筑　波　大　学

博 士 （医 学） 学 位 論 文

Study on Accessory Gene Regulator (AGR) Variants in *Staphylococcus aureus*

(黄色ブドウ球菌の病原性マスターレギュレータ Agr 系の相変異の研究)

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The contents previously published in mBio Aug 2019, 10 (4) e00796-19; DOI: 10.1128/mBio.00796-19 are re-used in this dissertation following the guidance of The American Society of Microbiology's Author Rights.

THESIS ABSTRACT

Purpose: *Staphylococcus aureus* is an opportunistic pathogen that creates a heavy economic and healthcare burden for humans. *S. aureus* is able to expertly establish infection in a variety of host environmental niches, and this ability is largely due to its impressive array of virulence factors that give it an edge for survival in any environment. The expression of these virulence factors must be finely controlled, and this is largely achieved by the central virulence regulatory network called the Accessory Gene Regulator (AGR) system. However, there are numerous reports of *S. aureus* clinical isolates that have a dysfunctional mutated Agr system. These mutants have a disadvantage in disseminating from the host and have thus been referred to as 'dead-end' mutants. Interestingly, previous reports have documented Agr-mutations that are reminiscent of Phase Variation, which is a bacterial mechanism of reversible gene expression. Thus, this study hypothesised that a fraction of Agr negative mutants may be phase variants that can repair and revert their Agr activity.

Methods: Potential Agr revertant strains were selected for by subjecting Agr negative variants to successive liquid cultures before plating on Sheep Blood Agar (SBA) and screening for haemolytic colonies. The Agr status of haemolytic colonies was confirmed by a modified CAMP test and by semi-quantitative RT-PCR. Phenotypic Agr revertant strains then had their Agr locus sequenced to identify any mutational mechanisms. A fluorescent reporter construct was used to monitor Agr activity in populations growing in planktonic and solid structured media. The reporter was also used to monitor Agr activity upon phagocytosis.

Results: Agr revertant strains were generated from two laboratory strains (MW2 and s0437) as well as two clinical strains (66r and 3082). Two underlying genetic Phase Variation mechanisms responsible for the phenotypic reversion were identified amongst MW2, s0437, and 66r. These were a duplication and inversion event as well as a ploy(A) tract alteration. The revertant strains did not activate their Agr system in planktonic culture but did when grown on solid media or when phagocytosed by macrophages.

Discussion: Agr negative clinical isolates have long been dismissed as having no evolutionary future. However, this thesis demonstrates that a fraction of Agr negative strains are Phase Variants that can selectively revert their Agr activity, recovering all phenotypes lost upon Agr dysfunction. Agr reversion in discrete local microenvironments upon growth on solid structured media could be important for the proper development of *S. aureus* biofilms, as separate works have shown that Agr-controlled exoproteins are known to be important in 3-dimensional biofilm structuring. Additionally, the ability of revertant cells to activate their Agr system upon phagocytosis could enable them to survive immune attack and could allow them to use host immune cells as trojan horses to disseminate to other sites of the body. Although we were unable to describe the prevalence of Agr phase variants in the clinical setting, we did identify a number of strains that could revert their haemolytic phenotype independently of Agr function. The mechanism of this reversion is yet to be elucidated.

Conclusion: Taken together, we propose a model whereby Agr Phase Variation acts as a predictive adaptation mechanism to insure against host-mediated immune stress. This thesis adds another layer to the complexity of *S. aureus'* lifestyle and takes us a step further in our understanding of this important pathogen.

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LIST OF ABBREVIATIONS USED

AGR: Accessory Gene Regulator

SBA: Sheep Blood Agar

CAMP test: Christie-Atkins-Munch-Peterson test

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

SSM: Slipped Strand Mispairing

SSR: Short Sequence Repeat

TCS: Two Component System

AIP: Autoinducing Peptide

RHK: Receptor Histidine Kinase

RR: Response Regulator

NET: Neutrophil Extracellular Trap

PSM: Phenol Soluble Modulin

TSB: Tryptic Soy Broth

TSA: Tryptic Soy Agar

WT: Wild Type

Cm: Chloramphenicol

RAV: Reversible Agr Variant

ORF: Open Reading Frame

AD: Atopic Dermatitis

TMAO: Trimethylamine Oxide

CCR: Carbon Catabolite Repression

TA: Toxin-Antitoxin

ACKNOWLEDGEMENTS

The journey of a PhD follows an arduous and tortuous road, intertwining a myriad of complex emotions resulting in a tumultuous psychological mire that constantly seeks expression. The preface of a thesis, unlike other literary works, may not necessarily provide the release that this tempest seeks and, though it is nigh impossible to prevent a little bleeding through, a more detailed account of my sentiments, and those of countless others, can be stumbled upon by cursory search on the internet. As for what follows, it is an accounting of the countless debts I have accrued through my PhD, and a mention of appreciation to those that stand out.

