment production by *S. aureus* can be accompanied by quantitative and qualitative differences in production of other virulence factors such as haemolysins (Parisi, 1966; Yoshikawa *et al.*, 1974), coagulase (Parisi, 1966), hyaluronidase (Parisi, 1966) and TSST-1 (Lee and Bergdoll, 1985). The decreased pigment production found under LSMMG may thus be accompanied by alterations in expression of important virulence factors such as toxin secretion.

The effect of LSMMG on haemolysin secretion by *S. aureus* was determined using sheep and rabbit erythrocytes.  $\alpha$ -toxin is the most active of the haemolysins when examined using rabbit blood and  $\beta$ -toxin is the most active against sheep erythrocytes (Fischetti *et al.*, 2006), which has been confirmed in the present study. The haemolytic activity of  $\beta$ -toxin is significantly enhanced by incubation at 37°C followed by a period at 4°C. For this reason, this toxin is often referred as “hot-cold” haemolysin (Wiseman, 1975). These temperature conditions were, therefore, used during the performance of the haemolysin assays.

LSMMG was found to reduce toxin secretion by all three *S. aureus* isolates studied compared to NG conditions. This finding is in agreement with the reduction of staphyloxanthin production found and strongly suggests that the virulence of this bacterium may be reduced under microgravity conditions since the production of virulence determinants by *S. aureus* is reduced. In contrast, it has been suggested that LSMMG enhances virulence of *S. enterica*, as previously reported by Nickerson and co-workers. The bacterium was grown during space flight (Wilson *et al.*, 2007) and under LSMMG (Nickerson *et al.*, 2000) and tested in a murine model. A decreased average host time to death and LD<sub>50</sub> was found compared to bacteria grown under NG. However, DNA microarray analysis showed no alteration in virulence genes (Wilson *et al.*, 2002b). This observation suggests that LSMMG may induce novel bacterial molecular mechanisms and that bacteria may respond differently to this unique environment. There is evidence that the phenotypic changes observed in bacteria cultured under reduced gravity are transitory and cells revert to the original state when subcultured in terrestrial environment (Tixador *et al.*, 1985b). Careful interpretation of experiments which require bacterial subculture should be thus undertaken.

Gene regulation in *S. aureus* is a complex process and can, in part, explain the interactions between the different virulence factors produced by this bacterium. Recently, considerable amount of research has been undertaken to determine the regulation of virulence genes in *S. aureus* (Novick, 2003; Lindsay, 2008). Interestingly, the alternative sigma factor  $\sigma^B$ , although particularly associated with bacterial responses to stress, has shown to function as a global gene regulator (Kullik *et al.*, 1998; Bischoff *et al.*, 2004) and has been linked with variations in pigment



production (Kullik *et al.*, 1998; Morikawa *et al.*, 2001), antibiotic susceptibility (Willis *et al.*, 1964; Morikawa *et al.*, 2001) and morphological changes to the staphylococcal cell wall (Morikawa *et al.*, 2001).

The *sigB* locus appears to negatively regulate *hla* expression (Cheung *et al.*, 1999; Bischoff *et al.*, 2004), the gene encoding  $\alpha$ -toxin production, and positively regulate pigment production by *S. aureus* (Kullik *et al.*, 1998; Morikawa *et al.*, 2001). Increased *sigB* expression has also been shown to be accompanied by increased antibiotic resistance as well as increased staphylococcal cell wall thickness by the bacterium (Morikawa *et al.*, 2001). Increased cell wall thickness may alter the penetration of the antibiotic into the cell and thus alter antibiotic susceptibility, as confirmed by other authors (Sieradzki and Tomasz, 2003).

As described in chapter two, LSMMG did not affect the antibiotic susceptibility or cell morphology of *S. aureus* isolates RF1, RF6 and RF11. However, growth under simulated microgravity conditions reduced pigment production and toxin secretion by all three isolates studied. These results do not correlate with the hypothesis that *sigB* expression may be altered, since decreased pigment production *sigB*-dependent has been described to be accompanied by increased Hla production (Kullik *et al.*, 1998; Cheung *et al.*, 1999; Bischoff *et al.*, 2004). This suggests that reduced production of virulence determinants by *S. aureus* under LSMMG must be due to other factors. Gene regulation in *S. aureus* will be discussed further in chapter five.

It has been shown in this chapter that LSMMG has an impact on the virulence properties of *S. aureus*, particularly on pigment production and toxin secretion (Table 3.1). Changes in bacterial virulence under microgravity conditions can have important implications for crew members and should thus be considered in space flight risk assessment. Moreover, this study provides important information on *S. aureus* behaviour in this unique environment and strongly suggests a decrease in the production of virulence factors. Therefore, subsequent work, described in the following chapters, focused to a large extent on the effect of changes in the gravitational field on broader aspects of the modulation of virulence effectors in *S. aureus*, to include global protein secretion and gene regulation.

**Table 3.1:** Summary of the effect of LSMMG on *S. aureus* production of virulence determinants.

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<b>Effect of LSMMG on <i>S. aureus</i></b>
↓ Reduction of pigment production
↓ Reduction of toxin secretion
↓ Reduction of production of virulence determinants

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## **CHAPTER FOUR**

### **Effect of LSMMG on the proteomic profile of *S. aureus***

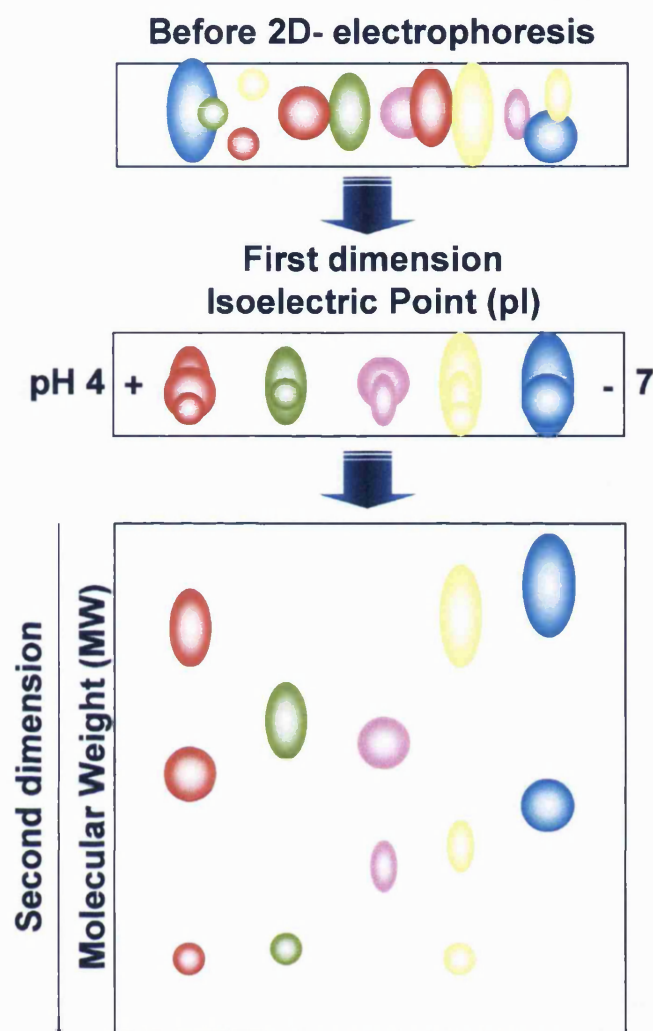
## 4.1 Introduction

The pathogenesis of *S. aureus* infection is a very complex process and involves the production of a variety of proteins, including many virulence factors, which have been described in general terms in chapter one. *S. aureus* has an extremely high capacity to adapt to environmental changes, and it is thus highly likely that growth under conditions of reduced gravity will trigger expression of new phenotypes. Alterations at transcriptional, translational or post-translational levels may impact on the final protein concentration, protein localization, protein function or even protein-protein interactions in the cell.

Proteomics is an excellent tool to obtain a more complete understanding of these modifications and enables large-scale screening of both intracellular and extracellular proteins. The procedure demands rigorously controlled steps of sample preparation, protein separation, image detection and analysis, spot identification, and database searches. The core technology of proteomics is two-dimensional electrophoresis.

### 4.1.1 Two-dimensional gel electrophoresis (2D electrophoresis)

2D electrophoresis, first described by O'Farrell (1975) and Klose (1975), is a powerful tool for the separation and analysis of complex protein mixtures. The method involves separation of individual proteins with differing physical properties in two dimensions: the first dimension uses isoelectric focusing (IEF) and separates proteins according to their isoelectric point (pI); the second dimension utilises sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and separates proteins according to their molecular weight (MW). By combining these two high-resolution systems, hundreds of proteins can be separated, detected, identified, quantified and characterised. In a typical 2D image, each spot will then correspond to a single protein (Fig. 4.1). Molecules are more effectively separated by 2D electrophoresis in comparison to 1D methods due to the low probability that any two proteins will have exactly the same pI and MW (Garfin and Heerdt, 2000; Berkelman and Stenstedt, 2004).



**Fig. 4.1:** Two-dimensional gel electrophoresis. The first dimension uses isoelectric focusing (IEF) and separates proteins according to their isoelectric point (pI); the second dimension utilises sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and separates proteins according to their molecular weight (MW).

The 2D process begins with sample preparation, which is essential for subsequent steps and optimal separation of proteins. As amphoteric molecules, proteins can carry positive, negative or neutral net charge, depending on the pH conditions. The pI thus refers to the pH at which a given protein carries neutral charge. Proteins will be charged at pH values other than the pI; at pH values below the pI they are positively charged and at pH values above the pI they are negatively charged (Garfin and Heerdt, 2000; Berkelman and Stenstedt, 2004).

The first step of 2D electrophoresis separates proteins according to their pI. During IEF, a pH gradient and an electrical potential are applied to a gel containing the protein sample. Thus, proteins will migrate through the gel according to their charge, until their overall charge is neutral, resulting in accumulation at a position in the gel corresponding to the pI: positively charged proteins will migrate towards the cathode and negatively charged molecules will migrate towards the anode. The slope of the pH gradient and the strength of the electrical field applied to the gel should be selected as appropriate to ensure good resolution of the 2D image.

The second step is based on conventional SDS-PAGE methodology, using gel slabs described by Laemmli (1970); in this procedure the proteins are separated according to their MW. Prior to separation, proteins are denatured and coated with negatively charged SDS molecules. An electrical potential is then applied to the polyacrylamide gel containing the sample and the protein-SDS complexes migrate through the gel matrix at different rates, depending on their size.

Proteins can then be detected using a variety of methods. The most commonly used procedure involves staining of the proteins in the gel with Coomassie blue, a process that is both simple to apply and relatively sensitive with regard to its capacity to detect proteins present in low concentration (lower limit is 10 to 100 ng). Other gel stains, such as Sypro Ruby and Silver staining, are more sensitive than Coomassie blue and can be used to detect lower protein concentrations (< 10 ng). Analysis of proteins visualised in the gel can be performed using complex software that facilitates spot-matching between similar samples and enables protein quantification.

Proteins of interest can be excised from the gel matrix and cleaved to smaller peptides using a proteolytic enzyme, usually trypsin. The resulting digest mixture can then be identified and characterised using mass spectrometry (MS) techniques and peptide identification performed by database or *de novo* search. For example, the Mascot (Matrix Science) search engine uses MS data for rapid protein identification from primary sequence databases. The database contains a variety of amino acid sequences predicted from genomic data, which is available for several *Staphylococcus* sp. If a given protein is present in the sequence database, it will be identified correctly since multiple matches are obtained for its peptides,

corresponding to a high MS/MS match score, based on a defined significance level; if the sequence database does not contain the protein obtained, homologous sequences, usually equivalent proteins from related species, are compared and the identification is based on the highest protein score. In contrast, *de novo* sequencing infers peptide sequences without knowledge of genomic data.

The use of proteomics associated with MS techniques enables the analysis and identification of several proteins present in a given sample, providing information on possible modifications at transcriptional or post-transcriptional modifications on a particular cell. This chapter describes the use of proteomic methods to study the effect of LSMMG on the extracellular and intracellular proteome of *S. aureus* and contributes to the general knowledge of *S. aureus* virulence mechanisms and pathogenicity.

## **4.2 Materials and Methods**

All materials, kits and instruments were purchased from Bio-Rad (Hemel Hempstead, Hertfordshire, UK) unless otherwise stated.

### **4.2.1 Preparation of samples**

#### **4.2.1.1 Preparation of proteins present in the supernatants**

After 24 h growth in NG or LSMMG, culture supernatants were obtained by centrifugation at 3,000 *g* for 10 min at 4°C, filtered using a 22 µm filter (Millipore Corporation, UK) and concentrated approximately 30-fold to a final volume of 1.5 ml using Vivaspın 20 ultracentrifugation spin columns with a 3,000 MWCO (Polyethersulfone) membrane (Generon, Berkshire, UK). The concentrated supernatants were stored in aliquots at -20°C.

#### 4.2.1.2 Preparation of intracellular proteins

After 24 h growth in NG or LSMMG, bacterial cells were recovered by centrifugation at 3,000 g for 10 min at 4°C, washed twice with Tris-EDTA buffer and suspended in 1 ml of sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2 protease inhibitor cocktail tablets) by pipetting. The suspended cells were transferred to a tube containing acid-washed glass beads ( $\leq 106\ \mu\text{m}$ ) (Sigma-Aldrich, Dorset, UK) and processed in a FastPrep Instrument (Qbiogene, Cambridge, UK) for 40 s at a setting of 6.0 for mechanical disruption of bacteria. The sample tube was centrifuged at 14,100 g for 10 min at RT and the supernatant ( $\sim 750\ \mu\text{l}$ ) transferred to a new microcentrifuge tube, avoiding any transfer of the debris pellet or lysing matrix. Samples were immediately prepared for proteomic analysis.

#### 4.2.1.3 Determination of protein content

The concentration of solubilised protein was determined using the protein assay based on the Bradford method according to manufacturer's instructions. Briefly, the dye reagent was diluted 1:5 in water and bovine serum albumin (BSA) standards prepared at concentrations of up to 0.8 mg/ml. Each standard and sample solution (30  $\mu\text{l}$ ) was added to 1.5 ml of diluted dye reagent, the reaction incubated for 5 min at RT and absorbance at  $\lambda_{595\text{nm}}$  determined using a Perkin Elmer Lambda 25 spectrophotometer. The concentrations of the extracted proteins were estimated using the BSA standard curve.

For determination of the protein content present in the supernatants, each of the three *S. aureus* isolates was grown in five independent HARV cultures. Five protein determinations per isolate (one for each HARV culture) were performed ( $n=5$ ). Duplicates were included in every protein determination experiment. Replicate data was combined to calculate mean values. Results were expressed as concentration in mg of protein/mg of cells (dry weight). The values obtained for each individual experiment were compared using paired Student's *t*-test ( $p < 0.01$ ).



For determination of intracellular protein content, each of the three *S. aureus* isolates was grown in three independent HARV cultures. Three protein determinations per isolate (one for each HARV culture) were performed (n=3).

### **4.2.2 One-dimensional gel electrophoresis**

Exoproteins were separated in 1D format by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) performed with a 10% (w/v) acrylamide / bis-acrylamide gel matrix. Samples were normalised using values based on the cell dry weight or total amount of protein. Normalised samples were loaded onto the gels and run at 150 V until the bromophenol blue dye reached the bottom of the gel. The proteins were visualised by staining with Coomassie brilliant blue (Sigma-Aldrich, Dorset, UK).

### **4.2.3 2D gel electrophoresis**

Proteins were separated by 2D electrophoresis on immobilised pH gradient strips with a pH range of 4–7 followed by separation based on MW using SDS-PAGE; experiments were performed using a 12% (w/v) acrylamide/bis-acrylamide gel matrix. Supernatant and intracellular proteins (300 µg) were prepared for IEF using Bio-Rad Clean-up Kit to eliminate contaminants such as salts, lipids and nucleic acids that may interfere with the IEF step.

#### **4.2.3.1 2D sample cleanup**

Samples for 2D electrophoresis were normalised using values based on the cell dry weight or total amount of protein. Protein samples were split into 1.5 ml microcentrifuge tubes in aliquots of 100 µl. For protein precipitation, 300 µl of Bio-Rad Precipitating Agent 1 were added to the protein sample, the mixture dispersed

using a vortex mixer and incubated on ice for 15 min. Similarly, 300 µl of Bio-Rad Precipitating Agent 2 were added to the mixture of protein and Bio-Rad Precipitating Agent 1 and thoroughly mixed by vortex. The tubes were centrifuged at 14,100 g for 5 min to form a tight pellet and the supernatant removed and discarded without disturbing the pellet. The protein pellet was washed with 40 µl of Bio-Rad Wash Reagent 1 added to the top of the pellet and the tube centrifuged at 14,100 g for 5 min. The supernatant was carefully removed and discarded and the proteins dispersed in 25 µl of ultrapure water, added to the top of the pellet and mixed for 10–20 s. Proteins were incubated at -20°C for at least 30 min in a mixture of 1 ml of Bio-Rad Wash Reagent 2 (pre-chilled at -20°C for at least 1 hour) and 5 µl of Bio-Rad Wash 2 Additive; the tube was then carefully vortexed for 30 s every 10 min during the incubation period. After incubation, the tube was centrifuged at 14,100 g for 5 min to form a tight pellet and the supernatant carefully and completely removed and discarded. The pellet was air-dried at RT for no more than 5 min and then suspended in an appropriate volume of 2D sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2 protease inhibitor cocktail tablets, 0.002% bromophenol blue, 50 mM DTT and 0.2% carrier ampholytes) to give a final volume of 300 µl. Each tube was mixed vigorously and centrifuged at 14,100 g for 5 min at RT to clarify the protein sample. The supernatant was applied immediately to IPG strips for IEF.

#### **4.2.3.2 First dimension: Isoelectric focusing (IEF)**

##### **4.2.3.2.1 Loading of IPG strips**

Each protein sample (300 µl) was loaded as a bed along the back edge of a channel in a rehydration tray. The IPG strips were carefully positioned over the sample, with the gel side facing down, taking care not to trap any air bubbles which could interfere with the even distribution of the sample in the strip. Mineral oil was used to overlay the strips to prevent evaporation of the samples. The rehydration tray was covered with a lid and the IPG strips left to rehydrate overnight in order to allow the proteins to load onto the IPG strips.

#### 4.2.3.2.2 Focusing of IPG strips

Electrode paper wicks were moistened with 8  $\mu$ l of ultra pure water and positioned on the electrodes of the focusing tray. After rehydration, the IPG strips were removed from the rehydration tray with a pair of forceps, placed on dried tissue paper with the gel side facing up and covered with wet tissue paper for 30 s in order to remove any excess mineral oil from the surface. The IPG strips were then positioned on the focusing tray with the gel side facing down. Mineral oil was layered over the IPG strips, completely covering them to prevent sample evaporation during focusing. The tray was positioned in the PROTEAN IEF cell and the cover closed. The focusing conditions used were:

- ⚡ Stage 1: 250 V for 15 min, rapid ramp;
- ⚡ Stage 2: 10,000 V for 2 h, slow ramp;
- ⚡ Stage 3: 10,000 V for 80,000 Vh, rapid ramp;
- ⚡ Stage 4: Hold at 500 V
- ⚡ Temperature: 20°C
- ⚡ Maximum current: 50  $\mu$ A/IPG strip.

Strips were immediately removed from the tray after completion of the focusing step, placed on dried tissue paper with the gel side facing up and covered with wet tissue paper for 30 s to remove any excess mineral oil from the surface. The IPG strips were then transferred to a clean tray with the gel side facing up and either immediately frozen at -80°C or prepared for gel electrophoresis.

#### 4.2.3.2.3 Equilibration of IPG strips

Before separation in the second dimension, IPG strips were equilibrated in two different SDS buffers. Equilibration buffers were prepared immediately prior to use by the addition of 125 mg DTT (equilibration buffer I) or 100 mg iodoacetamide (equilibration buffer II) to 5 ml of equilibration base buffer (6 M urea, 2% SDS, 0.05 M Tris/HCl pH 8.8, 20% glycerol) and the reagents vigorously mixed until the solids were completely dissolved. The IPG strips, placed on an equilibration tray with the gel side facing upwards, were covered with equilibration buffer I and shaken gently on a shaker table for 15 min. The liquid was then carefully decanted from the tray

and replaced by equilibration buffer II, followed by gentle shaking on a shaker table for 15 min. The liquid was again carefully decanted from the tray and the IPG strips prepared for gel electrophoresis.

#### **4.2.3.3 Second-dimension: SDS-PAGE**

IPG strips were removed from equilibration tray using forceps; both sides of the strip were trimmed to accommodate the strip on the gel cassette and the strip was washed by dipping briefly into Tris/Glycine/SDS (TGS) running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS at pH 8.3). Using forceps, strips were carefully pushed into the IPG well on a 12% (w/v) Tris-HCl gel, taking care not to trap air bubbles beneath the strip. Agarose solution was layered into the IPG well on the gel, filling the well to the top of the inner gel plate, and left to set. Gels were placed in a reservoir filled with TGS running buffer and electrophoresed at 45 mA until the bromophenol blue marker reached the bottom of the gel.

#### **4.2.3.4 Protein detection and analysis**

At the end of the SDS-PAGE run, gels were carefully removed from the cassette, fixed in acetic acid/methanol/water (1:4:5) for 30 min and stained with Sypro Ruby protein gel stain overnight. Gels were scanned using a Molecular Imager FX and analysed using PDQuest Advanced software. Gels for MS analysis were stained with Coomassie Blue stain (Sigma-Aldrich, Dorset, UK) for 2-3 h and then destained in acetic acid/methanol/water (1:4:5) until the background was removed.

#### **4.2.3.5 Protein identification by liquid chromatography-mass spectrometry (LC/MS-MS)**

The LC/MS-MS analysis was performed by Mr. Kevin Bailey at the School of Biomedical Sciences, University of Nottingham.

Spots of interest were excised from the gel using an EXQuest spot cutter with a picker head of 1.5 mm and transferred to 96-well microplates. Gel plugs were then subjected to automated in-gel chemical modification of cysteine residues using DTT and iodoacetamide, followed by tryptic digestion and peptide extraction (MassPrep robotic system, Waters/BioRad).

Extracted peptide mixtures were run on a Waters QTOF2 hybrid quadrupole mass spectrometer incorporating an integrated capillary LC system. A total of 5 µl of digest was introduced *via* an auto-sampler. The sample was initially loaded on to a small C18 pre-column for desalting and products of the tryptic digest were then eluted on to an analytical capillary C18 column (100 mm x 0.75 mm id). The LC system incorporated a flow splitting device to give a final flow through the column of 200 nl/min. A solvent gradient was run for a total of 1 h in order to elute peptides from the column and re-equilibrate prior to loading the next sample. Where samples were observed to contain high levels of peptides, a blank run was incorporated between sample runs to establish that there was no significant carry-over of the previous sample.

Peptides eluted from the analytical column were automatically transferred to the mass spectrometre *via* a nanospray device attached to the LC outflow, operating at 3 kV. A reference solution containing a peptide of known mass was sprayed into the mass spectrometer from a separate spray device. This ion source was sampled at regular intervals throughout the run to assist in maintaining accurate mass measurements of the ionised peptides from the analyte spray. The mass spectrometre was programmed to automatically switch to MS-MS mode and to generate fragmentation data whenever a peptide with an associated charge of 2+ or 3+ was detected above a pre-set threshold signal. The software was configured to enable scanning of multiple channels, in order to simultaneously fragment up to three co-eluting peptides and collect fragmentation data from each. A preset range of collision voltages was applied in order to fragment each peptide as efficiently as possible.

Data files were analysed using MassLynx 4.0 (incorporating BioLynx) (Waters, Hertfordshire, UK) and ProteinLynx Globalserver 2 (Waters, Hertfordshire, UK) in order to assess the identities of proteins present in the digest. Peak list files generated

from PLGS2 analysis were also used in an alternative search programme that accepted this data file format (Mascot, Matrix Science). In addition, some fragmentation data was analysed manually and *de novo* sequencing undertaken on selected peptides.

#### 4.2.3.6 Statistical analysis

Each of the three *S. aureus* isolates was grown in three independent HARV cultures. Three proteomic experiments per isolate (one for each HARV culture) were performed using the cells and supernatants collected from each growth culture (n=3). Normalisation and analysis was performed using PDQuest Advanced software to identify proteins with a greater than twofold change in gene expression, using Student's *t*-test ( $p < 0.05$ ).

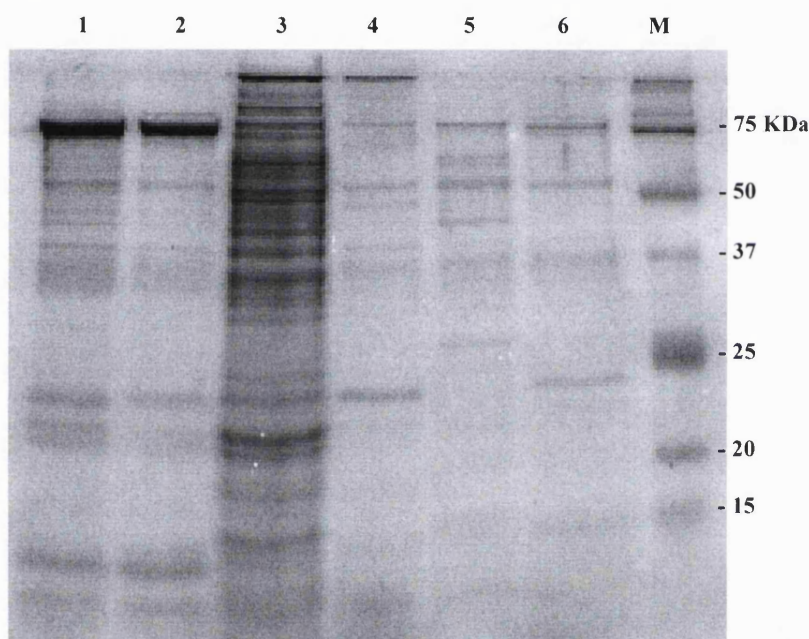
#### 4.2.4 Determination of protease activity using zymogram gels

Samples for zymogram assays were normalised using values based on the cell dry weight or total amount of protein. Samples were mixed with Bio-Rad Zymogram Sample Buffer in a 1:2 proportion to a final volume of 50  $\mu$ l and incubated at RT for 10 min. Protein samples were loaded on a Bio-Rad 12% Zymogram Casein Gel immediately after preparation. Trypsin was used as positive control. Zymogram gels were run at 125 V for approximately 90 minutes. After electrophoresis, gels were incubated in 1x Bio-Rad Zymogram Renaturation Buffer for 30 minutes at RT with gentle agitation and then equilibrated in 1x Bio-Rad Zymogram Development Buffer at RT for 30 minutes with gentle agitation. A final incubation was performed in fresh 1x Bio-Rad Zymogram Development Buffer at 37°C for at least 18 h. The gel was washed with distilled water, stained with 0.5% Coomassie Blue stain (Sigma-Aldrich, Dorset, UK) for 2-3 h and then destained in acetic acid/methanol/water (1:4:5) until areas of protease activity, identified as clear bands against a dark background, were visible.

## 4.3 Results

### 4.3.1 Effect of LSMMG on *S. aureus* supernatant proteins

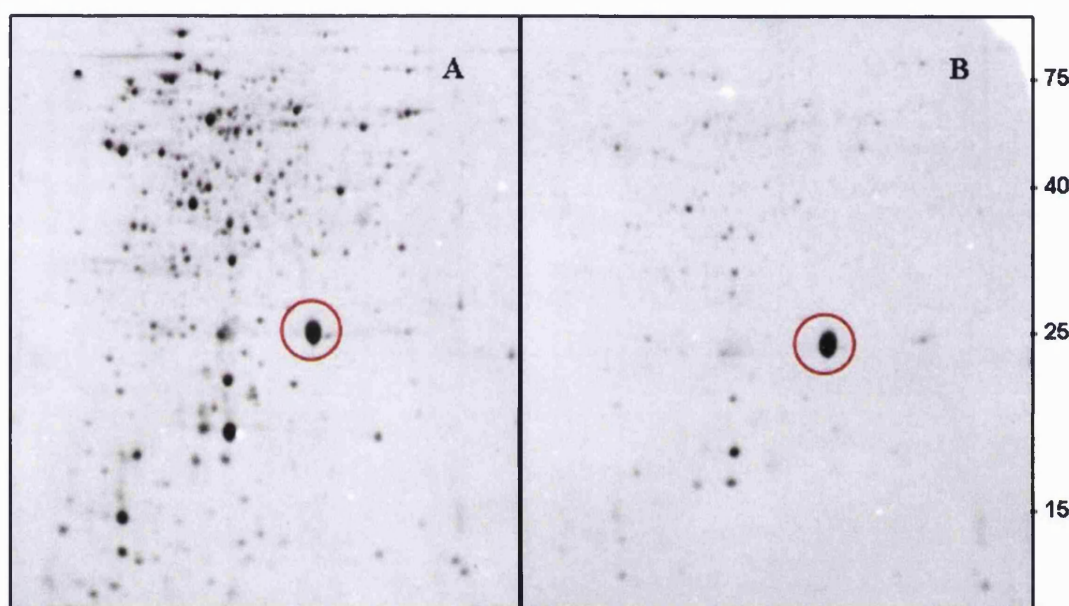
The effect of LSMMG on *S. aureus* protein secretion was initially determined by SDS-PAGE of solubilised proteins in the culture supernatant of all three isolates studied (Fig. 4.2). For this assay, samples were normalised according to the dry weight of the cells.



**Fig. 4.2:** SDS-PAGE of soluble proteins in the culture supernatant of *S. aureus* isolates, visualised with Coomassie blue. Lanes: RF1 grown under NG (1); RF1 grown under LSMMG (2); RF6 grown under NG (3); RF6 grown under LSMMG (4); RF11 grown under NG (5); RF11 grown under LSMMG (6); Molecular weight marker (M).

As shown in figure 4.2, it is clear that the three isolates secrete a large variety of proteins into the surrounding medium and there are clear differences in protein secretion patterns between them. Several differences between LSMMG- and NG-grown cells can also be observed. In general terms, the total protein secreted by LSMMG-grown cells is decreased in comparison to that secreted by bacteria grown under NG. The largest reduction was observed in the case of isolate RF6. Interestingly, the secretion of a limited number of proteins was maintained under LSMMG, for example, the secretion of a protein with a molecular mass of 24 kDa.

In order to investigate the protein profiles of the isolates in more detail, 2D electrophoresis was performed. The same sample normalisation procedure, using values based on cell dry weight, was adopted. Isolate RF6 was selected initially for this study as, from analysis of the 1D results, it appeared to secrete significantly lower amount of protein under LSMMG compared to NG. The results are shown in figure 4.3.

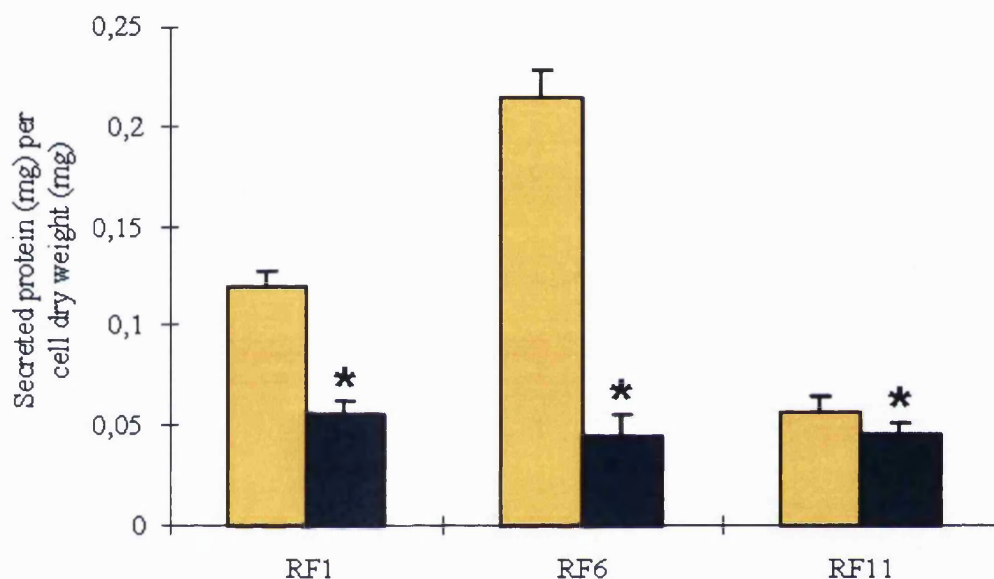


**Fig. 4.3:** 2D electrophoresis of soluble proteins in the culture supernatant of *S. aureus* isolate RF6 grown under NG (A) and LSMMG (B) visualised with Coomassie blue. The red circle marks a 24 kDa protein, the only secreted component found to be present in the same concentration under both NG and LSMMG.

In similar fashion to data obtained using 1D gel electrophoresis, a significant reduction in protein secretion was observed for isolate RF6 under conditions of LSMMG compared to NG. All proteins generally appeared to be secreted in lower amounts under LSMMG and the fluorescence signal of the majority of these proteins was below PDQuest Advanced software detection limit. With both 1D and 2D gels, a single protein with a molecular mass of 24 kDa (marked in red in figure 4.3) was found to be secreted in comparable amounts under NG and LSMMG, in contrast to the marked reductions found with other proteins when supernatants from LSMMG-grown cells were compared with their NG counterparts.



As the total amount of protein secreted by LSMMG-cells was found to be markedly reduced in comparison to NG, particularly with isolate RF6, a protein assay was performed in order to quantify the total amount of soluble protein in culture supernatants (Fig. 4.4).



**Fig. 4.4:** Quantification of soluble proteins in the culture supernatants of *S. aureus* isolates RF1, RF6 and RF11 grown under NG (■) and LSMMG (■). Error bars represent 1 SD; n=5; \*p<0.0001.

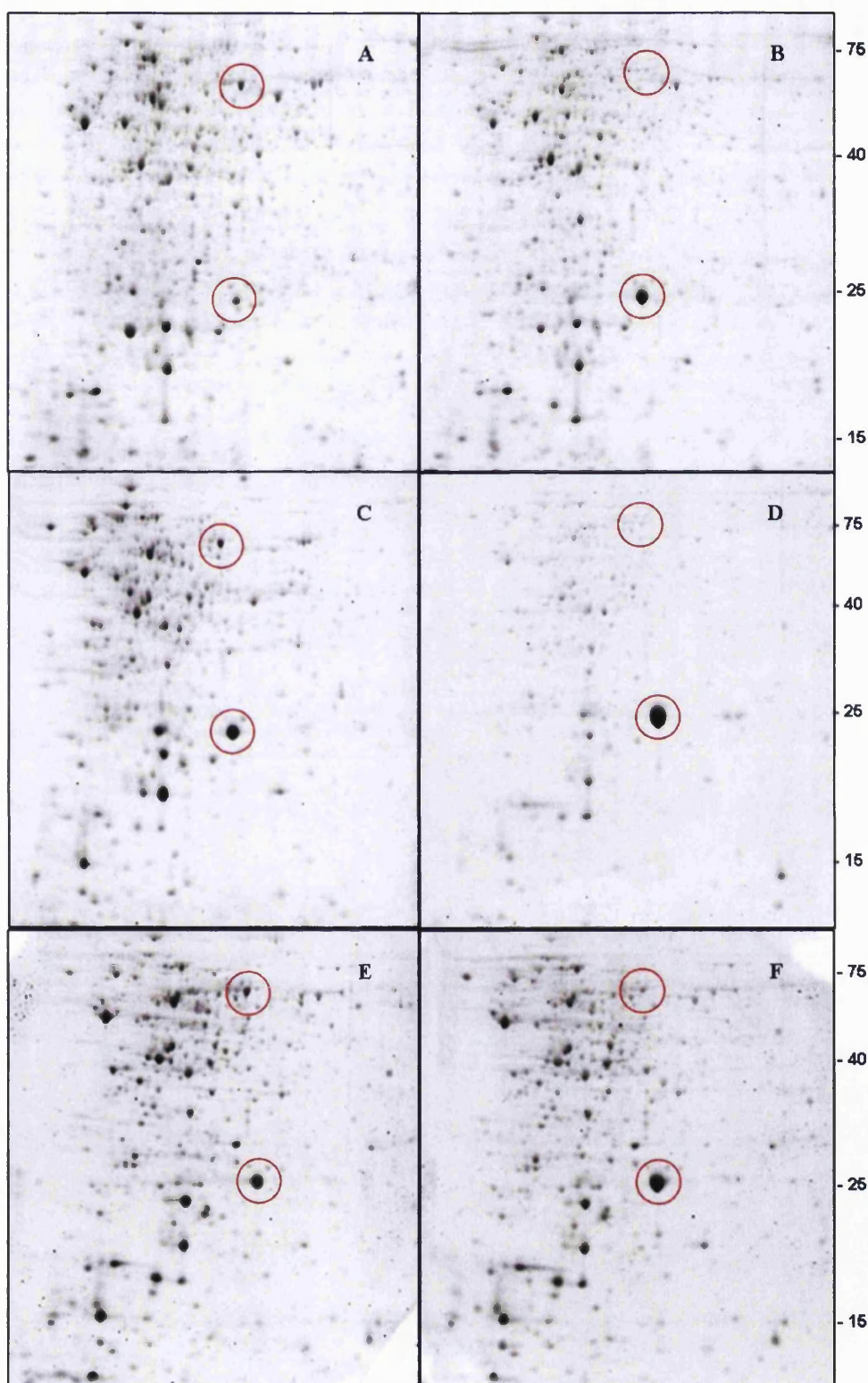
A significant reduction ( $p < 0.0001$ ) in protein secretion was found in all three isolates when they were grown in LSMMG compared to NG. In agreement with the results shown by SDS-PAGE, the largest reduction in protein secretion was observed in isolate RF6, where a 5.5-fold decrease was found. For isolates RF1 and RF11 respectively, a significant twofold and 1.2-fold reduction was noted. Interestingly, although inter-isolate variability was observed, the levels of protein secreted by all three isolates under LSMMG were similar, approximately 0.05 mg per mg of cells.

### 4.3.2 Effect of LSMMG on *S. aureus* supernatant proteome

2D electrophoresis was undertaken on secreted proteins from the three *S. aureus* isolates. Since the proteomic data generated by sample normalisation based on cell dry weight values was below the software detection limit (Fig. 4.3), samples were normalised according to the total amount of secreted protein previously determined (Fig. 4.4). A sample volume containing 300 µg of protein was loaded on to each gel; LSMMG protein levels were accordingly raised twofold, 5.5-fold and 1.2-fold respectively for *S. aureus* isolates RF1, RF6 and RF11 (Fig. 4.5).

Approximately 300 proteins secreted by *S. aureus* isolates RF1, RF6 and RF11 were analysed and quantified; the relative amounts secreted under NG and LSMMG growth conditions were compared using PDQuest Advanced software. Particularly interesting results were observed for isolate RF6, where a largest reduction in protein secretion was observed. Using this normalisation method, the relative amount of eighty proteins was significantly reduced twofold ( $p < 0.01$ ) in LSMMG compared to NG samples. Forty of these proteins were found reduced fourfold or more. For isolates RF1 and RF11, seven and ten proteins respectively were found to be reduced twofold ( $p < 0.05$ ) or more when grown under LSMMG in comparison to NG. Interestingly, with all three isolates, one protein of 24 kDa was secreted in readily detectable amounts and one protein of 58 kDa was secreted in very low amounts under LSMMG compared to NG.

The secretion level of NG and LSMMG proteins compared using PDQuest Advanced software was adjusted to the normalising factor utilised, previously determined by protein quantification (Fig. 4.4). Accordingly, protein secretion values were raised twofold, 5.5-fold and 1.2-fold respectively for isolate RF1, RF6 and RF11. The results are summarised in table 4.1, where protein secretion mean fold changes (LSMMG/NG) are indicated.

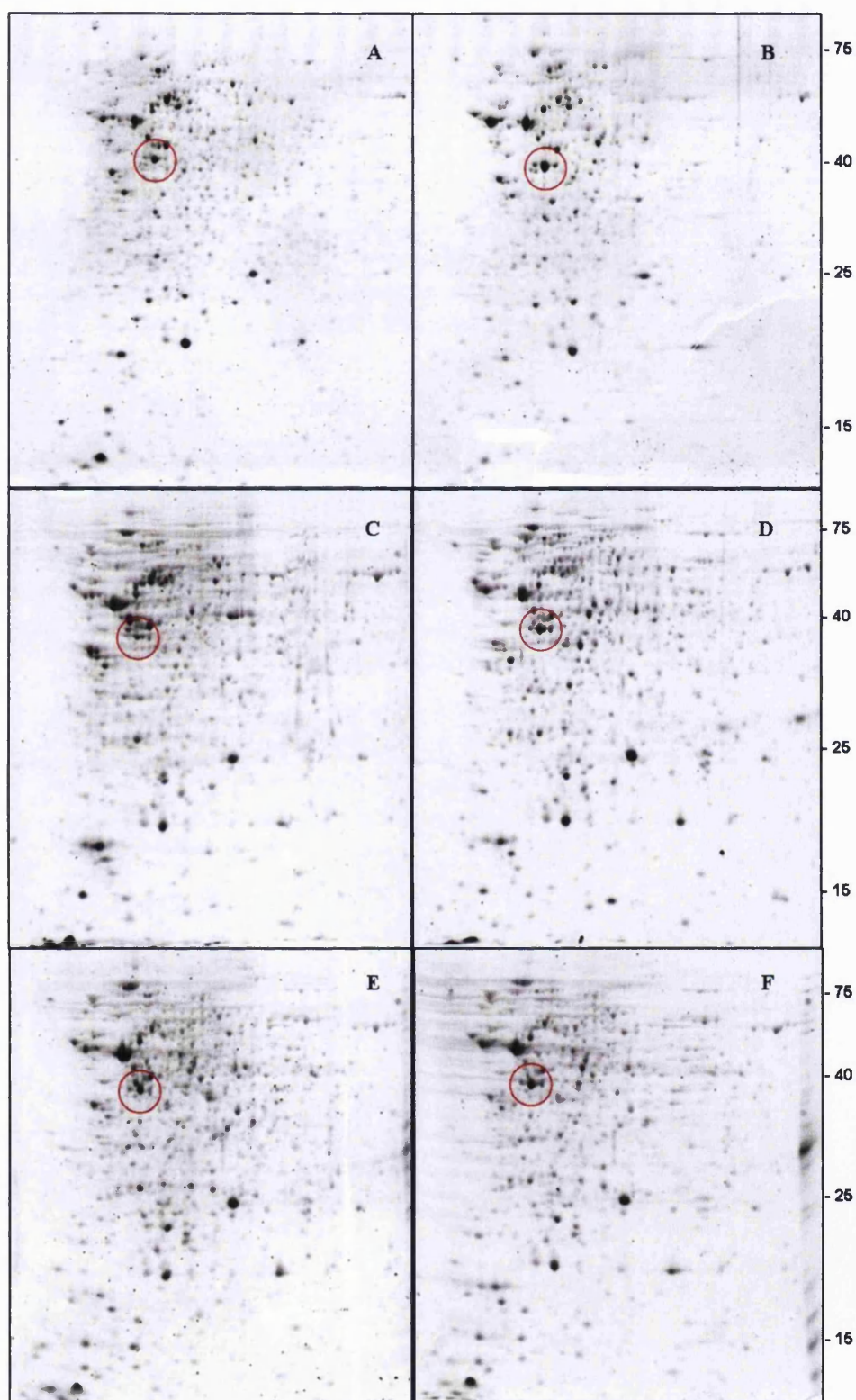


**Fig. 4.5:** 2D electrophoresis of soluble proteins in the culture supernatant of *S. aureus*, visualised with Sypro Ruby. RF1 grown under NG (A). RF1 grown under LSMMG (B). RF6 grown under NG (C). RF6 grown under LSMMG (D). RF11 grown under NG (E). RF11 grown under LSMMG (F). Red circles show proteins that were differentially expressed; their MWs were 24 and 58 kDa.

### 4.3.3 Effect of LSMMG on *S. aureus* intracellular proteome

2D electrophoresis was also used to study the intracellular proteome of *S. aureus* isolates RF1, RF6 and RF11 under NG and LSMMG (Fig. 4.6). Since comparable whole-cell protein concentrations were found between NG and LSMMG, samples were normalised according to the total amount of protein and a sample volume containing 300 µg of protein was loaded on to each gel.

It is clear from a comparison of figures 4.5 and 4.6 that the resolution of the intracellular proteins of *S. aureus* is technically more challenging in comparison to the extracellular proteome, due to both a higher protein concentration per unit cell mass and a greater number of individual proteins contained within the sample. Significant differences ( $p < 0.05$ ) in intracellular concentration of five proteins between NG- and LSMMG-grown bacteria were found in all three isolates. Interestingly, only one protein of 36 kDa was found in significantly higher concentrations under LSMMG compared to NG with all three isolates.