I start by acknowledging my parents, grandparents, and sister. My very existence is by dint of my parents' intentions and actions and while life offers a lot to curse at, even the most ardent pessimist would admit that it is a blessing. My mother has sacrificed much in her life to see me to where I am, and her contribution cannot be done justice to in a few short words. My sister has always been the optimistic spark who is quick to cheer me up, and to have been reciprocate in her early years of University when she needed it because I had come to such a far off place will always be one of my many regrets. My grandparents' support has always made me feel loved, and my grandfather was responsible for not only single-handedly financing my education up to my master's degree but also for teaching me about life and what it means to live it. That he suddenly died mere days after my PhD defence and without seeing me graduated is perhaps an expression of the frivolous irony of life, and though I shall ensure that the regret of distancing myself from him these last five

years by whimsically coming to Japan for my PhD does not develop into a festering miasma of turmoil, it will indeed be an uphill challenge. However, I rest easy with the assurance that I will do right by him, and live life to the best of his teachings as he would have wanted me to. Mum, Dad, Pappa, Mamma, and Rahee: your unstinting and unquestioning support during these long years given unequivocally (even though I know that you were sad to see me go so far away) has been a pillar holding me up and will forever be appreciated.

I am of course grateful to the Japanese taxpayers and the Japanese Ministry of Education, Culture, Sports, Science, and Technology (MEXT) for their generous scholarship and living allowance. Though every single Yen that I received, as well as countless thousands of pounds of my own savings from back home, were reinvested in small and large businesses, institutions, and corporations in Japan as a direct result of my coming here, I am nonetheless grateful for the opportunity provided to me by MEXT. Similarly, I thank The University of Tsukuba for accepting my application of further education and sponsoring my VISA status as a student. I am grateful to my PhD supervisor, Professor Morikawa, for accepting me in his lab and giving me a place to learn about the fascinating world of science while continuously stimulating the development of my critical and logical thinking and my scientific writing skills. I owe my graduation and success in my PhD to his supervision and am grateful for it. I would also like to thank my PhD defence committee for taking the time out of your schedules to go over my thesis in sufficient detail to initiate the interesting discussion that I experienced during my defence, and thank you for letting me pass as well.

A few lab members stand out to receive particular attention. The first, and perhaps most important, is Veronica. For the two years that we were in the lab together, you have been a much-needed friend, and an even more needed lab mentor (even though it was not your responsibility to shoulder). It is not an understatement to say that things would have turned out very differently had you not been around. I thank you very, very much for your teaching, your support, and most of all your friendship. Miyai, my Japanese kouhai; Your constant optimism was a cheerful boost, and your unrestrained kindness to a random boisterous foreigner will not be forgotten. All your jokes and your character have definitely rubbed off on me, and though it has not yet been a year since you left it feels much longer. Thank you as well for sharpening my Japanese skills. No amount of classroom learning could have prepared me for the torrent of Japanese I heard from you, and my only regret is that I could not reciprocate in helping you with English. Keep practising it (you are much better than you give yourself credit for) and I hope to see you and your family again in the future. The last mention goes out to Bobby, who brightened my lab life with his melodious Trinidadian accent and his equally musical personality. It's been good fun these last two years, and I thank you for all the laughs we had. Stay sane, always listen to Marsha, and come out of this with the same positive outlook and perfected dishwashing skills.

I would also like to take this opportunity to thank the various people around the university who went out of their way to help me. Thomas and Bryan, your constant offer of support and friendship has been very much enjoyed and appreciated. You do

a good job here, and I hope you understand the impact you have on our lives. Tom, I've always appreciated the tea and the chats and Bryan, the RPG nights were a hilarious diversion. Thank you both for the extra mile that you go to. I would also like to note my appreciation for the various non-academic staff around the University who have had dealings with me, mainly in the Academic Service Office in the medical area and in the Office for the Biomedical PhD program. Thank you for writing all the important emails in English and thank you for taking the time, on numerous occasions, to explain the baffling array of forms to me. Your help throughout the degree is one of the reasons I managed to graduate. I would also like to acknowledge the counsellors for foreign students at the university. I would perhaps be exaggerating if I said that my counselling sessions were very helpful but considering that every counsellor available was a volunteer who had chosen to step up to provide a service extraneous to their own employment responsibilities in reflection of the duty they feel towards students, their presence and intention is very worthy of gratitude. One might expect the university to employ dedicated psychologists to fill this wide gap but, in their absence, I thank those who stood up to try and bridge it.

After navigating through the plethora of acknowledgements, we now find ourselves coming towards the end. To any readers who have endured my logorrhoeic prose, I commend you and take this opportunity to acknowledge your dedication to the cause. Without further ado, and with the best having been saved for last, I shall begin wrapping matters up. It has been 5 long years that I have endured my PhD. Five years away from family, friends, familiarity, and fortune. Any discerning reader

would have picked up that the reality that I was subject to has been a far cry from the rose-tinted imaginations of the pre-PhD Vishal; the (relative) youth who still flirted with pessimism as an insulated abstract. I owe my graduation to my supervisor, and I owe my family for having supported me to the point where I was able to freely make the choice to come here to Japan. But I owe my persistence to a select group of individuals without whom I would have bailed ship long before stumbling to this point: The Sharmutas®. Chief Sharmut, El Conquistador, San Martín, and P-Money – El Bitch owes you a lot, and thanks you for slowing his descent on that slippery slope of regret that leads to the cesspit of self-pity. Pablo, though you departed post-haste I still appreciate the three years we had and the quirkiness you added to the mix. Toni, I thank you for always coming up with ideas of things to do, for the crazy drunk bicycle escapades, and for always being right. Martín, the patron saint of the Sharmutas®, I thank you for being a solid grounded individual who always steered us away from the brink of destruction and for being my unofficial therapist (a burden which I am eternally grateful you took upon yourself). Ralph, I'm not even sure how to begin appreciating what you mean to me in words, so I'm not even going to bother trying. Besides, you mean more to me than words CAN express. Thank you all for being forced into the role of my unofficial support group, thank you for the companionship, the crazy times, and, most of all, thank you for making it worth it; for making the walk through hell almost enjoyable; for being the dam holding back the flood of regret. I would be sorely remiss were I not to mention all the other people who have snowballed into my life and become very important characters and

influences. Christina for the constant coffees, cheerful brightness, and empathetic complaining session. Stina for the craziness, the loudness (matched only by mine) and the unexpected that was always a surprise. Taka and his family for the constant welcome, for the sense of inclusion, and (most importantly) for the introduction to Kinu no Yuu. Lara for the vibrant colours of both you and your hair, and for all the fun times that would brighten up the life in Japan. Thank you all. Lastly, of separate and special mention, thank you Petra for being a constant source of happiness and joy; for being a distraction from the darkness; for making me spend so much time thinking of ways to keep hearing that beautiful laughter; for being the one who has to endure all the repetitive moaning, complaining, ruminating, and excessive hypothesising of what might be; and for reminding me that life is worth putting in the effort. You are special not only to me, but because you can somehow deal with me, and I don't know how I would have faced the future if I didn't have you. Puno ti hvala.

Finally, I would like to thank the numerous people I met along the journey that I have not mentioned individually. I hope that I impacted your life as positively as you did mine. I end with a quote from that master author Tolkien that captures the emotions this journey has evoked: *"But in the end it's only a passing thing, this shadow; even darkness must pass"*.

CHAPTER 1: INTRODUCTION

Staphylococcus aureus characteristics and lifestyle

The Victorian era heralded significant advances in medical practice that were ushered in by diligent research across all scientific fields. Medical practice in the early 19th century was barbaric when compared to the modern-day standards, and it took leaps of scientific intuition to bring about a change for the better. One such example is the pioneering work of Alexander Ogston who, in 1880, determined that pus was the result of micrococci growing in the wound abscess (Ogston 1882). Ogston was inspired by the work of Jospeh Lister who is credited with the widespread introduction of antiseptic surgery, promoting the sterilisation of surgical equipment and wounds to dramatically reduce post-operative sepsis. Ogston was convinced that the cause of pus formation was a direct result of some form of "germ" within the pus itself, and to investigate this idea he observed smears of pus from abscesses of his patients under a microscope (Orenstein). Thus, he was able to observe clustered bunches of micrococci, which he named staphylococci (derived from the Greek staphyle meaning bunch of grapes). In 1884, a German surgeon by the name of Anton J. Rosenbach isolated two separate strains of staphylococci which he discriminated by the pigmentation of their colonies: *Staphylococcus albus* (later renamed *S. epidermidis* due to its ubiquitous presence on human skin) derived from the Latin albus for white, and *Staphylococcus aureus* derived from the Latin aurum for gold (Rosenbach 1884).