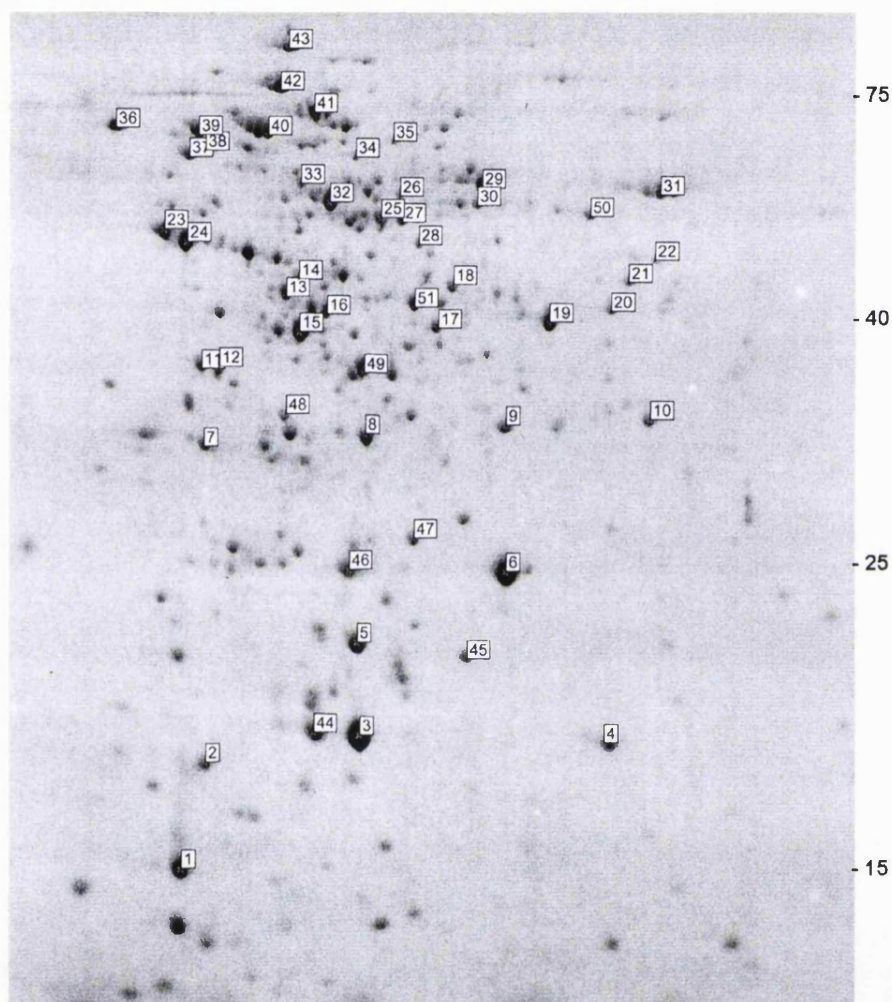


**Fig. 4.6:** 2D electrophoresis of intracellular proteins of *S. aureus*, visualised with Sypro Ruby. RF1 grown under NG (A). RF1 grown under LSMMG (B). RF6 grown under NG (C). RF6 grown under LSMMG (D). RF11 grown under NG (E). RF11 grown under LSMMG (F). The circles in red show a 36 kDa protein found in significantly higher concentrations in LSMMG compared to NG.



#### 4.3.4 Protein identification by LC/MS-MS

The most abundant proteins in *S. aureus* isolates RF1, RF6 and RF11 as identified by LC/MS-MS analysis are marked in figure 4.7.



**Fig. 4.7:** Proteins identified by LC/MS-MS. Spots numbered in the figure represent the most abundant proteins found in the supernatant proteome of *S. aureus* that were excised for MS analysis.

In total, fifty one proteins were analysed by LC/MS-MS. However, sixteen of these proteins were identified with low score and their sequences could not be matched with sequences in the Mascot (Matrix Science) protein database. The reasons for these anomalies were attributed to low protein quantity, mixtures of proteins or absence of matching sequences in the database. Proteins identified by LC/MS-MS are listed in table 4.1.

**Table 4.1:** The most abundant proteins found in the supernatant proteome of *S. aureus* isolates RF1, RF6 and RF11, identified by LC/MS-MS. Results are shown as mean fold changes (LSMMG/NG). Values were adjusted for protein content (Fig. 4.4) and proteomic analysis (Fig. 4.5). n=3; \*p<0.05, \*\*p<0.01.

Function	Protein identification	Mean fold change			
		RF1	RF6	RF11	
<i>Protein synthesis, folding and degradation</i>					
1	Dps	General stress protein 20U	-1.81	-16.23**	-1.45*
7	TufA	Translational elongation factor Tu	-3.54	-24.88**	-1.07
23	RpsA	30S ribosomal protein S1	-2.73*	-31.69**	1.49*
36	Tig	Trigger factor prolyl isomerase	-2.25	-32.32**	1.04
37	GroEL	60 kDa chaperonin	-	-21.80*	-
39	DnaK	Chaperone protein	-1.87	-39.64*	1.06
40	ClpL	Putative ATP-dependent Clp proteinase, proteolytic subunit ClpL	-2.09	-25.52	1.01
42	EF-G/FusA	Elongation factor G	-3.62*	-33.46*	1.93
48	HchA	Chaperone protein hchA (Hsp31)	-	-15.02**	1.06
49	EF-T/Tsf	Translation elongation factor Ts	-1.64	-14.75**	-2.10*
<i>Glycolysis</i>					
8	Fba	Fructose-bisphosphate aldolase	-	-10.33*	-
11	PdhB	Pyruvate dehydrogenase E1 component beta subunit	-1.80	-25.84**	-1.93**
15	Gap	Glyceraldehyde-3-phosphate dehydrogenase, type I	-1.59	-59.46*	-1.58
24	Eno	Phosphopyruvate hydratase – Enolase	-3.99**	-24.75**	-2.78**
38	PtsI	Phosphoenolpyruvate-protein phosphotransferase	-	-19.43**	-
51	Pgk	Phosphoglycerate kinase	-1.77	-19.14*	1.07
<i>TCA cycle</i>					
10	SucD	Succinyl-CoA ligase [ADP-forming] subunit alpha	-1.20	-45.53**	-1.01
13	CitC	Isocitrate dehydrogenase [NADP]	-4.09*	-68.39*	-
19	CitZ	Citrate synthase II	-17.90**	-322.82*	-1.61**
43	CitB	Aconitate hydratase I	-2.59	-33.99*	-1.75

Function	Protein identification	Mean fold change			
		RF1	RF1	RF1	
<i>Oxidative stress</i>					
5	SodA	Superoxide dismutase	-2.43*	-13.59**	-2.65**
25	AldA	Putative aldehyde dehydrogenase	-	-26.73**	-
29	KatA	Catalase	-13.83**	-36.09**	-2.16**
<i>Amino acid synthesis</i>					
9	CysK	Cysteine synthase	-2.95*	-21.49*	-1.13
17	ACOAT2	Acetylornithine aminotransferase 2	-1.47	-39.93*	-2.55
27	GlnA	Glutamine synthetase, type I	-2.84	-31.20**	-2.30
<i>Carbon metabolism</i>					
22	GlyA	Serine hydroxymethyltransferase	-4.22	-25.82**	-
31	Fhs	Formate-tetrahydrofolate ligase	-4.91*	-53.46**	-2.37*
<i>Acetate metabolism</i>					
21	AckA	Acetate kinase	-1.79	-49.41**	-2.35*
32	PdhD	Dihydrolipoamide dehydrogenase component of pyruvate dehydrogenase E3	-2.55**	-22.46**	-1.95
<i>Pyrimidine metabolism</i>					
14	PYNP	Pyrimidine nucleoside phosphorylase	-4.64**	-35.98**	-
<i>Iron transport and storage</i>					
2	FtnA	Ferritin	-1.52*	-10.02	1.18
<i>Unconfirmed function</i>					
3	Asp23	Alkaline shock protein	-1.95	-8.95	1.06*
6	IsaA	Immunodominant antigen A	1.16*	1.68*	2.53**
12	PpaC	Probable manganese-dependent inorganic pyrophosphatase	-4.42	-22.49**	-1.44



The proteins listed in table 4.1 represent the most abundant proteins found in the supernatant proteome of *S. aureus* isolates RF1, RF6 and RF11, identified by LC/MS-MS. A large fraction of these proteins are involved in metabolic processes such as glycolysis and tricarboxylic acid (TCA) cycle activity. Proteins involved in protein synthesis, folding and degradation are also well represented. The 24 kDa protein, secreted in comparable amounts under LSMMG and NG, was identified as immunodominant antigen A (IsaA); the 58 kDa protein, found to be secreted in lower amounts under LSMMG, was identified as catalase (KatA); the 36 kDa protein, found in higher intracellular concentration in LSMMG, was identified as glyceraldehyde-3-phosphate dehydrogenase (Gap).

The relative amounts of supernatant proteins in NG- and LSMMG-grown *S. aureus* isolates RF1, RF6 and RF11 were identified by LS/MS-MS. The highest reduction was found for isolate RF6. Interestingly, the large majority of proteins appeared to be secreted in lower amounts under LSMMG, with a few exceptions, such as IsaA, which was found to be actively secreted in comparable amounts under NG and LSMMG in all three isolates. The levels of enolase (Eno), citrate synthase II (CitZ), superoxide dismutase (SodA) and KatA were the most significantly reduced by LSMMG in all three isolates.

#### **4.3.5 Effect of LSMMG on *S. aureus* protease activity**

Protease activity was determined by zymography by the appearance of clear bands on the dark background, indicating zones of digestion of casein. No protease activity was detected using the method described.

### **4.4 Discussion**

The *S. aureus* genome encodes approximately 2800 proteins. However, only approximately 60% of these proteins have a known function (Lindsay, 2008). The study of the remaining 40% of proteins with unknown function is limited since under

standard laboratory culture conditions only part of the genome is active. For this reason, the study of the *S. aureus* proteome under different environmental conditions, such as LSMMG, can provide important information on staphylococcal pathogenicity by triggering the expression of new phenotypes and consequently altering the production and/or secretion of particular proteins. Analysis of these modifications can aid clarification of the function of unknown proteins encoded by the bacterial genome as well as elucidate the adaptation mechanism of the bacterium to different environments.

In general, proteomic analysis of Gram-positive bacteria is technically challenging due to the presence of large quantities of peptidoglycan and highly insoluble cell membrane proteins that have been shown to compromise the efficiency of the electrophoretic procedure (Hecker *et al.*, 2003; Scherl *et al.*, 2005). In addition, it is important to define and control the bacterial growth conditions prior to embarking on a proteomic study. The use of different strains, different growth media and different experimental conditions complicate comparison of proteomic profiles obtained by different research groups.

Useful proteomic reference maps have been previously published. Cordwell *et al.* (2002) analysed cytoplasmic proteins of *S. aureus* COL and NCTC 8325 grown in tryptic soya broth (TSB) at 37°C to exponential phase. Similarly, Kohler and co-workers (Kohler *et al.*, 2005) published an intracellular reference map of *S. aureus* COL grown to mid-exponential and stationary phases, in TSB at 37°C. Both intracellular and membrane-associated proteins of *S. aureus* N315 were studied by Scherl *et al.* after growth to stationary phase in MHB at 37°C (Scherl *et al.*, 2005). Ziebandt and co-workers (2001; 2004) utilised proteomic tools to determine the role of global regulatory genes, such as *agr*, *sarA* and *sigB* on the *S. aureus* extracellular proteome. The strains COL (methicillin-resistant), RN6390 (a derivative of the methicillin-sensitive NCTC 8325) and mutants of these strains were grown to post-exponential phase in Luria-Bertani (LB) broth (Ziebandt *et al.*, 2001). In a similar study performed by the same group, *S. aureus* was grown in TSB to exponential and stationary phase (Ziebandt *et al.*, 2004). Bernardo *et al.* (2002) examined the effect of temperature variations on toxin secretion by one *S. aureus* clinical isolate and two type strains, ATCC 43300 (methicillin-resistant) and ATCC 29213 (methicillin-

sensitive). Cells were grown to post-exponential phase in brain-heart-infusion (BHI) broth at 37°C and 42°C. Similar growth conditions were used in a later study by Koszól *et al.* (2006). The effect of subinhibitory concentrations of quinupristin/dalfopristin on *S. aureus* ATCC 29213 exoproteome were analysed after growth at 37°C to post-exponential phase in LB broth.

As described, although several proteomic studies have been published, the use of different strains and experimental conditions by the various research groups makes comparisons between different studies problematical and also results in difficulties in interpretation of data. Nevertheless, these proteomic analyses have provided unique information and are undoubtedly an excellent contribution to the knowledge of *S. aureus* virulence and pathogenicity mechanisms. To determine the effect of LSMMG on *S. aureus* proteome, cells were harvested and supernatants collected during the stationary phase, when the bulk of staphylococcal virulence factors are secreted and higher cell densities are obtained.

Virulence factors, manifested mainly as secreted or cell-wall associated proteins, play a major role in *S. aureus* pathogenicity (Fischetti *et al.*, 2006). Interestingly, LSMMG significantly reduced the overall levels of secreted protein by the three *S. aureus* isolates that were studied, and this was particularly marked in the case of isolate RF6. Such findings suggest that *S. aureus* virulence may be reduced under LSMMG.

The study of proteomic differences between strains or isolates, under similar or different environmental conditions, may be essential to understand *S. aureus* pathogenicity and LSMMG thus provides a new approach for a novel proteomic study. The proteins detected in the supernatant (Ziebandt *et al.*, 2001; Bernardo *et al.*, 2002; Ziebandt *et al.*, 2004; Rogasch *et al.*, 2006; Koszczól *et al.*, 2006) and intracellular (Bernardo *et al.*, 2002; Cordwell *et al.*, 2002; Kohler *et al.*, 2005; Scherl *et al.*, 2005) proteome of *S. aureus* isolates RF1, RF6 and RF11 are consistent with proteomic studies and reference maps published previously.

Although similar supernatant and intracellular proteomic profiles were obtained for isolates RF1, RF6 and RF11, clear qualitative and quantitative differences were

found. This observation is consistent with previous reports describing a number of differences in the exoproteome profile of various *S. aureus* strains (Ziebandt *et al.*, 2001; Rogasch *et al.*, 2006) and this was even more pronounced when clinical isolates were compared. In contrast, the intracellular proteome appeared to be rather more consistent amongst strains and isolates (Ziebandt *et al.*, 2001).

In general, 2D electrophoresis provides high resolution images in a very reproducible way. It is therefore a useful tool for proteomic analysis. The total amount of protein loaded on a gel influences the size of individual spots, their shape and may interfere with the location of the protein spot on the gel. This clearly affects the resolution of the image and it may compromise accurate matching of protein spots for comparative purposes (O'Farrell, 1975).

For this reason, it is extremely important that comparable amounts of protein are loaded on to gels in order to achieve meaningful analysis and an acceptable level of reproducibility. In general, the size of the spots increases with the amount of protein in the sample. However, better resolution is achieved when smaller amounts of protein are loaded on to the gel (O'Farrell, 1975). During the current proteomic study of *S. aureus* grown under NG and LSMMG, the best results were achieved using 300 µg of protein.

IPG strips can be selected with different lengths and pH ranges; these should be selected appropriately for the sample to be studied, as these parameters will affect the resolution of proteins in the 2D gel. When a wider pH gradient is used (for example from 3 to 10), proteins will cluster in small areas and resolution may be poor, even when spots are very small. Better resolution will be achieved using longer strips and narrower pH gradients (Berkelman and Stenstedt, 2004). However, it is unlikely that the complete bacterial proteome will be represented using a single IPG strip format.

The *S. aureus* proteome map predicted from genome sequencing shows that staphylococcal proteins have more neutral and alkaline pI than acidic pI (Hecker *et al.*, 2003; Kohler *et al.*, 2005). The 4 to 7 pH gradient selected for this study facilitated resolution of the main staphylococcal proteins, in particular cytoplasmic proteins which have a pI approximating neutral. Some secreted proteins, including

many of the staphylococcal virulence factors, are more alkaline, with pI values around 10. However, more alkaline gels are technically challenging and good resolution is more difficult to achieve compared to pH-neutral gels. For this reason, this proteomic study only resolves a fraction of the total *S. aureus* proteome and does not include strongly acidic or alkaline proteins. For example,  $\alpha$ -toxin has a theoretical pI of 8.7 and could not be separated and detected using IPG strips of pH range 4 to 7.

Although in general LSMMG reduced the protein amount present in *S. aureus* supernatant, the levels of IsaA produced in relation to the dry weight of the bacterial cells were comparable to yields from NG. The function of IsaA is still unknown; however, it has been associated with *S. aureus* virulence, as high levels of this specific antigen have been found in patients with endocarditis and sepsis (Lorenz *et al.*, 2000a; Lorenz *et al.*, 2000b). It has thus been considered as a potential vaccine candidate (Vytvytska *et al.*, 2002; Hecker *et al.*, 2003). In terms of cellular localization, the levels of IsaA found in *S. aureus* isolates RF1, RF6 and RF11 are consistent with previous intracellular (Cordwell *et al.*, 2002) and extracellular (Ziebandt *et al.*, 2001; Ziebandt *et al.*, 2004) proteomic studies involving other staphylococcal stains.

A significant reduction in secretion levels of KatA by LSMMG was found in all three *S. aureus* isolates investigated. The role of catalase in oxidative stress responses is well known, as most bacteria employ this enzyme in metabolic processes following exposure to oxygen (Dubbs and Mongkolsuk, 2007). An increased resistance to oxidative stress due to greater KatA expression has been previously described in *S. aureus* (Cosgrove *et al.*, 2007). Reduction of KatA levels in the supernatant proteome under LSMMG could be induced by lower exposure of the staphylococcal cells to oxidative stress, since HARV cultures were not aerated during their growth period. However, the intracellular levels of this enzyme were found to be unaltered, suggesting that a transcriptional or post-transcriptional modification may have been induced by LSMMG.

Interestingly, and in addition to catalase, other proteins with typical cytoplasmic functions such EF-G, enolase, Gap, GroEL and superoxide dismutase, were found in

the *S. aureus* supernatant proteome. These proteins do not appear to carry a secretion signal but have been previously described as being amongst the most abundant cytoplasmic proteins, particularly in the later stages of cell growth (Kohler *et al.*, 2005). Several other authors have also found intracellular proteins in culture supernatant (Bernardo *et al.*, 2002; Ziebandt *et al.*, 2004; Rogasch *et al.*, 2006; Koszczol *et al.*, 2006); these findings could be attributable to cell lysis. In addition, the hypothesis that intracellular proteins are exported in to the surrounding medium by an unknown export pathway has been previously proposed (Sibbald *et al.*, 2006) and is consistent with the differences found between the intracellular and supernatant protein concentrations of *S. aureus* isolates RF1, RF6 and RF11 grown under NG and LSMMG.

The production of virulence factors in *S. aureus* is growth-phase dependent and involves a number of regulatory systems, such as *agr*, *sar*, *sae* and *sigB* (Novick, 2003; Lindsay, 2008). During the exponential phase, and up to the post-exponential phase, surface proteins such as IsaA are in the main produced; during the post-exponential phase and the stationary phase, the expression of extracellular proteins, such as Hla, increases and the production of surface proteins is repressed (Vandenesch *et al.*, 1991; Ziebandt *et al.*, 2004).

Extensive research has been performed in an attempt to understand the gene regulation of *S. aureus* virulence (Novick, 2003; Lindsay, 2008) and the recent use of proteomics, in association with transcriptomics, has brought new insights to the mechanics of staphylococcal gene regulation (Gertz *et al.*, 2000; Scherl *et al.*, 2005). The effects of LSMMG on the *S. aureus* proteome, in particular reduced levels of protein secretion, could be explained by a novel gene regulation mechanism induced by modification of the gravitational force.

It has been suggested that transcription of *isaA* (Ziebandt *et al.*, 2004), *kata* (Horsburgh *et al.*, 2002) and *hla* (Cheung *et al.*, 1999; Ziebandt *et al.*, 2001; Bischoff *et al.*, 2004; Karlsson-Kanth *et al.*, 2006) are under the regulatory activity of *sigB*. This locus is predominantly associated with the *S. aureus* stress response (Kullik and Giachino, 1997; Chan *et al.*, 1998; Horsburgh *et al.*, 2002). However, there is strong

evidence that *sigB* is also involved in virulence regulation in this bacterium (Kullik *et al.*, 1998; Gertz *et al.*, 2000; Horsburgh *et al.*, 2002; Bischoff *et al.*, 2004).

Although  $\alpha$ -toxin was not detected by 2D electrophoresis in the culture supernatants of isolates RF1, RF6 and RF11 due to the pI range 4-7 used, the data described in chapter three show a clear reduction in secretion of this protein under LSMMG. In addition, *sigB* has been shown to positively regulate IsaA (Ziebandt *et al.*, 2004) and negatively regulate Hla (Cheung *et al.*, 1999; Ziebandt *et al.*, 2001; Karlsson-Kanth *et al.*, 2006). Such observations are consistent with the proteomic data obtained for isolates RF1, RF6 and RF11, suggesting an up-regulation of *sigB* under conditions of LSMMG compared to NG. However, the reduction in pigment production induced by LSMMG does not correlate with *sigB* over-expression (Kullik *et al.*, 1998; Nicholas *et al.*, 1999; Morikawa *et al.*, 2001; Giachino *et al.*, 2001; Karlsson-Kanth *et al.*, 2006), suggesting that other mechanisms *sigB*-independent may be involved in response to LSMMG.

In addition to *sigB*, other systems are associated with the regulation of transcriptional levels of *isaA* and *hla*: *agr* appears to positively regulate *hla* (Ziebandt *et al.*, 2004) and negatively regulate *isaA* (Ziebandt *et al.*, 2004) while *sarA* appears to positively regulate *isaA* (Oscarsson *et al.*, 2006) and negatively regulate *hla* (Ziebandt *et al.*, 2001). Thus, down-regulation of *agr* and/or up-regulation of *sarA* induced by LSMMG would theoretically increase the levels of IsaA and decrease the levels of Hla detected in the culture supernatant. Table 4.2 summarises some of *S. aureus* global regulatory systems and correlates these with the effects of LSMMG on *S. aureus* gene expression. These issues will be analysed and discussed further in chapter five.

The mechanisms of virulence regulation in *S. aureus* are very complex and, for that reason, alterations in gene expression may not explain completely the effects of LSMMG on protein secretion. In fact, other authors have observed reduced protein concentrations in the *S. aureus* exoproteome, although protein transcription levels were maintained (Scherl *et al.*, 2005).

**Table 4.2:** Summary of *S. aureus* global regulatory mechanisms which may be involved in response to LSMMG environment. (+) positive regulation, (-) negative regulation, (?) undescribed regulatory mechanism.