Since then, *S. aureus* has become somewhat infamous in the medical theatre. It is a Gram-positive member of the phylum Firmicutes (characterised by a low GC content in the DNA nucleotide makeup). It is non-motile, non-sporulating, facultative anaerobe that grows optimally between 30 to 37^{o}C and pH 6-7 and can be differentiated from other staphylococci by its production of both catalase and coagulase enzymes (Harris, Foster et al. 2002, Valero, Pérez-Rodríguez et al. 2009).

Although pathogenic, *S. aureus* is also a commensal organism. *S. aureus* can colonise multiple sites of the human body, but the main niche of commensal colonisation is the anterior nares. There are three main carrier-patterns of *S. aureus* amongst healthy individuals: persistent carriers (~20%), intermittent carriers (~30%), and non-carriers (~50%) (Wertheim, Melles et al. 2005). Nasal carriage of *S. aureus* has been linked to a higher chance of contracting *S. aureus* infections (Wertheim, Melles et al. 2005), but the factors that enable colonisation of the nasal passages by the bacterium are as yet unclear. The commensalism of *S. aureus* does not make it unique, as there are many examples of opportunistic pathogens. What does set it aside, however, is the astounding diversity of infections it can cause. Although benign infections such as skin infections and abscesses are common more serious conditions can also develop. *S. aureus* is the leading cause of infective endocarditis, osteoarticular infections, and surgical site infections (Tong, Davis et al. 2015). *S. aureus* bacteraemia is also prevalent, as well as pneumonia and respiratory infections (especially amongst persons living with cystic fibrosis) (Tong, Davis et al. 2015). Furthermore, *S. aureus* is supremely adept at infecting alien surfaces within

the host body, examples of which include, but are not limited to, catheters, artificial heart valves, and prosthetic joints (Tong, Davis et al. 2015).

S. aureus can cause this plethora of infections, and yet can also remain as a commensal causing relatively little harm. Furthermore, each infectious niche would call for a specifically tuned and tailored phenotype. In addition, *S. aureus* is notable for its rapid development of resistance to antibiotics, and recurrent chronic infections that are difficult to cure are common. This extremely complicated lifestyle is possible largely due to two aspects: a vast arsenal of virulence factors (Oliveira, Borges et al. 2018, Tam and Torres 2019) that can be deployed for a variety of infectious niches and an innate ability to be sensitive to environmental changes and react accordingly.

The hallmark of environmental sensitivity is conferred upon *S. aureus* by its complement of Two-Component Systems (TCSs). At its simplest, a TCS is composed of two components (as the name suggests): a transmembrane histidine kinase protein with an extracellular sensory domain that is sensitive to specific environmental changes and a corresponding response regulator which is activated upon detection of an environmental change and elicits a cellular reaction in response. *S. aureus* carries 16 TCSs which, together with a suite of cytoplasmic gene expression regulators, are responsible in large part to *S. aureus'* remarkable adaptability, the details of which are out of the scope of this thesis. It is, however, important to note that although this large number of TCSs allows reaction to a diverse array of environmental cues and stimuli, it is nevertheless a *reaction*, and is

thus an adaptation strategy to tangible environmental fluctuations. To be truly flexible in any given environment, *S. aureus* must evolve strategies that do not merely react to detectable changes but enable it to adapt beyond a simple stress response. This can be achieved by the phenomenon of phenotypic variation amongst the bacterial population.

Phenotypic heterogeneity as an adaptation mechanism to improve population fitness

Increasing attention is being paid to phenotypic variation and heterogeneity in bacteria and there is mounting evidence contrasting the traditionally held view of bacterial populations being homogenic. Heterogeneity in a population can be generated by a number of mechanisms and allows for the implementation of population-level strategies, such as bet-hedging and division of labour, to improve the survivability and fitness of the bacterial species (Fig. 1).