	Global regulatory gene system						LSMMG
	<i>sigB</i>	References	<i>agr</i>	References	<i>sarA</i>	References	
<b>IsaA</b>	+	(Ziebandt <i>et al.</i> , 2004)	-	(Ziebandt <i>et al.</i> , 2004)	+	(Oscarsson <i>et al.</i> , 2006)	≈
<b>KatA</b>	-	(Horsburgh <i>et al.</i> , 2002)	?	Not described	?	Not described	-
<b>Hla</b>	-	(Cheung <i>et al.</i> , 1999) (Ziebandt <i>et al.</i> , 2001) (Bischoff <i>et al.</i> , 2004) (Karlsson-Kanth <i>et al.</i> , 2006)	+	(Dunman <i>et al.</i> , 2001) (Ziebandt <i>et al.</i> , 2004)	-	(Ziebandt <i>et al.</i> , 2001) (Oscarsson <i>et al.</i> , 2006)	-
<b>Pigment</b>	+	(Kullik <i>et al.</i> , 1998) (Nicholas <i>et al.</i> , 1999) (Morikawa <i>et al.</i> , 2001) (Giachino <i>et al.</i> , 2001) (Karlsson-Kanth <i>et al.</i> , 2006)	?	Not described	?	Not described	-



The effect of LSMMG on the *S. aureus* intracellular proteome of isolates RF1, RF6 and RF11 was also investigated in the current study. Intracellular levels of the large majority of proteins were found to be unaltered, but a protein involved in glycolysis, glyceraldehyde-3-phosphate dehydrogenase (Gap), was observed to be expressed at significantly higher concentrations in all three isolates. Nevertheless, fewer qualitative and quantitative differences in intracellular protein levels were found between NG- and LSMMG-grown bacteria compared to the supernatant proteome, suggesting that LSMMG might favourably alter cell permeability and/or protein transport across the membrane. It has been suggested that proteins destined for export to the extracellular environment may be retained in the *S. aureus* membrane during mid-exponential phase (Gatlin *et al.*, 2006), and this goes some way towards explaining the results obtained in the current study.

Expression of various staphylococcal proteases has been described and is associated with *S. aureus* virulence, as these enzymes are essential for the inactivation of host defence proteins (Fischetti *et al.*, 2006). Proteolytic degradation of staphylococcal exoproteins has been previously described and studied by 2D gel electrophoresis (Kawano *et al.*, 2001; Nakano *et al.*, 2002); proteolysis has been found to be particularly significant in high MW proteins expressed during late-stationary phase (Kawano *et al.*, 2001). For this reason, protease activity was determined by Zymography using the culture supernatants of NG- and LSMMG-grown *S. aureus*. Presence of proteases in the samples analysed was not detected using the method described. Although NG- and LSMMG-cells were grown for 24 h, a maximum OD 3 was reached, possibly indicating that the cells were in early-stationary phase. Moreover, protease inhibitors were used during the 2D process in order to minimise these effects. Proteolysis is thus considered unlikely to account for the observed differences in protein secretion under NG and LSMMG; it should, however, be taken into account when considering interpretation of data. Interestingly, 2D electrophoretic analysis of early stationary phase *S. aureus* demonstrated the appearance of multiple spots in the gels and the effects of proteolysis were considered insignificant (Kawano *et al.*, 2001; Nakano *et al.*, 2002).

The study of the effects of LSMMG on the bacterial proteome is a novel area of research and consequently there is a very limited amount of data available. Nickerson and co-workers (2000) analysed *S. enterica* whole-cell protein under altered gravitational vector and found a threefold or more down-regulation of 38 proteins under LSMMG compared to NG, from which a group of six proteins was more than tenfold down-regulated. The nature of these proteins has not been yet identified, leaving data interpretation in abeyance.

Similarly, preliminary studies performed by Orihuela and co-workers (2001) report that levels of *S. pneumoniae* protein synthesis were also altered under conditions of LSMMG. Studies performed in *P. aeruginosa* show no alteration in protein secretion by this bacterium under simulated microgravity conditions (Guadarrama, 2006). These studies contrast with the findings described in the present study, although different bacterial species and experimental conditions have been used by these research groups. Nevertheless, the data presented in this chapter provides strong evidence that LSMMG alters protein secretion with the potential to reduce *S. aureus* virulence. This study facilitated the identification of potential targets for staphylococcal vaccines and novel alternative therapeutics.

Although in general the 2D method provides high resolution images in a very reproducible way, technical problems may occur during proteomics procedures. The quality of sample preparation is critical for production of good 2D gels and, properly applied, should result in total protein solubilisation, disaggregation, denaturation and reduction. The procedure should therefore be kept simple to avoid conditions that might lead to protein loss or modification, resulting in single proteins giving more than one spot or gels with poor resolution.

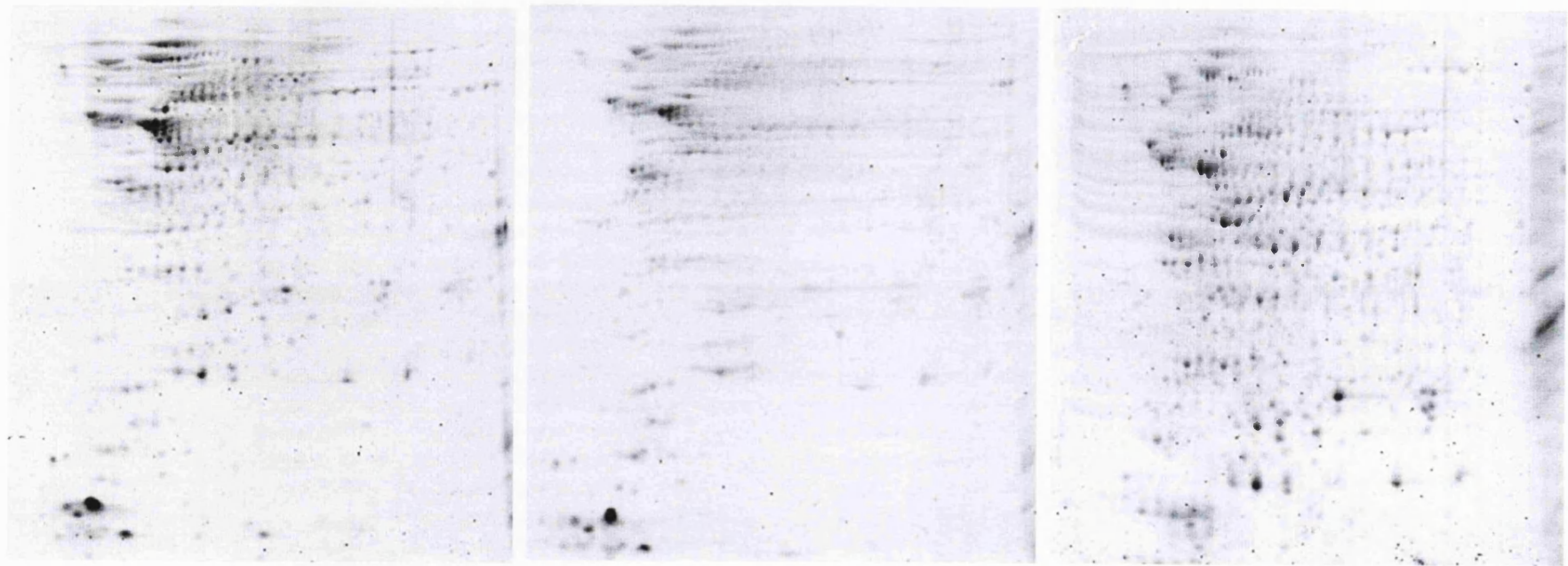
The use of specific reagents is recommended during this stage: urea is used as a solubilising and denaturing agent; thiourea improves solubilisation; CHAPS and carrier ampholytes enhance protein solubilisation and prevent aggregation; DTT preserves the fully reduced denatured state, aiding solubilisation (it is essential that DTT is added immediately before use). It is also important to avoid protein proteolysis, which interferes greatly with protein resolution on the gels, particularly

when cells are lysed. For this reason, sample preparation should be performed at low temperature and agents such as urea and other protease inhibitors should be used. Once the sample is prepared, it is important to proceed immediately with 2D gel electrophoresis so that final image resolution is not compromised. Figure 4.8 shows decreased gel resolution when the prepared samples were frozen before IEF.

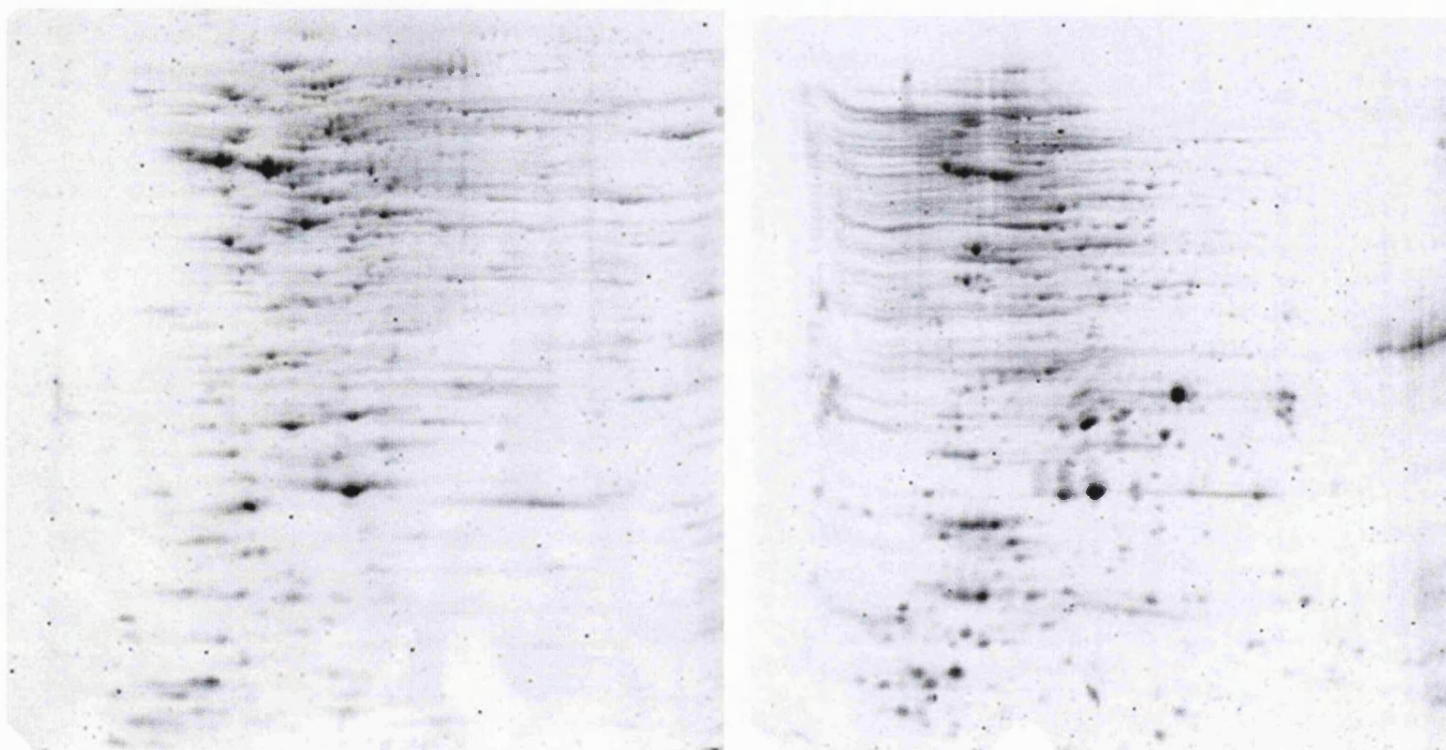
Appropriate IEF conditions are essential for good resolution of gels. During this step, the pH gradient should be uniform and this can be achieved by the use of carrier ampholytes. The electrical potential should also be rigorously controlled. Figure 4.9 shows examples of poor quality gels resulting from a non-optimal IEF, where severe streaking can be observed. It is also important to note that changes in IEF conditions may not necessarily interfere with the quality of the gel, but may affect the relative positions of protein spots (O'Farrell, 1975).

After running the samples in the first dimension, they should then be equilibrated and immediately loaded into a gel for protein separation in the second dimension. During the equilibration step, the use of particular reagents is recommended: the alkaline pH equilibration buffer reduces protein aggregation and maintains the IPG strip in a pH range appropriate for electrophoresis; the urea is used as denaturant and solubiliser; the glycerol, together with urea, reduces the effects of electroendosmosis; SDS denatures proteins and forms negatively charged protein-SDS complexes for electrophoresis; the DTT preserves the reduced state of proteins and iodoacetamide prevents their re-oxidation (it is essential that DTT and iodoacetamide are added just before use).

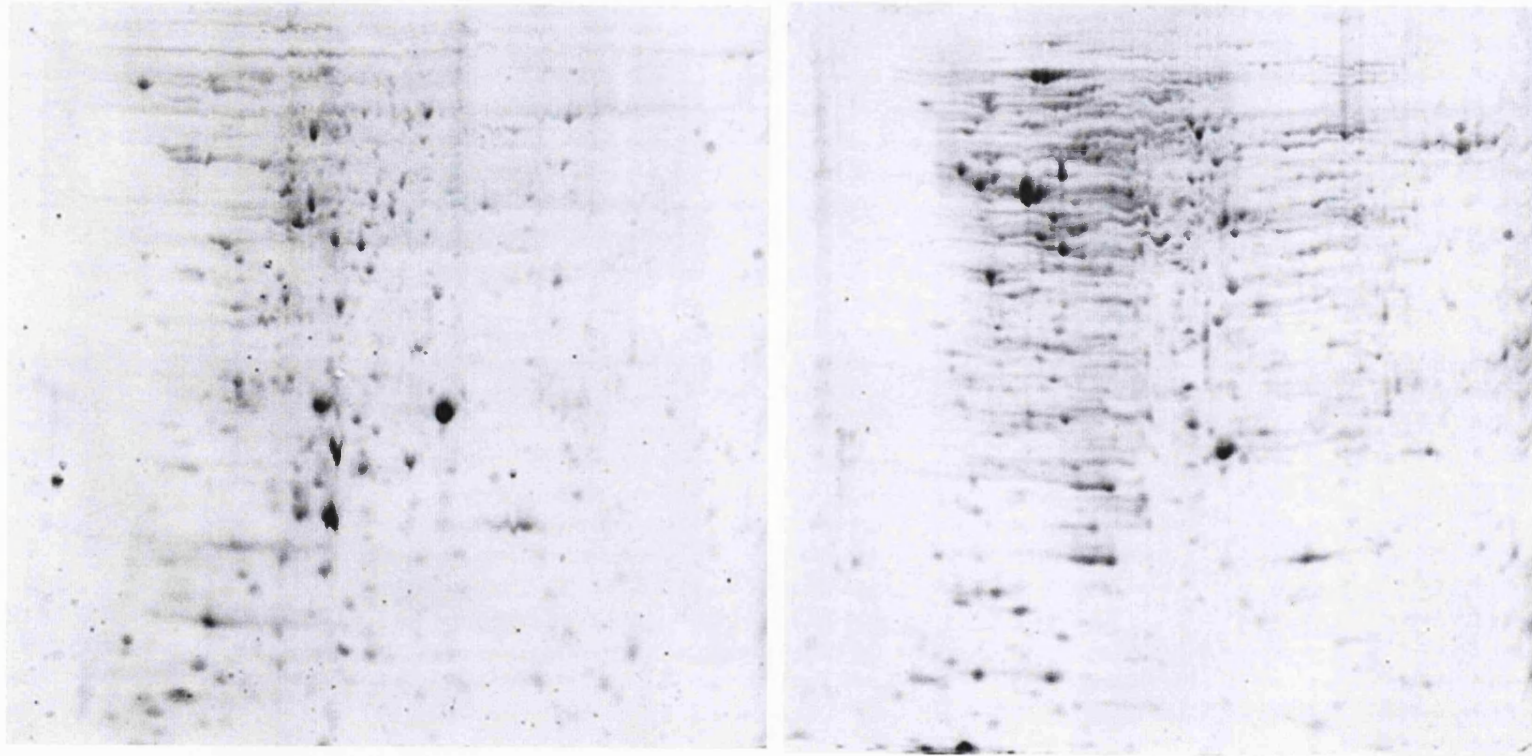
Careful selection of SDS-PAGE conditions is essential for a good final gel resolution. Gels should be left to polymerise for an optimal period and run at low voltage in a cool environment. Figure 4.10 shows the effects of gel polymerisation on 2D protein separation; spot shape and size are shown to be altered.



**Fig. 4.8:** Examples of poor gel resolution due to prior sample freezing before IEF.



**Fig. 4.9:** Examples of poor gel resolution due to non-optimal IEF conditions.



**Fig. 4.10:** Examples of poor gel resolution due to gel polymerisation.

Table 4.3 summarises the findings described in this chapter.

**Table 4.3:** Summary of the effect of LSMMG on *S. aureus* proteome.

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<b>Effect of LSMMG on <i>S. aureus</i></b>
⬇ Reduction in total amount of protein present in the supernatants
⬇ Quantitative and qualitative changes in the supernatant proteome
⬇ Comparable levels of IsaA in NG and LSMMG supernatants
⬇ Reduced levels of CitZ, Eno, SodA and KatA in LSMMG supernatants compared to NG
⬇ No effect on the intracellular proteome
⬇ No effect on protease activity

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## **CHAPTER FIVE**

**Effect of LSMMG on the transcriptomic profile of *S. aureus***



## 5.1 Introduction

The regulation of virulence in *S. aureus* is a complex and co-ordinated process, involving the expression and interaction of a number of genes and enables the pathogen to establish and maintain infection (Novick, 2003; Lindsay, 2008). Considerable effort has been made to understand gene regulation in *S. aureus*. The first publication of the complete *S. aureus* genome sequence of the two MRSA strains N315 and Mu50 (Kuroda *et al.*, 2001) and the development of new technologies, such as genomics and transcriptomics, has given new insights into the molecular basis of *S. aureus* adaptation, evolution, virulence and pathogenesis. So far, twelve *S. aureus* strains have been sequenced and annotated, eleven of which are human isolates (Kuroda *et al.*, 2001; Baba *et al.*, 2002; Holden *et al.*, 2004; Gill *et al.*, 2005; Diep *et al.*, 2006; Gillaspay *et al.*, 2006; Mwangi *et al.*, 2007; Baba *et al.*, 2008).

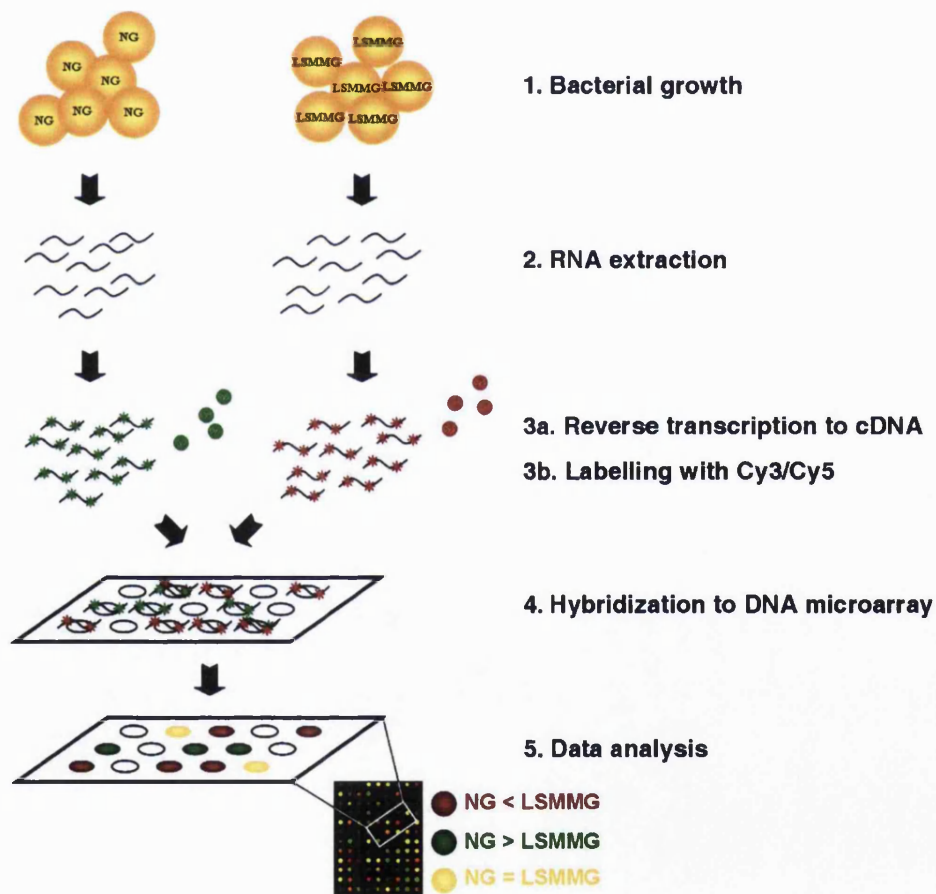
Although bacterial cells may carry an identical DNA blueprint, the transcription levels of a given gene or group of genes may vary, particularly if adaptation of the bacterium to a new environment is required. It is known that the *S. aureus* genome encodes approximately 2800 genes; more than 20% of these are variable among various strains and isolates and approximately 40% of genes found to be active encode proteins with an unknown function (Lindsay, 2008). Changes in gravity could offer a fundamentally new approach to the study of the regulatory processes of *S. aureus* virulence.

This chapter is a contribution to the study of the gene regulation of staphylococcal virulence and, in particular, of the impact of LSMMG as an environmental signal for gene expression in this bacterium.

### 5.1.1 DNA microarrays and transcriptomics technology

Transcriptomics is a recently developed technology that enables simultaneous analysis of thousands of genes of a given organism, in a particular environment, at a

given time (Shalon *et al.*, 1996). To determine which genes are expressed under a specific set of conditions (Fig. 5.1), RNA is extracted from a control and a sample, the RNA is reverse transcribed to cDNA, and the bacterial cDNA is differentially labelled with fluorescent dyes, most commonly Cy3 (green channel excitation) and Cy5 (red channel excitation). The labelled products are then allowed to competitively hybridise to a *S. aureus* DNA microarray slide, which contains thousands of PCR products of the genes identified by *S. aureus* genome sequencing (Schena, 1999; Baldi and Hatfield, 2002; Witney *et al.*, 2005).

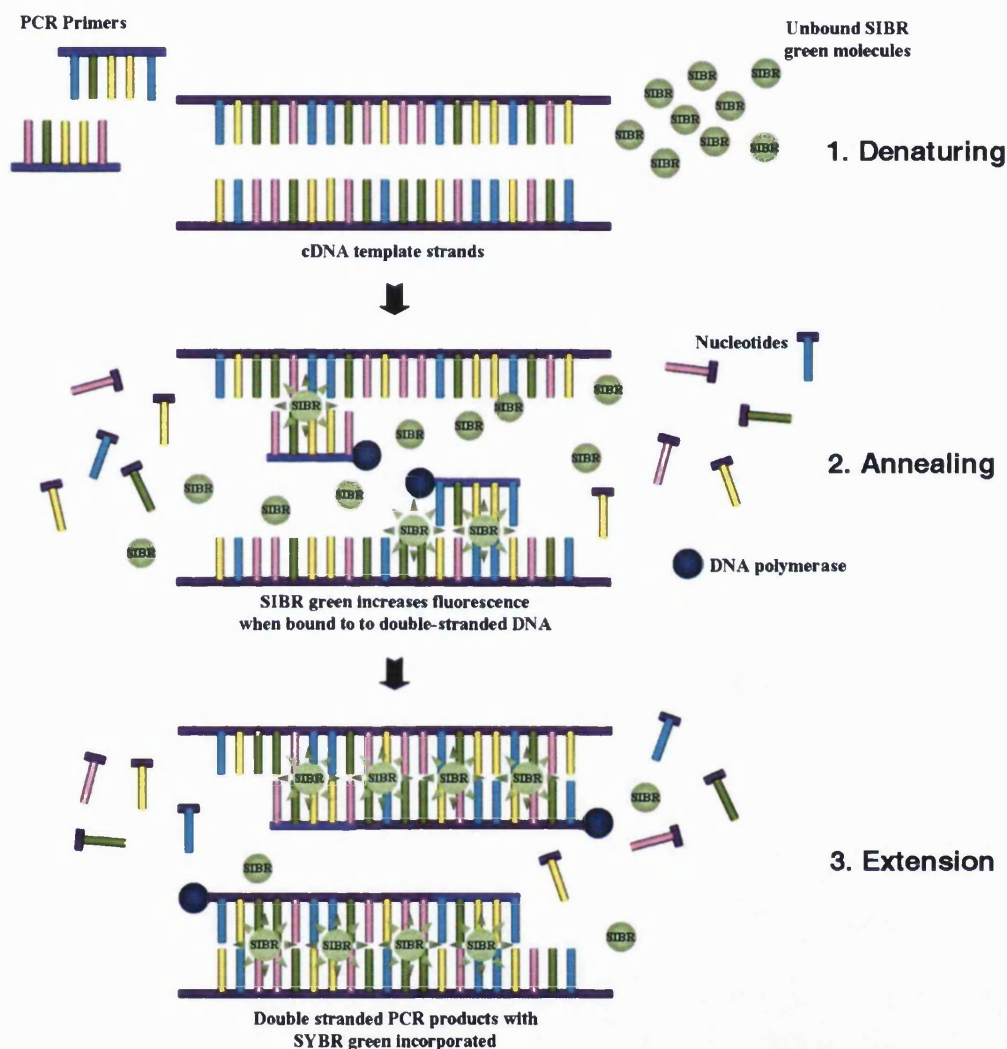


**Fig. 5.1:** Typical sequence of procedures used for microarray analysis. Bacteria are grown in different environmental conditions which may induce alterations in gene expression (1). RNA is extracted from both samples (2) and reverse transcribed to cDNA (3a). Each sample is then labelled with a fluorescent dye (3b). The labelled products are hybridised to a DNA microarray (4) and gene expression levels compared (5).

If a particular gene is highly expressed at the time samples are collected, it produces many RNA molecules, and the respective cDNA hybridises to the microarray slide to give a very strong signal from the corresponding fluorescent dye. This signal is detected and analysed with specialised software and gene expression in the sample can then be compared with the control (Schena, 1999; Baldi and Hatfield, 2002).

DNA microarrays provide a large amount of information which can be very complex to analyse. It is thus typically recommended to validate the data obtained by microarrays with a quantitative method such as qRT-PCR. This is a highly sensitive technique for the detection and quantification of RNA that also enables analysis of gene expression at any given time in a cell culture.

For qRT-PCR, cDNA copies of transcribed RNA molecules are produced by reverse transcriptase using oligonucleotide primers. The cDNA product is then used as a template in a modified PCR. DNA is amplified using specific primers for the gene one wishes to study, and is quantified as it accumulates in the reaction following each amplification cycle. Quantitative detection of gene expression is possible using a variety of methods. A common and simple detection method uses the fluorescent dye SYBR green (Fig. 5.2). This fluorophore intercalates into double-stranded DNA, enabling detection of PCR products as they accumulate during PCR cycles in qRT-PCR reactions. Detection is monitored by measuring the increase in fluorescence intensity; this is proportional to the quantity of PCR product accumulated throughout the procedure. Results can be displayed as amplification plots reflecting changes in fluorescence during cycling (Dieffenbach and Dveksler, 2003).



**Fig. 5.2:** Sequential steps during qRT-PCR quantification with SYBR green dye. cDNA molecules are denatured at 95°C in order to separate the two strands (1). The reaction is cooled to 55°C and specific primers for the gene of interest are annealed (2). The temperature is then raised to 72°C, the DNA polymerase extends the DNA from the primers and SYBR green molecules intercalate into the newly formed double-stranded DNA, increasing its fluorescence and enabling quantification (3).

## 5.2 Materials and Methods

### 5.2.1 RNA extraction

RNA was extracted using RNA bacteria protect (Qiagen Ltd., West Sussex, UK) and FastRNA Pro Blue Kit (Q-BIOgene, Cambridge, UK) according to the manufacturers' instructions. After 24 h growth under conditions of NG and LSMMG, the bacterial cultures were centrifuged at 3,000 *g* for 10 min at 4°C. Bacteria were then suspended in 1 ml of RNA protect bacteria reagent and the mixture incubated for 5 min at RT. After centrifugation under the same conditions, the pellet was suspended in 1 ml of RNAPro™ solution and transferred to a blue-cap tube containing Lysing Matrix B (Q-BIOgene, Cambridge, UK). For mechanical disruption of the bacteria, the sample tube was processed in the FastPrep® Instrument (Q-BIOgene, Cambridge, UK) for 40 s at a setting of 6.0 and centrifuged at 14,100 *g* for 5 min at RT. The supernatant (~750 µl) was transferred to a new microcentrifuge tube, care being taken to avoid the transfer of both the pellet and the lysing matrix. The sample was then incubated for 5 min at RT in order to increase the yield of RNA. Chloroform (300 µl) was added to the sample, the mixture agitated for 10 s and the sample incubated for 5 min at RT to permit nucleoprotein dissociation and to increase RNA purity. The tubes were centrifuged at 14,100 *g* for 5 min at RT and the upper phase transferred to a fresh microcentrifuge tube without disturbing the interphase. For precipitation of RNA, 500 µl of cold absolute ethanol were added to the sample, the contents of the tube mixed by inversion 5 times and the sample held at -20°C for at least 30 min. The RNA appeared as a white pellet in the tube. After centrifugation at 14,100 *g* for 15 min at 4°C, the supernatant was removed and the RNA pellet washed with 500 µl of cold 75% ethanol (made with DEPC water); the solvent was then removed by centrifugation and the residue air dried for 5 min at RT. Finally, the RNA was dissolved in 100 µl of DEPC water and incubated for 5 min at RT to facilitate RNA dissolution.

### 5.2.2 Purification of total RNA and DNase digestion

For microarray analysis, extracted RNA was further purified using RNeasy mini kit with on-column DNase digestion (Qiagen Ltd., West Sussex, UK) according to the manufacturer's instructions. The RNA sample (100 µl) was diluted with 350 µl of Buffer RLT followed by addition of 250 µl of ethanol (96–100%) and the sample mixed well using a pipette. The sample (~700 µl) was transferred to an RNeasy mini column positioned in a 2 ml collection tube, centrifuged for 15 s at 14,100 g and the eluant discarded. The RNeasy spin column membrane was washed with 350 µl of Buffer RW1, centrifuged for 15 s at 14,100 g and the eluant again discarded. DNase digestion was performed by adding 80 µl of DNase I incubation mix to the RNeasy spin column membrane followed by incubation at RT for 15 min. The RNeasy spin column membrane was washed with 350 µl of Buffer RW1, centrifuged for 15 s at 14,100 g and the eluant discarded. Two further washes of the RNeasy column were performed with 500 µl Buffer RPE (in ethanol). To elute the RNA, 30 µl of RNase-free water was added to the RNeasy silica gel membrane. The RNA concentration was determined with a nanodrop (Thermo Fisher Scientific Inc., Wilmington, USA) and the purity assessed using a bioanalyser (Agilent Technologies, Cheshire, UK). The RNA solutions were stored at -80°C until required.

For qRT-PCR, extracted RNA was digested with Turbo DNase (Ambion Inc., Austin, TX) according to the manufacturer's instructions. Briefly, samples were diluted to 10 µg of RNA per 50 µl with DEPC-treated water prior to addition of the DNase incubation mix. After incubation at 37°C for 20–30 min, DNase Inactivation Reagent was added and the mixture incubated for 2 min at RT. The RNA solution was recovered by centrifugation at 14,100 g for 1.5 min and transferred to a fresh tube. The RNA concentration was determined using a Perkin Elmer Lambda 25 spectrophotometre and the purity was determined by RNA native agarose gel electrophoresis. The RNA solutions were stored at -80°C until required.

RNA native agarose gel electrophoresis can be used to assess, in semi-quantitative fashion, the integrity of total RNA samples by visualisation of ribosomal RNAs. RNA samples were diluted 1:6 by mixing 2 µl of RNA with 2 µl of 6x gel loading dye (Promega, Madison, USA) and nuclease-free water to a total volume of 12 µl.

RNA samples were separated by electrophoresis through a 1% agarose gel prepared in Tris-acetate-EDTA (TAE) buffer (Bio-Rad, Hertfordshire, UK) and containing 0.5 µg/ml of ethidium bromide (Sigma-Aldrich, Dorset, UK). Gels were run at 100 V until the dyes separated. Binding of the ethidium bromide to the RNA allowed visualisation of the separated RNA molecules when the gel was exposed to ultraviolet (UV) light.

### 5.2.3 Microarray analysis

RNA samples (5 µg) from bacteria grown under NG and LSMMG were reverse transcribed to cDNA with SuperScript II Reverse Transcriptase (Invitrogen Ltd., Paisley, UK) and fluorescently labelled with Cy3 / Cy5 (Amersham Biosciences, Buckinghamshire, UK). The excess Cy3 and Cy5 dCTP was removed from labelled cDNA samples using a MinElute Reaction Cleanup Kit (Qiagen Ltd., West Sussex, UK). The paired samples were pooled and hybridised overnight to a BµG@S SAv1.1.0 *S. aureus* microarray that has been described previously (Witney *et al.*, 2005), followed by washing and scanning. Image acquisition was attained using an Affymetrix 428 scanner and Cy3/Cy5 fluorescence signal data was measured using ImaGene 5.5 (BioDiscovery, California, USA) software.

For statistical analysis, each *S. aureus* isolate was grown in three independent HARV cultures. Six microarray slides per isolate (two for each HARV culture) were hybridised and analysed (n=6). Arrays were performed both forward- and reverse-labelled to account for dye hybridisation differences: NG samples were first labelled with Cy3 and LSMMG samples with Cy5; the experiment was then repeated swapping the dyes, with NG samples labelled with Cy5 and LSMMG samples with Cy3. Replicate data was combined to calculate mean expression ratios and mean fold changes. Normalisation and analysis was performed using GeneSpring 7.3 (Agilent Technologies, Cheshire, UK) to identify genes with a greater than twofold change in gene expression, using Student's *t*-test ( $p < 0.05$ ) with Benjamini and Hochberg false discovery rate correction.

## 5.2.4 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Oligonucleotide primers (Eurofins MWG operon, London, UK) were designed with Clone manager 6.0 (Sci-Ed Software central, Cary, USA) and are listed in table 5.1.

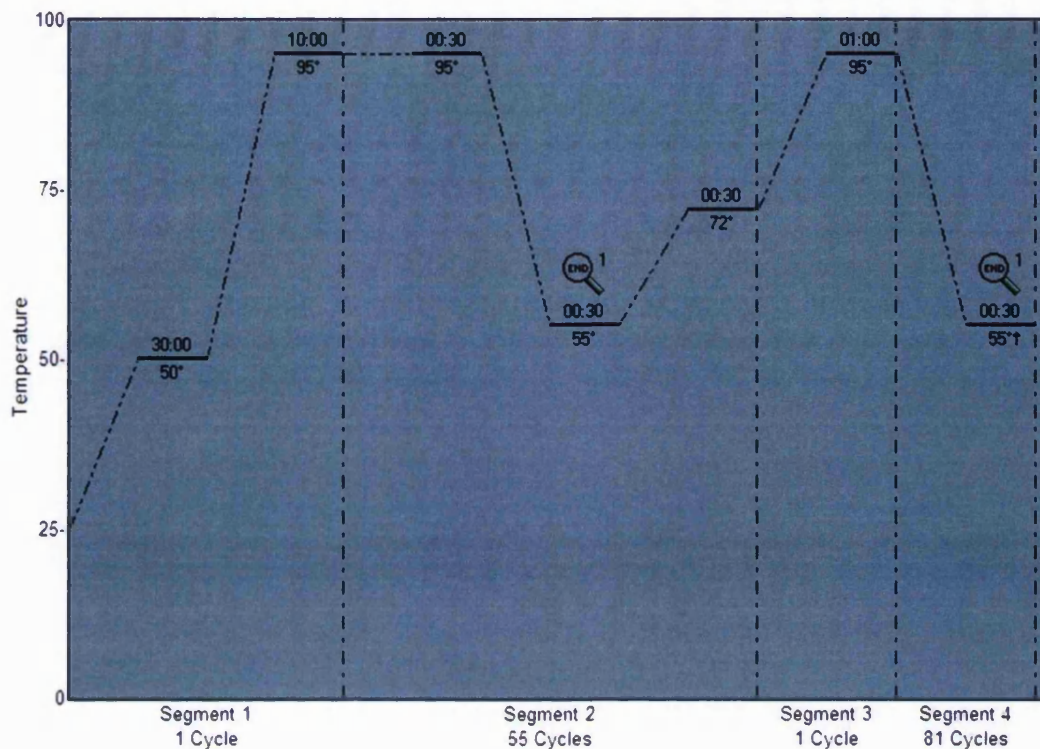
**Table 5.1:** Oligonucleotide primers designed for qRT-PCR.

Gene	Forward primer	Reverse primer
<b>16s rRNA</b>	ACT GAG ACA CGG TCC AGA CT	CGG ATA ACG CTT GCC ACC TA
<b><i>hla</i></b>	CTG TCG CTA ATG CCG CAG AT	AAG GCT GAA GGC CAG GCT AA
<b><i>saeRS</i></b>	TGC CAA CCA TTG GAG CAA CG	GCC AAT ACC TCC ATC GCT AA
<b><i>gapA2</i></b>	GCT AGT TAT CCA CCC GAA AC	GGC AAG CTT TCA GGA TTA CG
<b><i>clpL</i></b>	CTT AAC CGC TTC AAC GGC AT	GCG TGC GCC TAA TTC TTC AT
<b><i>dnaK</i></b>	CCT ACA CGC CAA GCA ATG AA	GTG ATA ACG CCA CCT TGG AT
<b><i>sarA</i></b>	ACA TGG CAA TTA CAA AAA TCA ATG AT	TCT TTC TCT TTG TTT TCG CTG ATG
<b><i>sigB</i></b>	CGA TGA ACT AAC CGC TGA AT	GCG TAA CAG TTG AAC CAT CT

For primer design, a few considerations were taken into account: primers were no more than 18 to 30 bp of length, with a guanine-cytosine (GC) content of 45-55% and a melting temperature between 55-60°C. The predicted PCR product was no more than 100-200 bp in length (Dieffenbach and Dveksler, 2003).

qRT-PCR was performed using Brilliant SYBR Green RT-QPCR Master Mix Kit, one step (Stratagene Inc., La Jolla, CA) according to manufacturer's instructions. The reactions were run in an Mx3000P thermocycler (Stratagene Inc., La Jolla, CA) using the thermal profile setup shown in figure 5.3.





**Fig. 5.3:** Thermal profile used for qRT-PCR.

During the first stage of the qRT-PCR process, RNA molecules were copied to cDNA by reverse transcriptase using oligonucleotide primers at 50°C. The cDNA was denatured at 95°C in order that the two strands were able to separate. The sample was cooled to 55°C and specific primers for the gene of interest were annealed. The temperature was then raised to 72°C and the heat-stable Taq DNA polymerase used to extend the DNA from the primers. DNA was amplified and quantified using SYBR green during accumulation in the reaction after each amplification cycle.

For statistical analysis, each *S. aureus* isolate was grown in three independent HARV cultures. Six qRT-PCR experiments per isolate (two for each HARV culture) were performed (n=6). Duplicates were included in every qRT-PCR experiment. Replicate data was combined to calculate mean expression ratios. Normalisation and analysis were performed using the comparative Ct method to identify genes with greater than twofold change in gene expression, using Student's *t*-test ( $p < 0.05$ ).

## 5.3 Results

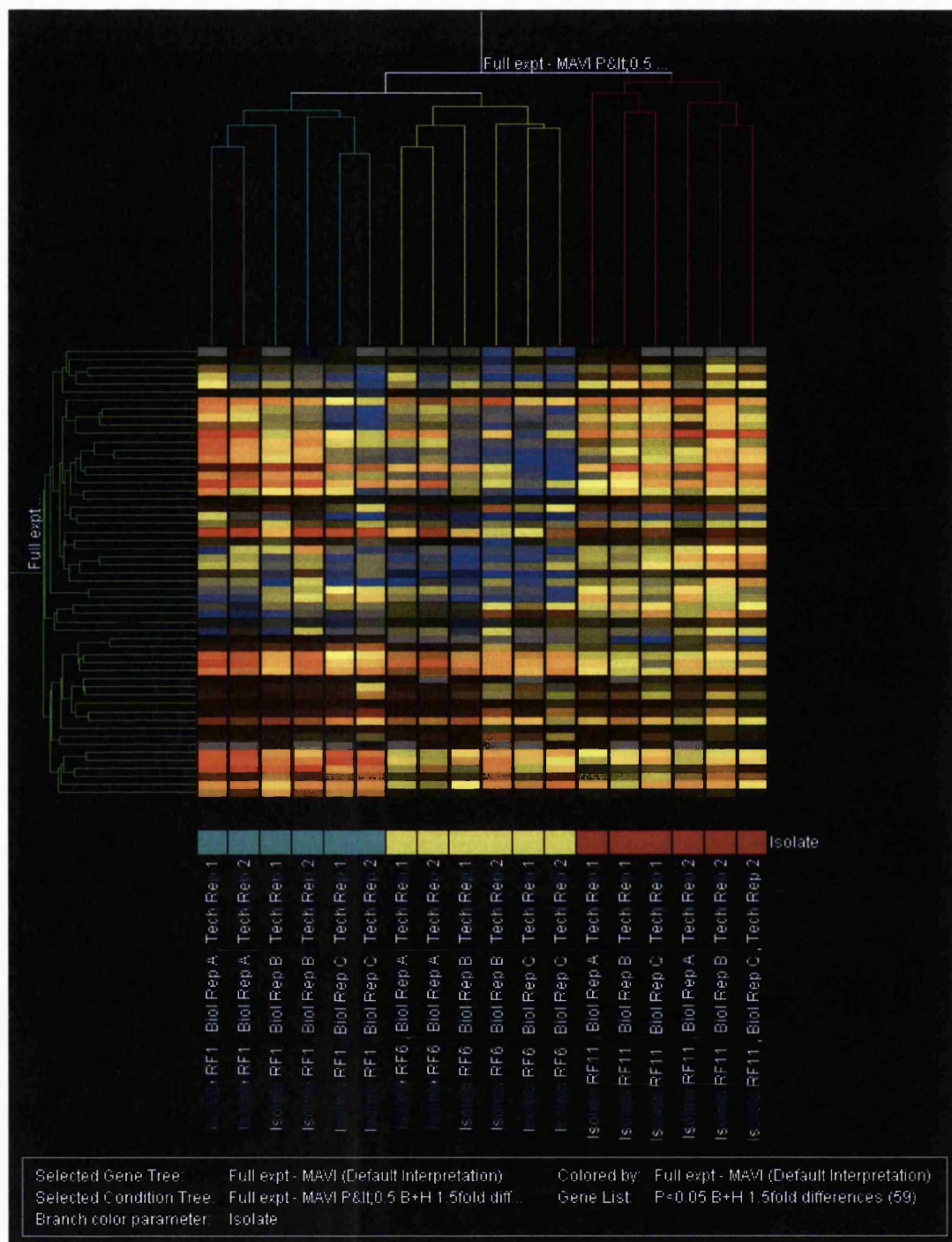
As described in chapter four, LSMMG modulates protein secretion by the *S. aureus* isolates used in this study, providing a strong indication of alteration at the level of gene transcription. I have, therefore, undertaken DNA microarrays in order to elucidate the global transcriptional response of *S. aureus* under LSMMG. Validation of microarray data was performed by qRT-PCR.

### 5.3.1 Microarrays

Gene expression by the three *S. aureus* isolates RF1, RF6 and RF11 under NG and LSMMG was studied and compared. GeneSpring enabled data representation in colour-coded images for the expression of each gene, during growth under NG and LSMMG conditions, based on fluorescence intensity ratio. The yellow code represents genes that are expressed during both NG and LSMMG, whereas the blue code represents genes that have been down-regulated following growth under LSMMG and the red genes up-regulated under LSMMG. Poor-quality data or with signals less than twofold above background appear in gray code.

GeneSpring also enabled data display in different formats facilitating analysis and interpretation of gene expression levels between NG and LSMMG. In a condition tree (Fig. 5.4), samples were grouped together based on the similarity of their gene expression profiles, so that samples with similar gene expression profiles were grouped closer to each other in the tree. Each column represents one replicate of each *S. aureus* isolate studied. The genes are represented by the coloured rectangles (using the colour code previously described) to the right of the tree structure, displayed in green. Similarly coloured genes tend to cluster together.

Condition trees can thus be used to perform quality control on samples since replicates should group closer. Deviation from this assumption could be due to poor quality samples or true biological variation. Figure 5.4 shows replicate grouping and highlights significant variability between the three *S. aureus* isolates studied, visible through variations in the colour of the genes expressed.

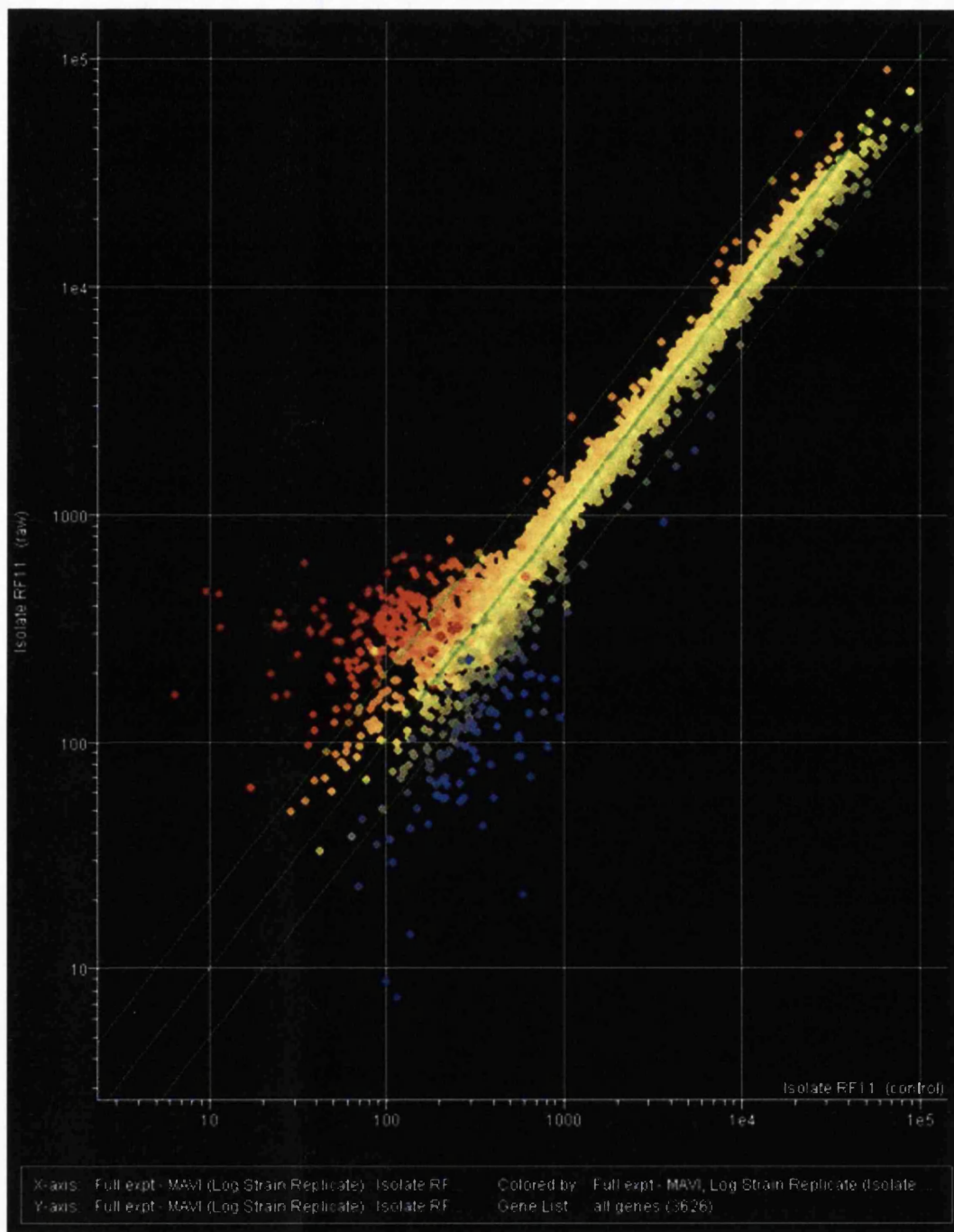


**Fig. 5.4:** Condition tree of *S. aureus* isolates RF1, RF6 and RF11 grown under NG and LSMMG. Samples are grouped according to the degree of similarity of their expression profiles so that samples with similar gene expression profiles are grouped closer to each other in the tree. Each column represents one replicate NG- and LSMMG-grown *S. aureus* isolates RF1 (■), RF6 (■) and RF11 (■).

A scatter plot view is useful in comparing global gene expression between two samples or two experimental conditions. In the present study, a scatter plot (Fig. 5.5) was created to compare global gene expression between NG- ( $x$  axis) and LSMMG- ( $y$  axis) grown cells.

The scatter plot allows qualitative identification of genes whose expression is significantly different between NG and LSMMG. Each dot represents one gene and follows the colour code previously described. Genes expressed in identical fashion under NG and LSMMG fall on the central green line between the  $x$  and  $y$  axes and genes whose expression was altered by factor of twofold or less, represented in figure 5.5 as yellow spots, fall within the outer green lines and are not considered significantly altered by a change in the gravitational field. Values falling above the twofold line, in red, are those considered to be up-regulated under the influence of LSMMG and those below the twofold line, in blue, are considered down-regulated. Genes identified with very low expression intensity (with very low  $x$  and  $y$  values) are below the detection limit of the software detection and are usually not analysed.

*S. aureus* RF1, RF6 and RF11 genes found to be up- or down-regulated by a factor of at least twofold when grown under LSMMG and compared to NG are listed in table 5.2. Results are shown as mean fold change (LSMMG/NG). Genes with expression ratios  $< 0.5$  were considered down-regulated and  $> 2$  up-regulated.



**Fig. 5.5:** Comparative gene expression of *S. aureus* isolate RF11 under NG and LSMMG. Similar results were observed for *S. aureus* isolates RF1 and RF6. This scatter plot allows qualitative identification of genes whose expression is significantly different between NG and LSMMG. Each dot represents one gene. Genes represented in yellow are expressed in identical fashion under NG and LSMMG and fall within the twofold lines between the  $x$  and  $y$  axes. Values falling above the twofold line, in red, are those considered to be up-regulated and those below the twofold line, in blue, are considered down-regulated under the influence of LSMMG.



**Table 5.2:** *S. aureus* RF1, RF6 and RF11 genes up- and down-regulated following growth under LSMMG, compared to NG. Results are shown as mean fold changes for LSMMG-grown cells, compared to NG-grown cells (LSMMG/NG). \* $p < 0.05$ .

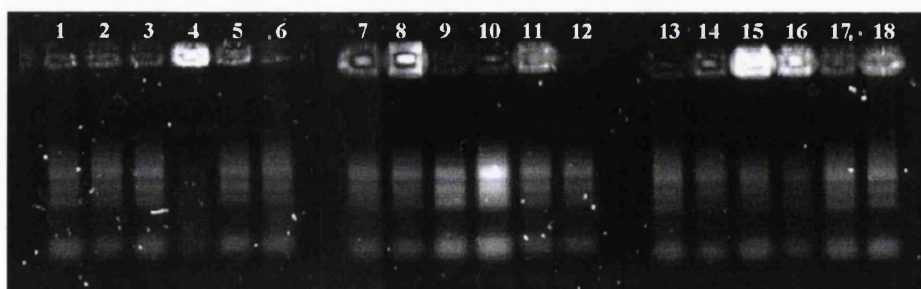
<i>S. aureus</i> isolate	Genes	Description	Mean fold change*
RF1	SAR1545	hypothetical phage protein	5.52
	<i>gap2</i>	glyceraldehyde 3-phosphate dehydrogenase 2	4.95
	SAR1150	antibacterial protein	2.86
	SAR1370	hypothetical protein	2.83
	SAR2264	conserved hypothetical protein	2.80
	SAV0855	hypothetical protein	2.79
	<i>clpL</i>	ATP-dependent protease ATP-binding subunit ClpL	2.51
	<i>hutU</i>	urocanate hydratase	2.49
	<i>narT</i>	nitrite transport protein	2.48
	<i>blaZ</i>	beta-lactamase precursor	2.44
	SAR2096	putative anti repressor	2.34
	COL2004	-	2.33
	SAR0624	putative esterase	2.11
	SAR1459	conserved hypothetical protein	2.10
	SAR1458	conserved hypothetical protein	2.09
	SAV0912	hypothetical protein	-3.75
	<i>nuc</i>	thermonuclease precursor	-2.98
	SAR2001	staphopain protease	-2.85
	SAR0189	putative thiamine pyrophosphate enzyme	-2.75
	SAR0435	Exotoxin	-2.28
	<i>vraX</i>	hypothetical protein	-2.23
	SAR0390	putative lipoprotein	-2.13
	<i>hsdS</i>	-	-2.09
	SAR1266	conserved hypothetical protein	-2.09
	SAR0583	hypothetical protein	-2.06
RF6	<i>gapA2</i>	glyceraldehyde 3-phosphate dehydrogenase 2	2.94
	COL3568	-	2.14
	<i>hla</i>	alpha-haemolysin precursor (pseudogene)	-5.25
	<i>vraX</i>	hypothetical protein	-4.53
	SAR1651	putative membrane protein	-2.56
	SAR0279	conserved hypothetical protein	-2.39
	<i>saeS</i>	histidine kinase protein	-2.37
	<i>saeR</i>	response regulator protein	-2.34
	<i>fadD</i>	putative acyl-CoA dehydrogenase	-2.31
	<i>geh</i>	lipase precursor	-2.11
	SAR0760	putative membrane protein	-2.07
	SAN2285	putative cell wall-anchored protein	-2.01
RF11	<i>clpL</i>	ATP-dependent protease ATP-binding subunit ClpL	2.43
	SAR0217	formate acetyltransferase	2.20
	<i>dnaK</i>	chaperone protein	-3.13
	<i>vraX</i>	hypothetical protein	-2.41

Statistically significant changes were found with 25 genes in the case of isolate RF1, 12 genes with isolate RF6 and 4 genes with isolate RF11. The mapping of these genes on the *S. aureus* genome showed that they were distributed along the entire chromosome and it was not possible to identify clusters on the same operon. Although there was significant variability when comparing results between the three isolates, glyceraldehyde 3-phosphate dehydrogenase 2 (*gapA2*) was up-regulated in both RF1 and RF6 and putative ATP-dependent protease ATP-binding subunit ClpL (*clpL*) was up-regulated in both RF1 and RF11. One gene, *vraX*, described as encoding a hypothetical protein, was found to be consistently down-regulated in all three isolates. In fact, other hypothetical proteins were found to be up- or down-regulated under LSMMG but their function has not been yet determined.

Particularly interesting results were found for isolate RF6. The microarray analysis indicates a 2.5-fold down-regulation on the expression of the regulatory system *saeRS*. In addition, the gene which encodes the production of the major virulence factor  $\alpha$ -toxin, *hla*, was also found to be fivefold down-regulated in this isolate.

### 5.3.2 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

To validate the data obtained by DNA microarrays, qRT-PCR was performed. RNA was extracted from NG- and LSMMG-samples and quality was assessed by RNA native agarose gel electrophoresis (Fig. 5.6).



**Fig. 5.6:** RNA quality assessment by RNA native agarose gel electrophoresis of *S. aureus* isolates RF1, RF6 and RF11 grown in three independent HARV cultures. RF1 grown under NG (1, 3, 5). RF1 grown under LSMMG (2, 4, 6). RF6 grown under NG (7, 9, 11). RF6 grown under LSMMG (8, 10, 12). RF11 grown under NG (13, 15, 17). RF11 grown under LSMMG (14, 16, 18).

qRT-PCR amplification is an exponential reaction reflected by changes in SYBR green fluorescence during cycling, creating a sigmoid amplification plot with a final plateau indicative of the termination of the reaction (Fig. 5.8 and 5.9). Quantification of qRT-PCR data can be achieved with the comparative threshold (Ct) method, which is also referred to as the  $2^{-\Delta\Delta Ct}$  method. This method involves the comparison of the Ct values of the NG control with those of the LSMMG sample normalised against the 16s standard. The threshold cycle (Ct) value corresponds to the cycle at which the sample fluorescence is determined to be statistically significant above background and is inversely proportional to the log of the initial copy number. The threshold cycle has a default value assigned by the software programme (Dieffenbach and Dveksler, 2003). For the  $\Delta\Delta Ct$  calculation, the following formula was used:

$$\Delta\Delta Ct = \Delta Ct_{LSMMG} - \Delta Ct_{NG}$$

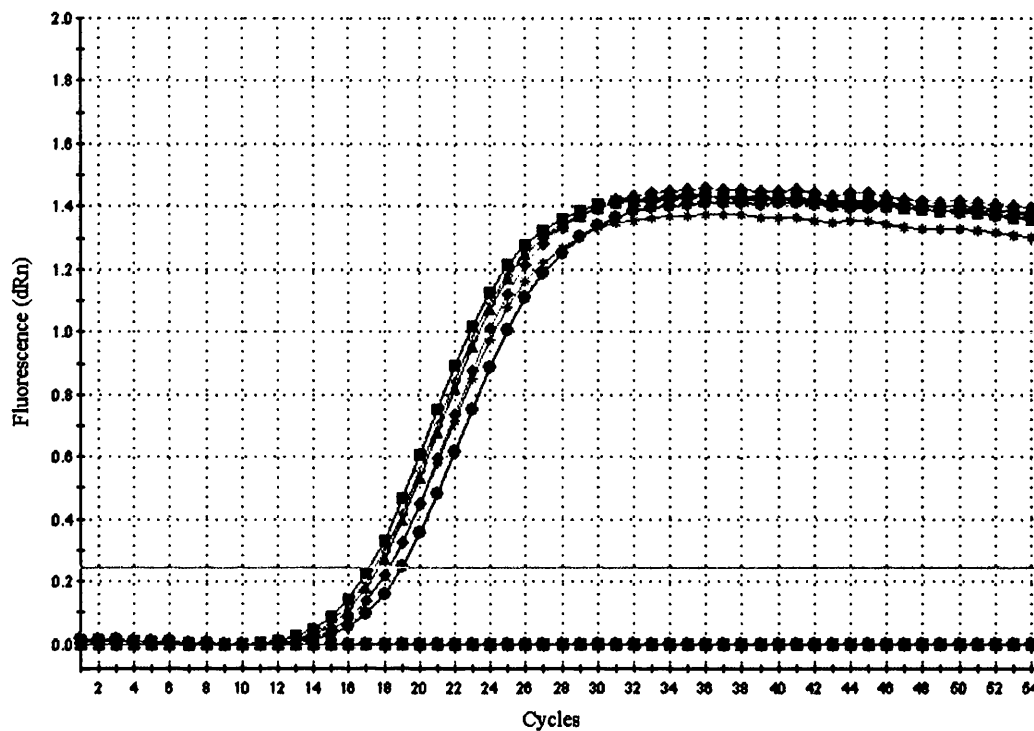
where  $\Delta Ct_{LSMMG}$  is the Ct value for the LSMMG sample normalised to the 16s standard and  $\Delta Ct_{NG}$  is the Ct value for the NG control normalised to the 16s standard. The comparative expression level is then given by  $2^{-\Delta\Delta Ct}$ . The more template that is initially present in the PCR reaction, the fewer the number of cycles it will take to reach the point where the fluorescence signal is detectable above background and, accordingly, the lower the Ct value will be. The Ct values of the samples can be obtained by software analysis as indicated in figure 5.7.

The 16s rRNA was selected as an internal standard for qRT-PCR reactions. This housekeeping gene is not influenced by the experimental conditions and is therefore expressed to a comparable level in all cells, controls and samples, enabling final data normalisation.



All	1	2	3	4	5	6	7	8	9	10	11	12
A	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator
	Ref 1 15.46 A	Ref 3 14.35 C	Ref 5 14.94 E	Ref 13 29.15 A	Ref 15 28.06 C	Ref 17 30.49 E	Ref 25 29.58 A	Ref 27 27.88 C	Ref 29 30.78 E	Ref 37 39.37 A	Ref 39 36.83 C	Ref 41 36.47 E
B	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator
	Ref 1 15.46 A	Ref 3 14.35 C	Ref 5 14.94 E	Ref 13 29.15 A	Ref 15 28.06 C	Ref 17 30.49 E	Ref 25 29.58 A	Ref 27 27.88 C	Ref 29 30.78 E	Ref 37 39.37 A	Ref 39 36.83 C	Ref 41 36.47 E
C	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
	Ref 2 13.77 B	Ref 4 14.85 D	Ref 6 14.11 F	Ref 14 31.81 B	Ref 16 32.21 D	Ref 18 30.50 F	Ref 26 26.96 B	Ref 28 28.74 D	Ref 30 29.07 F	Ref 38 33.08 B	Ref 40 34.71 D	Ref 42 35.22 F
D	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
	Ref 2 13.77 B	Ref 4 14.85 D	Ref 6 14.11 F	Ref 14 31.81 B	Ref 16 32.21 D	Ref 18 30.50 F	Ref 26 26.96 B	Ref 28 28.74 D	Ref 30 29.07 F	Ref 38 33.08 B	Ref 40 34.71 D	Ref 42 35.22 F
E	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator
	Ref 7 37.14 A	Ref 9 33.70 C	Ref 11 30.80 E	Ref 19 38.40 A	Ref 21 33.30 C	Ref 23 35.09 E	Ref 31 32.11 A	Ref 33 30.51 C	Ref 35 31.61 E	Ref 43 38.68 A	Ref 45 36.04 C	Ref 47 44.37 E
F	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator	Calibrator
	Ref 7 37.14 A	Ref 9 33.70 C	Ref 11 30.80 E	Ref 19 38.40 A	Ref 21 33.30 C	Ref 23 35.09 E	Ref 31 32.11 A	Ref 33 30.51 C	Ref 35 31.61 E	Ref 43 38.68 A	Ref 45 36.04 C	Ref 47 44.37 E
G	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
	Ref 8 34.33 B	Ref 10 31.35 D	Ref 12 33.38 F	Ref 20 35.48 B	Ref 22 35.40 D	Ref 24 38.13 F	Ref 32 28.07 B	Ref 34 22.81 D	Ref 36 29.44 F	Ref 44 38.54 B	Ref 46 39.11 D	Ref 48 46.00 F
H	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
	Ref 8 34.33 B	Ref 10 31.35 D	Ref 12 33.38 F	Ref 20 35.48 B	Ref 22 35.40 D	Ref 24 38.13 F	Ref 32 28.07 B	Ref 34 22.81 D	Ref 36 29.44 F	Ref 44 38.54 B	Ref 46 39.11 D	Ref 48 46.00 F

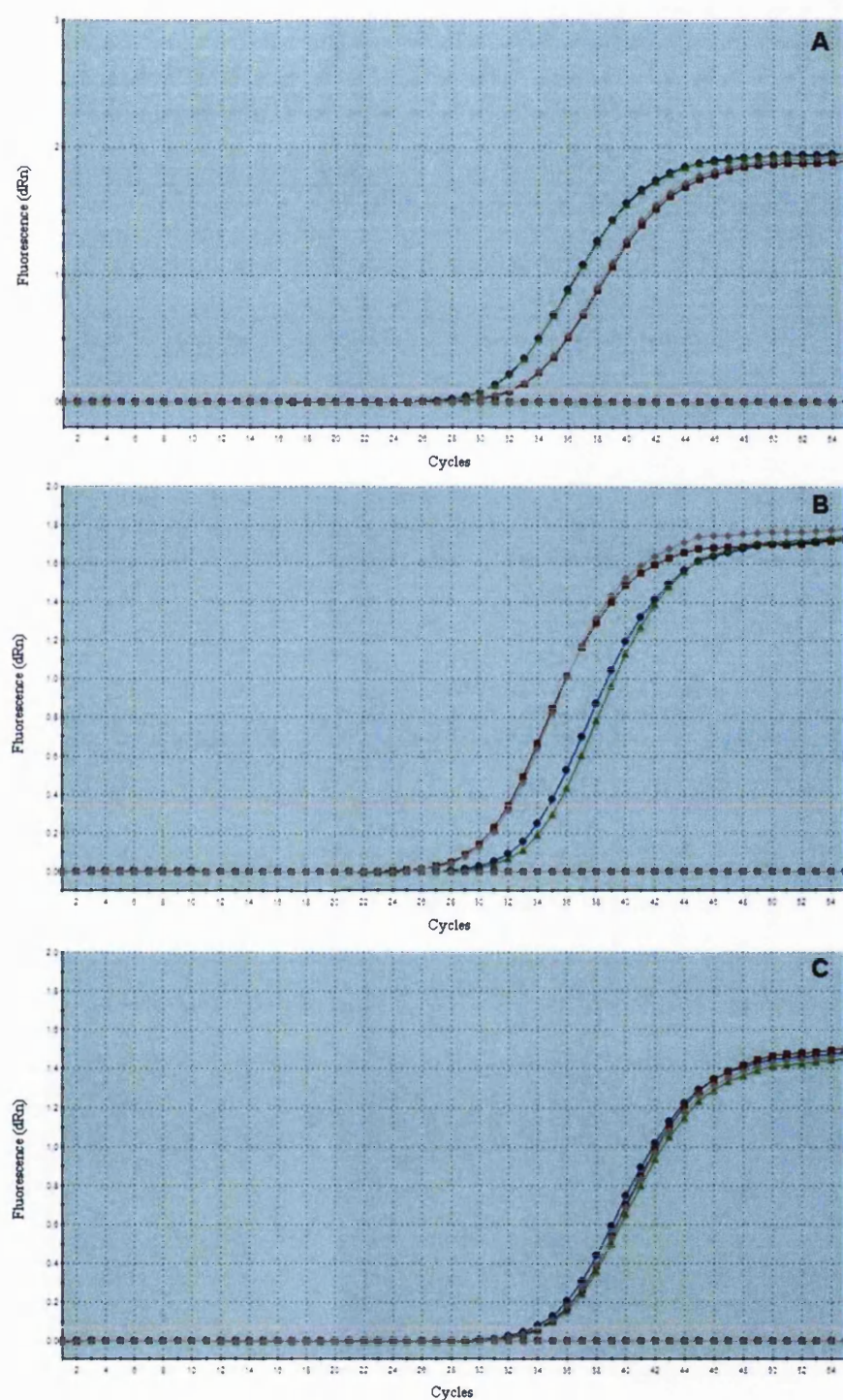
Fig. 5.7: Ct values obtained as qRT-PCR data (as marked by red circle).



**Fig. 5.8:** qRT-PCR amplification plots obtained with specific primers for 16s rRNA. Each colour represents the mean of duplicates performed for each replicate of *S. aureus* isolate. RF1 under NG (●), RF1 under LSMMG (■), RF6 under NG (▲), RF6 under LSMMG (○), RF11 under NG (\*), RF11 under LSMMG (◆).

Figure 5.8 shows qRT-PCR amplification plots, obtained using specific 16s rRNA primers, for all NG and LSMMG replicates of *S. aureus* isolates RF1, RF6 and RF11. Each colour represents the mean value of duplicates performed for each of RF1, RF6 and RF11 replicates, in a single experiment. The figure shows good correlation between isolates and replicates, as amplification was initiated approximately at the same cycle and all the curves have a similar sigmoid shape. Similar Ct values were obtained during this reaction for all samples and replicates, including NG and LSMMG samples, indicating that 16s rRNA represents a good internal standard for gene expression analysis for this particular study.

A similar procedure was adopted to analyse the levels of expression of the genes found to be altered under LSMMG conditions, as identified by microarray analysis. Figure 5.9 shows the qRT-PCR amplification plots obtained using a specific primer for *hla*, the gene encoding  $\alpha$ -toxin, in *S. aureus* isolates RF1, RF6 and RF11.



**Fig. 5.9:** Amplification plots obtained from comparison of *hla* expression in NG and LSMMG, showing a twofold difference with isolate RF1 (A), a sevenfold difference with isolate RF6 (B) and no significant difference for isolate RF11 (C).

A comparison of *hla* expression under NG and LSMMG using the Ct method demonstrated a twofold reduction for isolate RF1 under reduced gravity conditions (A), a sevenfold difference for this gene in RF6 (B) but no significant difference in the case of isolate RF11 (C). These trends were confirmed since differences are semi-quantitatively observed comparing the amplification plots obtained: the larger the distance between NG and LSMMG amplification plots, the higher the difference observed in *hla* expression levels. In all figures, it is clear that there is good correlation between replicates; in figure 5.9 C, in particular, the sigmoid curves overlap indicating no differences between NG and LSMMG. In general, LSMMG down-regulated *hla* transcription in *S. aureus*.

The same procedure was adopted for the other relevant genes in study. The qRT-PCR values were compared with those obtained by microarray analysis; the genes that were found to be up- or down-regulated under LSMMG conditions are listed in table 5.3 together with mean fold changes.

A good correlation between DNA microarrays and qRT-PCR was obtained and the microarray data was, therefore, considered to be validated. The results obtained using qRT-PCR methods were more consistent than microarrays. Accordingly, *hla* and *saeRS* expression was found to be significantly down-regulated in strains RF1 and RF6 and *gapA2* and *clpL* significantly up-regulated in all three isolates when they were grown under LSMMG. No differences were found in *sarA* and *sigB* expression.

Because SYBR green can bind to double-stranded DNA regardless of sequence, it has the capacity to bind to non-specific reaction products such as contaminants, mispriming (PCR products resulting from annealing of primers to complementary or partially complementary sequences on non-target DNAs) or primer-dimer artifacts (self-annealing of the primers, thereby creating small templates for PCR amplification), generating false positive signals (Dieffenbach and Dveksler, 2003).

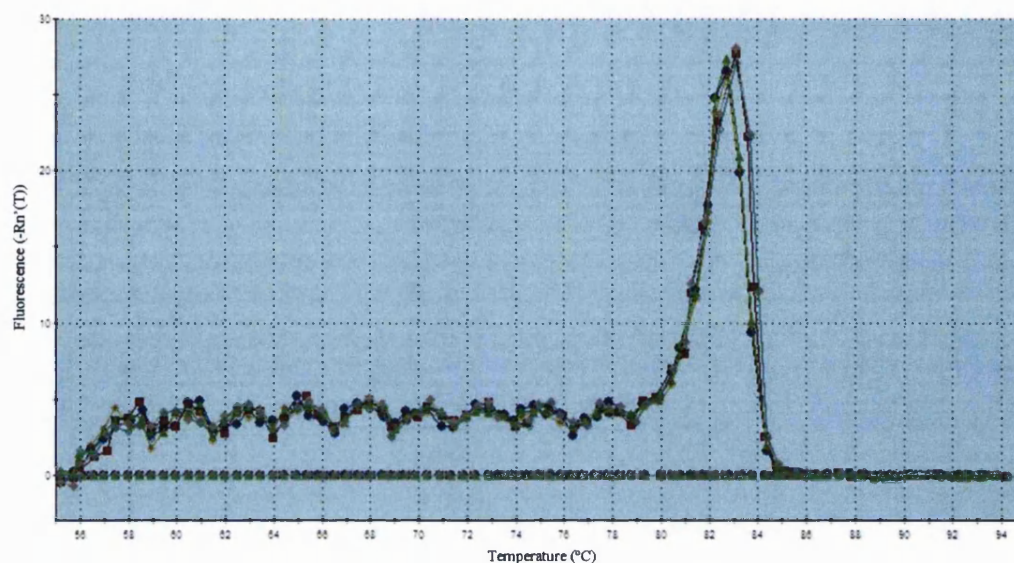


**Table 5.3:** Comparison of up- and down-regulated genes of *S. aureus* isolates RF1, RF6 and RF11 grown under LSMMG and compared to NG-grown cells. Results are shown as mean fold changes for LSMMG-grown cells, compared to NG-grown cells (LSMMG/NG).

\* $p < 0.05$ , \*\* $p < 0.01$ .

Gene	Isolate	Mean fold change	
		Microarrays	qRT-PCR
<i>hla</i>	RF1	-1.35	-2.17**
	RF6	-5.26	-6.67*
	RF11	1.18	1.15
<i>saeRS</i>	RF1	1.03	-2.94*
	RF6	-2.38	-5.56*
	RF11	-1.05	-1.02
<i>gapA2</i>	RF1	4.95	7.66**
	RF6	2.94	9.49**
	RF11	1.34	6.20**
<i>clpL</i>	RF1	2.52	2.43*
	RF6	1.23	2.73*
	RF11	2.43	4.40*
<i>dnaK</i>	RF1	1.39	-1.37
	RF6	1.41	1.09
	RF11	-3.13	-3.57*
<i>sarA</i>	RF1	-	-1.33*
	RF6	-	1.38
	RF11	-	1.76
<i>sigB</i>	RF1	-	-1.47
	RF6	-	-1.59
	RF11	-	1.12

An optimised reaction procedure is an essential prerequisite for the acquisition of meaningful data. One way to ensure the quality of PCR products is to check their melting point by performing a melting curve at the end of the amplification process. The temperature is raised by a fraction of a degree and the change in fluorescence is measured. When the temperature reaches the melting temperature ( $T_m$ ) of the product, the two strands of DNA separate and fluorescence rapidly decreases. The  $T_m$  is dependent on the composition and length of the DNA double helix (Dieffenbach and Dveksler, 2003). Therefore, all PCR products for a particular set of primers should have the same melting temperature and only one peak should be found in the dissociation curve, such as that shown in the example in figure 5.10.



**Fig. 5.10:** Dissociation curve ( $T_m$ ) for determination of the quality of PCR products produced during the qRT-PCR reaction.

The data indicates that PCR products produced during the qRT-PCR reaction have the same melting temperature and that the quality of these data is sufficient to facilitate meaningful interpretation.

## 5.4 Discussion

The complete genome sequencing and annotation of eleven human *S. aureus* isolates (Kuroda *et al.*, 2001; Baba *et al.*, 2002; Holden *et al.*, 2004; Gill *et al.*, 2005; Diep *et al.*, 2006; Gillaspay *et al.*, 2006; Mwangi *et al.*, 2007; Baba *et al.*, 2008) and the design, printing and validation of multi-strain *S. aureus* microarrays comprised of PCR products for all previously identified genes (Witney *et al.*, 2005), have provided the opportunity for a comprehensive study of the opportunistic pathogen *S. aureus*.

In the current study, DNA microarray analysis has facilitated the characterisation of gene transcription and regulation by *S. aureus* cells grown under NG and LSMMG and has led to the identification of up- and down-regulation of gene expression in

three clinical isolates. The *S. aureus* microarray used for this study has been described previously (Witney *et al.*, 2005) and contains 3,623 PCR products representing all predicted open reading frames identified in seven whole-genome sequencing projects, incorporating sequences from the following *S. aureus* strains: Mu50, N315, MW2, MSSA476, MRSA252, COL and NCTC8325. Comparisons of the genomes of these seven strains have indicated that approximately 20% of the genes are variable; for this reason, more than 1500 of the genes present in this multistrain DNA microarray are variable, allowing a global study of *S. aureus* gene regulation (Witney *et al.*, 2005).

In agreement with previously published data (Dufour *et al.*, 2002; Gomes *et al.*, 2005; Rogasch *et al.*, 2006), the transcriptome analysis reported here indicates a degree of variability between the three *S. aureus* isolates investigated. The study of strain-specific differences in gene regulation and virulence is extremely important as they can have a significant but variable impact on bacterial pathogenicity.

The effect of LSMMG on *S. aureus* gene expression was most profound in the case of isolate RF6. Microarray analysis indicated a 2.5-fold down-regulation of the expression of the regulatory system *saeRS* under LSMMG conditions. In addition, the gene which encodes the production of the major virulence factor  $\alpha$ -toxin, *hla*, was also found to be down-regulated fivefold. These results concord with the findings that LSMMG significantly reduced global protein secretion, particularly of haemolysins such as  $\alpha$ -toxin, as described in chapters three and four.

The *sae* locus was first described in 1994 (Giraud *et al.*, 1994) and was identified as one of the major global regulators of *S. aureus* (Giraud *et al.*, 1999). The locus contains four ORFs; two of these encode the two-component *saeRS*, where *saeR* is a response regulator and *saeS* a histidine protein kinase (Giraud *et al.*, 1999; Novick and Jiang, 2003; Steinhuber *et al.*, 2003).

Giraud and co-workers (Giraud *et al.*, 1994; Giraud *et al.*, 1997) have demonstrated that *sae* regulates the expression of virulence genes in *S. aureus*. Levels of  $\alpha$ -toxin secretion, as well as levels of other virulence factors, were

drastically reduced in a *sae*- mutant compared to the parental strain (Giraudo *et al.*, 1994). Northern blot analysis (Giraudo *et al.*, 1997), DNA microarray and qRT-PCR studies (Liang *et al.*, 2006) later confirmed that *hla* was not transcribed in the absence of the regulatory locus.

Interestingly, *in vivo* assays have demonstrated that *sae* is required for *S. aureus* virulence due to its capacity to regulate the expression of virulence factors such as *hla* (Rampone *et al.*, 1996; Goerke *et al.*, 2001; Benton *et al.*, 2004; Goerke *et al.*, 2005; Liang *et al.*, 2006). Such findings are compatible with the microarray data obtained in this study for *S. aureus* isolate RF6 grown under LSMMG, where expression levels of *saeRS* and *hla* were highly reduced.

In addition to *sae*, other key global virulence regulatory systems have been described in *S. aureus*. Probably the best characterised are the accessory gene regulator, *agr* (Recsei *et al.*, 1986), the staphylococcal accessory regulator, *sar* (Cheung *et al.*, 1992) and the alternative sigma factor, *sigB* (Wu *et al.*, 1996). These three loci are considered fundamental to the expression of *S. aureus* virulence genes, such as *hla* (Novick, 2003; Lindsay, 2008), and are associated with virulence regulation by *sae* (Giraudo *et al.*, 1997; Goerke *et al.*, 2001; Novick and Jiang, 2003; Goerke *et al.*, 2005; Liang *et al.*, 2006).

The *agr* locus has been previously characterised in *S. aureus* (Peng *et al.*, 1988; Janzon *et al.*, 1989; Novick *et al.*, 1993) and shown to regulate the transcription of several staphylococcal virulence genes such as *hla* (Recsei *et al.*, 1986; Peng *et al.*, 1988; Janzon and Arvidson, 1990; Novick *et al.*, 1993). The transcription of the *agr* operon produces an RNA molecule, RNAIII, which was found to be the effector molecule of the *agr* locus (Novick *et al.*, 1993) and overlaps the *hld* gene encoding  $\delta$ -toxin (Janzon *et al.*, 1989).

It has been suggested that *agr* negatively regulates the transcription of surface proteins during exponential phase and positively regulates the transcription of extracellular proteins expressed in late late-log phase and stationary phase (Recsei *et al.*, 1986; Peng *et al.*, 1988; Janzon and Arvidson, 1990; Novick *et al.*, 1993;



Morfeldt *et al.*, 1995; Saravia-Otten *et al.*, 1997; Ziebandt *et al.*, 2004). Accordingly, *agr* appears to positively regulate *hla* (Dunman *et al.*, 2001; Ziebandt *et al.*, 2004) and negatively regulate *isaA* (Ziebandt *et al.*, 2004) which, from the proteomics analysis described in chapter four, indicates down-regulation of this regulatory system under LSMMG. However, no differences were found in *agr* expression levels.

The *sar* locus encodes a DNA-binding protein, SarA (Cheung and Projan, 1994), that is thought to regulate staphylococcal gene transcription in two ways: directly by SarA binding to the promoter regions of virulence genes (Cheung *et al.*, 1999; Chien *et al.*, 1999; Blevins *et al.*, 1999; Wolz *et al.*, 2000; Oscarsson *et al.*, 2006), and indirectly by regulation of RNAPIII expression (Cheung and Projan, 1994; Heinrichs *et al.*, 1996; Morfeldt *et al.*, 1996; Cheung *et al.*, 1997; Chien *et al.*, 1998; Chien and Cheung, 1998; Rechtin *et al.*, 1999; Chakrabarti and Misra, 2000). For this reason, the *sar* locus appears to be closely associated to the *agr* system in *S. aureus*.

The virulence regulation by *sarA* is complex and there is evidence that this locus positively regulates *isaA* (Oscarsson *et al.*, 2006) and negatively regulates *hla* (Ziebandt *et al.*, 2001; Oscarsson *et al.*, 2006). As described in chapter four, the analysis of the *S. aureus* proteomic profile under conditions of LSMMG suggests up-regulation of *sarA*; however, no differences were found in SarA levels between NG- and LSMMG-grown cells.

It has been suggested that the expression of *sar* may be influenced by the alternative sigma factor of RNA polymerase,  $\sigma^B$  (Deora *et al.*, 1997). The *sigB* locus has been identified in *S. aureus* and encodes the alternative sigma factor  $\sigma^B$  (Wu *et al.*, 1996; Kullik and Giachino, 1997) and the alkaline shock protein Asp23 (Kullik *et al.*, 1998; Giachino *et al.*, 2001). Although the expression of *sigB* is strongly associated with *S. aureus* responses to stress (Kullik and Giachino, 1997; Chan *et al.*, 1998; Horsburgh *et al.*, 2002) there is good evidence that it might also be involved in virulence regulation (Kullik *et al.*, 1998; Gertz *et al.*, 2000; Horsburgh *et al.*, 2002; Bischoff *et al.*, 2004). However, it is still not clear if *sigB*-dependent regulation occurs directly to modulate particular virulence genes, or indirectly by interaction

with regulatory systems such as *sar* and *agr* (Bayer *et al.*, 1996; Deora *et al.*, 1997; Manna *et al.*, 1998; Chan *et al.*, 1998; Cheung *et al.*, 1999; Nicholas *et al.*, 1999; Gertz *et al.*, 2000; Giachino *et al.*, 2001; Bischoff *et al.*, 2001).

In similar fashion to *sarA*, the proteomic profiles of the three *S. aureus* isolates under LSMMG indicate up-regulation of *sigB*, as this locus appears to positively regulate *isaA* (Ziebandt *et al.*, 2004) and negatively regulate *hla* (Cheung *et al.*, 1999; Ziebandt *et al.*, 2001; Bischoff *et al.*, 2004; Karlsson-Kanth *et al.*, 2006). However, this hypothesis does not correlate with the finding that LSMMG reduces staphyloxanthin production by *S. aureus*, as inactivation of *sigB* has been shown to reduce pigment production by the bacterium (Kullik *et al.*, 1998; Nicholas *et al.*, 1999; Morikawa *et al.*, 2001; Giachino *et al.*, 2001; Karlsson-Kanth *et al.*, 2006). Nevertheless, no differences were found in *sigB* expression levels between NG- and LSMMG-grown cells.

As with *sigB*, the expression of *dnaK* is associated with the *S. aureus* stress response and with pigment production (Singh *et al.*, 2007). This gene has been identified in *S. aureus* and it encodes the heat shock protein DnaK (Hsp70) (Ohta *et al.*, 1994). Interestingly, *dnaK* expression was found to be significantly reduced under condition of LSMMG in isolate RF11 which could, in part, explain the reduction in pigment production in this isolate, described previously in chapter three. However, these results are not compatible with *dnaK* levels of expression in isolates RF1 and RF6, since they were very similar between NG- and LSMMG-grown cells. These results suggest that the reduction in pigment production induced by LSMMG is not *dnaK*- or *sigB*-dependent and may be attributable to regulation by *sae* or by other factors.

Heat-shock proteins (Hsps) in addition to DnaK have been described in *S. aureus*; these include the caseinolytic proteases (Clps). As with other molecular chaperones and proteases, Clps are known to have major functions in bacterial stress responses, preventing the accumulation of misfolded proteins (Frees *et al.*, 2003; Frees *et al.*, 2004). They are components of energy dependent proteolytic complexes and are formed by the association of a proteolytic subunit and an ATPase specificity factor (Lindsay, 2008). It has been suggested that Clp proteins are involved in virulence

regulation in *S. aureus*, probably through interaction with major regulatory systems such as *agr* and *sar*, and they therefore interfere with *hla* transcription (Frees *et al.*, 2003; Frees *et al.*, 2005; Michel *et al.*, 2006). Dunman and co-workers (2001) suggest a negative regulation of *clpL* by *sarA*. This evidence does not correlate with the findings that LSMMG reduced expression of *clpL* while *sarA* transcription remained unaltered. An investigation undertaken by Gertz and co-workers (Gertz *et al.*, 2000) suggests that expression of Clp proteins may be under the regulation of *sigB*. Interestingly, the expression of *clpL* was found to be up-regulated under conditions of LSMMG, compared to NG, in all three isolates. This suggests that enhanced expression of *clpL* in *S. aureus* could impact on final levels of Hla. Several studies have recently been performed involving Clp proteins, particularly with ClpP and ClpX (Frees *et al.*, 2003; Frees *et al.*, 2005; Michel *et al.*, 2006). A recent work by Chatterjee and co-workers (2009) suggests that ClpC may be involved in gene regulation of metabolic pathways such as gluconeogenesis, nucleotide metabolism and oxidative stress. However, the role of the ATP-binding subunit ClpL in *S. aureus* virulence is currently not defined and should be further studied.

*gap*, like *clpL*, was found to be up-regulated under LSMMG in all three isolates; this is consistent with the higher intracellular levels of Gap found during the proteomic study described in chapter four. The role of glyceraldehyde 3-phosphate dehydrogenase in glycolysis has been extensively characterised in several organisms (Kormanec *et al.*, 1995; Kundu and Roy, 2007; Piatigorsky, 2007; Lama *et al.*, 2009; Lu *et al.*, 2009). This enzyme is versatile and multifunctional, participating in membrane transport as well as in transcriptional and translational control (Piatigorsky, 2007). Since *gap* is usually expressed at high levels and is quite stable, it is considered as a housekeeping gene and is frequently used as an internal standard in qRT-PCR reactions. However, since the microarray experiment showed a significant difference between NG and LSMMG samples, this gene could not be used as qRT-PCR internal standard. The basis of the modulation of *gap* expression by LSMMG is not clear.

Transcriptomic analysis also revealed down-regulation of *vraX* under LSMMG in all three isolates and was more pronounced in isolate RF6. There is evidence that the

*vraSR* (vancomycin resistance associated) locus is involved in the transcriptional regulation of antibiotic resistance in *S. aureus* (Kuroda *et al.*, 2000), in the control of cell wall peptidoglycan biosynthesis and in stress responses (Kuroda *et al.*, 2003). The low transcription levels of *vra* may lead to alterations in *S. aureus* membrane permeability which could impact on the final levels of protein secretion. There is insufficient information on *vra* function in virulence regulation; a more detailed study of *vraX* in *S. aureus* pathogenicity is therefore proposed.

Extensive studies have been undertaken in an attempt to understand the complex and co-ordinated process by which these global regulatory systems function and interact with each other. However, the fine detail of this mechanism remains unclear (Novick, 2003; Lindsay, 2008). Although alterations in gene expression as detailed in this chapter may explain, to some extent, the reduced virulence of *S. aureus* under LSMMG, it is probable that post-transcriptional modifications also occur. This is consistent with recent reports from Scherl and co-workers (2005), who demonstrated that protein secretion may be reduced whilst protein transcription levels remain unaltered. Table 5.4 summarises some of *S. aureus* global regulatory systems and correlates them with the effects of LSMMG on *S. aureus* gene expression.

The effects of simulated microgravity (Wilson *et al.*, 2002b) and space flight (Wilson *et al.*, 2007) on bacterial gene expression have been previously described. Using the HARV and DNA microarray analysis, Wilson and co-workers (2002b) have determined that LSMMG differentially regulates the expression of a large number of functionally diverse genes in *S. enterica*; although the regulatory elements of the LSMMG-mediated phenotype have yet to be identified, there is evidence that the ferric uptake regulator *fur* is involved in the LSMMG response. More recently, the same group has shown alterations in *S. enterica* gene expression and virulence during space flight. They suggested that a pathway involving the conserved RNA-binding regulatory protein Hfq plays a role in the response of *S. enterica* to a reduced gravitational field (Wilson *et al.*, 2007). It is not possible to compare these data with the microarray results obtained from *S. aureus* isolates RF1, RF6 and RF11 since such genes were not identified.

**Table 5.4:** Summary of *S. aureus* global regulatory mechanisms which may be involved in response to LSMMG environment. (+) positive regulation, (-) negative regulation, (?) undescribed regulatory mechanism.

	IsaA	References	Hla	References	KatA	References	Pigment	LSMMG	
<i>agr</i>	-	(Ziebandt <i>et al.</i> , 2004)	+	(Dunman <i>et al.</i> , 2001) (Ziebandt <i>et al.</i> , 2004)	?	Not described	?	Not described	≈
<i>sae</i>	?		+	(Giraud <i>et al.</i> , 1994) (Giraud <i>et al.</i> , 1997) (Liang <i>et al.</i> , 2006)	?	Not described	?	Not described	-
<i>sarA</i>	+	(Oscarsson <i>et al.</i> , 2006)	+	(Ziebandt <i>et al.</i> , 2001) - (Oscarsson <i>et al.</i> , 2006)	?	Not described	?	Not described	≈
<i>sigB</i>	+	(Ziebandt <i>et al.</i> , 2004)	-	(Cheung <i>et al.</i> , 1999) (Ziebandt <i>et al.</i> , 2001) (Bischoff <i>et al.</i> , 2004) (Karlsson-Kanth <i>et al.</i> , 2006)	-	(Horsburgh <i>et al.</i> , 2002)	+	(Kullik <i>et al.</i> , 1998) (Nicholas <i>et al.</i> , 1999) (Morikawa <i>et al.</i> , 2001) (Giachino <i>et al.</i> , 2001) (Karlsson-Kanth <i>et al.</i> , 2006)	≈
<i>dnaK</i>	?	Not described	?	Not described	?	Not described	-	(Singh <i>et al.</i> , 2007)	-
<i>clpL</i>	?	Not described	?	Not described	?	Not described	?	Not described	+
<i>gap</i>	?	Not described	?	Not described	?	Not described	?	Not described	+
<i>vraX</i>	?	Not described	?	Not described	?	Not described	?	Not described	-

It is important to indicate that comparison between transcriptomics experiments performed by different research groups can sometimes be hard to accomplish and demands considerable interpretation efforts due to technical and experimental issues. In particular, in *S. aureus* studies, the activation of the different gene regulatory systems is growth-phase dependent and is thought to be dependent on cell density, energy availability and environmental signals (Vandenesch *et al.*, 1991; Ji *et al.*, 1995; Novick, 2003).

*S. aureus* quorum sensing is *agr*-dependent (Novick and Geisinger, 2008); as transcription increases during exponential phase and reaches a maximum in post-exponential phase (Vandenesch *et al.*, 1991), it is likely that gene regulation is mainly due to RNAIII-mediated processes. In consequence, suppression of transcription of surface proteins and enhancement of transcription of extracellular proteins during the transition from the exponential-phase to the stationary phase of bacterial growth is regulated by the *agr* locus (Recsei *et al.*, 1986; Peng *et al.*, 1988; Janzon and Arvidson, 1990; Novick *et al.*, 1993; Morfeldt *et al.*, 1995; Saravia-Otten *et al.*, 1997). In addition, it has been proposed that *agr*-independent signals are responsible for the transcriptional regulation of virulence genes in *S. aureus* (Vandenesch *et al.*, 1991; Saravia-Otten *et al.*, 1997; Wolz *et al.*, 2000). The available data suggest that other regulatory systems, such as *sae* (Giraud *et al.*, 2003; Novick and Jiang, 2003), *sar* (Bayer *et al.*, 1996; Blevins *et al.*, 1999) and that *sigB* (Kullik and Giachino, 1997; Senn *et al.*, 2005) may also be involved in *S. aureus* quorum sensing.

It is, therefore, clear that the phase of bacterial growth is of critical importance in the determination of gene expression in *S. aureus* and that transcriptional (and proteomic) analysis will be markedly influenced by the growth environment extant at the time of cell harvesting. To determine the effect of LSMMG on *S. aureus* virulence, cells were harvested during stationary phase, when the bulk of staphylococcal virulence factors are undergoing active secretion. In addition, during the stationary phase the cell density is at a level sufficiently high to facilitate the collection of sufficient amounts of RNA for DNA microarray analysis. Nevertheless,

during the stationary phase, some staphylococcal genes may not be expressed and will impact on studies of gene expression

Other technical issues concerning DNA microarray technology include RNA stability and experimental design. RNA is induced rapidly (in minutes) and has a short half-life (90% of *S. aureus* mRNA has a half-life of less than 5 min) (Roberts *et al.*, 2006a). These issues are minimised by the use of RNA protect reagent and by maintenance of samples in the frozen state prior to analysis. In terms of experimental design, several microarray platforms are available, data can be produced in different formats using different normalisation processes, and this represents a challenge with respect to data interpretation and for comparison of transcriptional profiles between different groups of bacteria.

The characterisation of transcriptional regulation using microarray technology has provided the means to determine the effects of LSMMG on *S. aureus* gene expression, has led to the identification of genes whose expression has been altered by novel growth conditions and has contributed to the elucidation of virulence gene regulation and pathogenicity in clinical isolates of *S. aureus*. The main findings described in this chapter are summarised in table 5.5. The mechanism by which *S. aureus* virulence is altered in a reduced gravitational field should be studied in greater detail. Notwithstanding, this study has facilitated the identification of new potential targets for vaccine and drug discovery for the treatment and control of staphylococcal infections.

**Table 5.5:** Summary of the effect of LSMMG on *S. aureus* transcriptome.

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**Effect of LSMMG on *S. aureus***

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- ⬇ Differential gene expression
  - ⬇ Down-regulation of *saeRS* and *hla* in isolates RF1 and RF6
  - ⬇ Significant down-regulation of *vraX* in all three isolates
-

## **CHAPTER SIX**

### **General discussion**



Space exploration, which began in earnest in the late 1950s, has been one of mankind's greatest achievements. In the current era of advanced space technology, exceptional space transportation systems and the unique ISS, the space agencies of the nations involved in the exploration of space are increasingly broadening their horizons towards deep space, for both manned and unmanned missions. As the distance and duration of manned space missions progressively extend, the probabilities that astronauts' health may be compromised markedly increase. Risk assessment strategies and careful mission planning are therefore essential.

Reduction in immune competence (Sonnenfeld and Shearer, 2002) and changes to the saprophytic flora (Ilyin, 2005) of crew members have been described following prolonged exposure to a weightless environment. The control of bacterial and viral pathogens during space missions may thus be compromised, thereby increasing the risk of microbial infection. Moreover, identification of several microorganisms aboard space vessels indicates that they are able to survive and proliferate during space flight. *S. aureus*, in particular, has been identified as one of the most predominant contaminants of air and surfaces on spacecraft and space stations and is thus a potential risk to astronauts (Novikova, 2004; Novikova *et al.*, 2006).

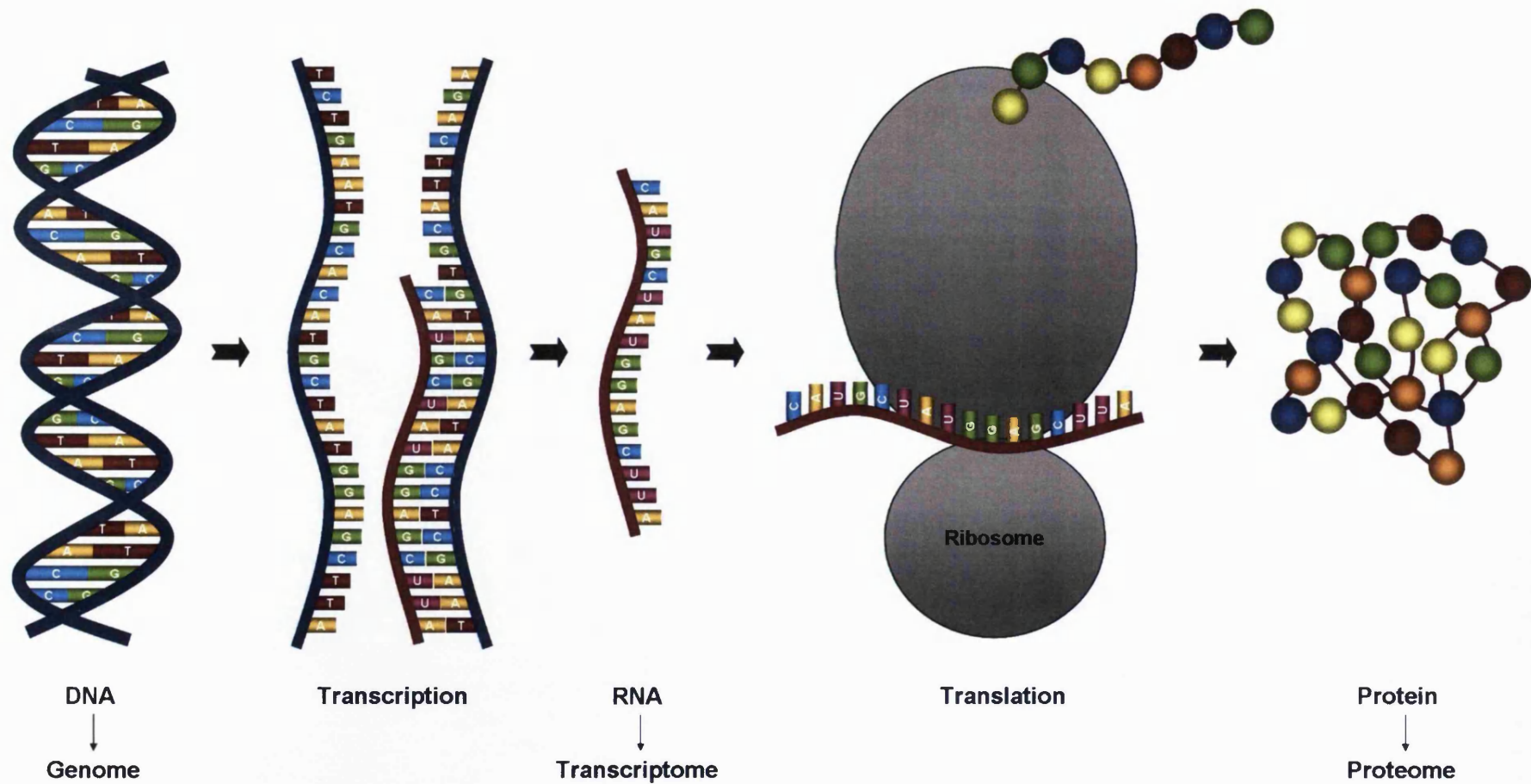
Rather than relying on in-flight solutions, there has been growing interest in the study of the effect of the spaceflight environment on the key properties of various microorganisms, in the attempt to develop means to prevent and control microbial infection during spaceflight. Pathogens such as *S. enterica* and *E. coli* have been examined both during spaceflight and under modelled microgravity (Nickerson *et al.*, 2004); however, these pathogens pose little or no risk to astronauts, whereas *S. aureus*, an opportunistic pathogen and a major contaminant of space stations, represents a substantial health threat.

Opportunistic pathogens such as *S. aureus* possess a remarkable capacity to adapt to extreme environmental conditions. Growth of bacteria in extraterrestrial and simulated microgravity environments is thus likely to result in profound changes in cell physiology and behaviour. For example, it has been reported that spaceflight (Wilson *et al.*, 2007) and culture under LSMMG conditions (Nickerson *et al.*, 2000;

Nickerson *et al.*, 2003) increase the virulence and alter both the proteomic and transcriptomic profiles of *S. enterica*. This environmental adaptation is driven by mechanisms that are not well understood. Investigation of the adaptation processes of bacteria to different environmental conditions, such as LSMMG, may provide important information on bacterial pathogenicity and virulence mechanisms. For this reason, I examined the effect of LSMMG on the virulence properties of *S. aureus*.

The success of *S. aureus* as an opportunistic pathogen is mainly due to its capacity to produce an extensive array of secreted and surface-associated virulence factors, subject to complex and well-balanced regulatory processes, that contribute to colonisation, dissemination and host damage (Fig. 6.1). The information contained within cellular DNA is transcribed in line with the requirement of the bacterium to synthesise proteins that facilitate survival and growth in a given environment; this process begins when the relevant sections of the DNA helix are unwound to expose the genetic information, which is then transcribed into RNA. The RNA is directed to ribosomes, where translation occurs through a process characterised by the orderly alignment of amino acids to the extending polypeptide chain. A range of chemical, structural and/or conformational post-translational modifications may occur during the later stages of protein biosynthesis and these may determine protein localization, function and interaction with other components of the cell.

After translation, the linear chain of amino acids produced from a sequence of RNA must fold correctly, generating its native and functional state. In addition, post-translational modification of the amino acids extends the range of functions of the protein by attachment of other biochemical functional groups, such as phosphate (phosphorylation) or carbohydrate (glycosylation). The resulting three-dimensional structure is essential for the protein to function.



**Fig. 6.1:** From DNA to protein. DNA is transcribed into RNA this molecule is directed to a ribosome where translation occurs to produce a polypeptide chain. Genomic, transcriptomic and proteomic tools contribute significantly to the global study of an organism.

Approximately 25% to 30% of the bacterial proteins (secreted or cell-surface associated proteins) have evolved to be translocated across the cell membrane following assembly in the cytosol (Driessen and Nouwen, 2008). The major protein transport pathway in bacteria is the general secretory (Sec) pathway, which was first described in *E. coli* (Rusch and Kendall, 2007) and *B. subtilis* (Ling *et al.*, 2007); recently, some data has become available for *S. aureus* (Sibbald *et al.*, 2006). Proteins are synthesised with an N-terminal extension, a signal peptide which distinguishes them from resident cytoplasmic proteins. Protein unfolding enables their translocation across the cell membrane; the signal peptide is then removed by a signal peptidase (SPase) and the protein folds into the correct conformation once its site within the cell has been located (Driessen and Nouwen, 2008).

Correct and prompt post-translational modification is essential for proteins to achieve a fully functional and protease-resistant conformation (Sarvas *et al.*, 2004). Failure to fold into the intended shape usually produces inactive or toxic proteins. It is known that, if cells are subject to certain types of stress, such as extreme temperature or pH, secretory proteins may misfold; this affects their localization, function and interaction with other proteins. These effects are mainly due to malfunction of chaperone proteins, which facilitate correct protein folding and are compromised by these environmental stresses (Lund, 2001; Henderson *et al.*, 2006). It is therefore extremely important to study cell modification driven by environmental stresses such as LSMMG. Interestingly, the analysis of the effect of LSMMG on the *S. aureus* proteomic profile indicates significant reduction in several proteins associated with protein synthesis, folding and degradation mechanisms, suggesting that an altered gravitational field may affect the translational and post-translational events described above.

The complete genome sequencing and annotation of eleven human *S. aureus* isolates have provided the opportunity for a comprehensive study of staphylococcal virulence and pathogenicity mechanisms (Lindsay, 2008). Although it is known that the *S. aureus* genome encodes approximately 2800 proteins, only 60% of the genes are transcribed in to proteins of known function (Lindsay, 2008). Whilst the genome of an organism is somewhat static, the proteome differs from cell to cell and is

constantly responding to changes in the environment. For this reason, in the post-genomic era, research is increasingly focusing on functional studies of gene products, a process that is particularly important for genes and proteins of unknown function. Recent advances in combined transcriptomic and proteomic studies have contributed significantly to the discovery of the function of several genes and proteins and consequently to the understanding of bacterial mechanisms of virulence and pathogenicity, enabling the identification of potential targets for development of novel vaccines and therapeutics (Mader *et al.*, 2002a; Mader *et al.*, 2002b; Lee *et al.*, 2003; Rogasch *et al.*, 2006).

Transcriptomics can provide an array of information with respect to global gene expression. However, the analysis at transcription level may only estimate the degree of gene expression into a protein. After transcription, RNA may be degraded rapidly or translated inefficiently, resulting in the synthesis of only small amounts of transcribed protein. Moreover, many proteins may undergo post-translational modifications that are likely to impact on their function and localization. For this reason, proteomics is becoming a powerful tool to facilitate better understanding of bacterial behaviour in response to environmental change. The cell proteome is dynamic and for this reason, the scope of proteomics is broadening to the study of post-translational modifications of proteins, that include phosphorylation (phosphoproteomics) (Soufi *et al.*, 2008) and glycosylation (glycoproteomics) (Hitchen and Dell, 2006) as well as cell localization and protein-protein interaction (Monti *et al.*, 2005; Hooker *et al.*, 2007).



















The use of environmental triggers to induce the expression of new phenotypes, with concomitant alterations in protein production and/or protein secretion, may provide important information on bacterial virulence and pathogenic mechanisms. LSMMG provides a unique environment, the effect of which has not so far been described for *S. aureus*. Analysis of the behavioural modifications of the bacterium under an altered gravitational field may aid clarification of the function of unknown proteins encoded by the staphylococcal genome, which would not be transcribed under standard laboratory conditions, as well as provide insights into the adaptation of the bacteria to LSMMG.

The present work describes the effect of LSMMG as an environmental signal affecting the phenotype and the virulence properties of *S. aureus*. This study represents, as far as I know, the first investigation of the effect of simulated microgravity on the behaviour of *S. aureus*. The use of a low-shear low-turbulence environment differentiates this work from the majority of laboratory-based studies of the impact of environmental stresses, such as alteration in temperature, pH or changes to the growth medium.

The HARV was utilised to simulate the microgravity conditions that pertain during spaceflight, providing a low-shear low-turbulence environment for cell growth. Nevertheless, other parameters such as hydrodynamics, oxygen tension and shear stress may influence cell growth in the HARV and should thus be considered for data interpretation in studies such as the one described here. Although it has proven to be a model which provides unique environmental conditions that trigger phenotypic changes in bacteria, the level of correlation between ground-based studies and spaceflight experiments remains to be elucidated since results to date, described in general throughout this thesis, have been inconsistent. Nevertheless, the RCCS is a readily accessible tool, in contrast to the limited opportunities for conducting experiments during spaceflight. These experiments should contribute to the general understanding of the risks posed to crew members, and to other individuals exposed to a self-contained environment, by opportunistic pathogens such as *S. aureus*. Moreover, the study of the effect of microgravity as an environmental stress affecting the bacterial phenotype and virulence may contribute to a fuller understanding of infectious disease processes.

In contrast to previous studies that have described an increase in the virulence of *S. enterica* during spaceflight (Wilson *et al.*, 2007) and in microgravity simulators (Nickerson *et al.*, 2000), this study strongly suggests that expression of virulence determinants by *S. aureus* is reduced under LSMMG conditions, as indicated by reduced production of virulence determinants. Table 6.1 summarizes the effect of LSMMG on the properties of *S. aureus* described in this thesis.

**Table 6.1:** Effect of LSMMG on *S. aureus* and bacterial targets with potential application for development of vaccines and antibacterial agents identified in this project.

Effect of LSMMG on <i>S. aureus</i>	Potential vaccine/drug targets identified
 No effect on growth kinetics	 <i>cit</i>
 No effect on antibiotic susceptibility	 <i>cpl</i>
 No effect on cell morphology	 <i>eno</i>
 Reduction of pigment production	 <i>gap</i>
 Reduction of toxin secretion	 <i>isa</i>
 Reduction of production of virulence determinants	 <i>kat</i>
 Reduction of protein secretion	 <i>hla</i>
 Differential gene expression	 <i>sae</i>
	 <i>sod</i>
	 <i>vra</i>

LSMMG did not induce major modifications in to the kinetics of growth, the antibiotic susceptibility or the cell morphology of *S. aureus*. However, large reductions in pigment production and protein secretion, including staphylococcal toxins, were found. The global reduction in staphylococcal protein present in the supernatant induced by LSMMG can be partially explained by alterations in gene expression. Importantly, the global regulatory system, *sae*, and the major virulence factor, *hla*, were significantly down-regulated. The expression of other genes such as *clp*, *gap* and *vra* were also found to be modulated under conditions of LSMMG, compared to NG. Although expression of *isa* was not found to be substantially altered by LSMMG, this protein has been identified as a potential target for vaccine and antibacterial drug development (Vytvytska *et al.*, 2002; Hecker *et al.*, 2003) and should therefore be further characterised. In addition, the roles of *cit*, *eno*, *kat* and *sod* should also be subjected to more extensive studies in relation to *S. aureus* virulence and pathogenicity mechanisms.

Although recent work has shed some light on the complex regulatory networks that control gene expression in staphylococci (Giraudo *et al.*, 2003; Xiong *et al.*, 2006; Geisinger *et al.*, 2006; Chen and Novick, 2007), the environmental cues that stimulate the synthesis and transport of virulence and antibiotic resistance determinants are poorly understood (Novick, 2003). Regulatory components that lead to down-regulation of such determinants would represent attractive targets for chemotherapy. This work is a contribution to the identification of the key step(s) in the regulatory pathway that determine(s) a marked reduction in the synthesis and secretion of major virulence factors by *S. aureus*. One strong candidate for a role in the regulation of the LSMMG “trigger” is the gene product of *vraX*. This small polypeptide has been associated with transcriptional regulation of antibiotic resistance in *S. aureus*, control of cell wall peptidoglycan biosynthesis and stress responses (Kuroda *et al.*, 2003). Further analysis on the impact of mutations in this and other particular genes such as *saeRS*, may provide important information on the regulatory mechanisms of the *S. aureus* pathogenic phenotype and contribute to the identification of new targets for chemotherapeutic attack. These studies are currently being undertaken and may provide further mechanistic information for the differences in protein profiles observed in cultures grown under LSMMG compared to NG.

The somewhat limited impact of LSMMG on gene expression suggests that levels of protein secretion may be affected by post-transcriptional modifications or by changes in the rate and extent transport across the cytoplasmic membrane. The use of recent proteomic techniques, such as phosphoproteomics and glycoproteomics, could aid clarification of the processes involved and should be considered for future study.

Nevertheless, factors other than variation of the gravitational vector, such as aeration, growth medium and shear stress, may also contribute to some of the effects reported in this study. The use of different experimental conditions during the execution of future studies should thus be considered. I have provided evidence that different *S. aureus* clinical isolates may respond differently to changes in the gravitational vector. Together with previous studies also describing differences amongst *S. aureus* strains as well as clinical isolates in response to environmental changes (Ziebandt *et al.*, 2001; Gomes *et al.*, 2005; Rogasch *et al.*, 2006), this information highlights the



need to extend LSMMG studies to other *S. aureus* strains, such as life-threatening MRSA clinical isolates. It would also be of value to repeat the experiments described in this thesis using different ground-based models and aboard the ISS to better understand how changes in gravity, as well as other parameters associated with the space environment, may affect *S. aureus* behaviour.

The observation that LSMMG may reduce *S. aureus* virulence raises the possibility that pharmacological modulation of the “microgravity trigger” that produces this avirulent phenotype would “disarm” the pathogen and resolve the infection. This work constitutes the first step in a search for inhibitors that would prevent the secretion of a family of proteins necessary for infection to take place. The LSMMG-regulons identified in this study could be considered as novel targets for antibacterial therapeutics and should be further studied with this in mind, possibly using mutagenesis techniques as described by others (Cheung *et al.*, 1994; Rampone *et al.*, 1996; Giraudo *et al.*, 1996). The attenuated phenotype generated by a LSMMG-like pharmacological intervention would not survive systemically and would be removed by the host’s immune surveillance or by antibacterial agents not effective against the NG-generated phenotype. In contrast to conventional antibiotics, such therapeutic agents would modify rather than kill the target pathogen and, as a consequence, apply less direct selective pressure on bacterial populations.

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**Matrix Science:** [www.matrixscience.com](http://www.matrixscience.com)

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## **PUBLICATIONS, PRESENTATIONS AND AWARDS**

## PUBLICATIONS

**Rosado, H., Doyle, M., Hinds J., Taylor, P.W.** 2009. Low-shear modelled microgravity alters expression of virulence determinants by *Staphylococcus aureus*. Acta Astronautica. Accepted for publication.

Taylor, P.W., **Rosado, H.** 2008. Manned missions to Mars – the likely impact of microbial infection. pp 165-185 In *Planet Mars Research Focus*, ed. L.A. Costas, Nova Science Publishers, Hauppauge, NY.

**Rosado, H., Doyle, M., Hinds J., Taylor, P.W.** 2008. Low-shear modelled microgravity alters expression of virulence determinants by *Staphylococcus aureus*. Proceedings of the 59th International Astronautical Congress IAC-08-A1.7.-A2.7.5.

**Rosado, H., Stapleton, P.D., Taylor, P.W.** 2006. Effect of simulated microgravity on the virulence properties of the opportunistic bacterial pathogen *Staphylococcus aureus*. Proceedings of the 57th International Astronautical Congress IAC-06-A1.7/A2.7.06.

## COMMUNICATIONS

**Rosado, H., Doyle, M., Hinds J., Taylor, P.W.** 2008. Low-shear modelled microgravity alters expression of virulence determinants by *Staphylococcus aureus*. 59th International Astronautical Congress. Glasgow, Scotland.

**Rosado, H., Doyle, M., Hinds J., Taylor, P.W.** 2008. Effects of simulated microgravity on *Staphylococcus aureus*. Staphylococcus GBI. Leeds, England.

**Rosado, H., Doyle, M., Hinds J., Taylor, P.W.** 2008. Simulated microgravity alters global gene expression and protein secretion by *Staphylococcus aureus*. 108th General Meeting of the American Society for Microbiology. Boston, Massachusetts, USA.



**Rosado, H.**, Doyle, M., Hinds J., Taylor, P.W. 2008. Simulated microgravity alters global gene expression and protein secretion by *Staphylococcus aureus*. PhD research day. The School of Pharmacy, University of London, UK.

**Rosado H.**, Doyle M., Hinds J., Taylor P.W. 2007. Modelled microgravity down-regulates expression of *Staphylococcus aureus* virulence determinants. European Low Gravity Research Association (ELGRA) Biennial Symposium. Florence, Italy.

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## POSTERS

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## AWARDS

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Best poster presentation at The School of Pharmacy, University of London, PhD Research Day 2007.