Microbial bet-hedging is when an organism spreads risk across cells in order to improve overall fitness in an unpredictably fluctuating environment (Grimbergen, Siebring et al. 2015). To achieve this, specialised subpopulations of cells, each with a different gene and protein expression profile, will arise in a heterogenous manner in the overall population. These sub-populations are maladapted to the current growth environment, reducing the overall fitness of the population, but may be *pre-adapted* for a future environment. This is beneficial if the environment changes suddenly or extremely, imposing a severe burden for maladaptation in the future environment. In such a situation, the majority of the maladapted population will likely be wiped out,

but if there is a sub-population of cells that was pre-adapted then they will survive and eventually restore the population, generating more heterogenous sub-populations while doing so. In this way, the geometric mean fitness of the population is improved; though each generation is less fit than an isogenic one, over time there will be less fitness variation through generations (de Jong, Haccou et al. 2011, Grimbergen, Siebring et al. 2015).

Bet-hedging is an inherently stochastic process and thus depends on "noise" in the bacterial cell. Noise in the biological context refers to errors in cellular processes occurring as a result of unplanned or unexpected interaction between component molecules (Elowitz, Levine et al. 2002, Veening, Smits et al. 2008). Biochemical reactions often experience a relatively surprising degree of error due to random fluctuations and thus biological signals are rarely discrete and always have a low background level of noise. Bacteria can exploit this to increase the chance of stochastically generated phenotypic variation. For instance, biological noise is thought to be highest when the number of component molecules is small, such as in the processes of transcription and translation (Veening, Smits et al. 2008). This can be exploited by the evolution of positive feedback within regulatory systems, which amplifies low level noise and further bifurcates the population. At the population level, this amplification would convert the graded-response gene expression that would be predicted to result from low-level noise into a cleanly bimodal pattern of expression referred to as a bistable expression pattern (one in which cells can exist in two stable states: an "ON" and an "OFF" state).

Heterogeneity in a bacterial population can be generated by phase variation. Phase variation is an evolutionarily stable mechanism of stochastic gene expression switching that allows bacteria to “turn off” gene expression at a time when it is not needed (Henderson, Owen et al. 1999). It plays a role in adaptation to different environments and can be thought of as similar to bet hedging in the respect that it creates a bifurcated population of specialist cells. Phase variation can occur through several mechanisms including, but not limited to, genetic rearrangements, Slipped Strand Mispairing (SSM) in Short Sequence Repeat (SSR) regions (Fig.2), and genetic disruption by insertion sequences (van der Woude and Baumler 2004, van der Woude 2011). Buckling et. al. (Buckling, Neilson et al. 2005) demonstrate a case where SSM’s in a poly(A) tract within the coding sequence of the *S. aureus mapW* gene can shift the coding frame to prematurely terminate protein translation. An example of a controlled phase variation mechanism is described by Valle et. al. (Valle, Vergara-Irigaray et al. 2007) in the biofilm phenotype of *S. aureus*. It had been reported that the insertion sequence IS256 contributes to phase variation in biofilm-associated genes by disrupting the *sarA* transcriptional regulator, resulting in a biofilm negative phenotype. Valle et. al. added a new layer of understanding to this IS256-mediated phase variation by showing that it is regulated, to some extent, by σ^B.

Taken together, it is strikingly clear that phenotypic heterogeneity plays a robust role in bacterial lifestyles, allowing population-level measures to be implemented to improve the fitness of a species across several generations, not just in the present

timeframe. Yet, there is still much to discover about phenotypic heterogeneity and its underlying mechanisms and causes, and this is a gap that is constantly being filled by new and exciting studies.

Background of study

As mentioned above, one of the key contributors to the effectiveness of S. aureus as a pathogen is its vast arsenal of virulence factors that facilitate the establishment of infection (Jenkins, Diep et al. 2015). The transition from a commensal to a pathogenic state is driven by the expression of these virulence factors and thus they are tightly controlled by the Agr system (Fig. 3). The Agr locus is composed of two divergently transcribed units: the *agrBDCA* operon under the control of the P2 promoter and the *RNAIII*/*hld* gene under the control of the P3 promoter (Wang and Muir 2016) (Fig. 4). The AgrD pre-cursor peptide undergoes two modifications to convert it into mature autoinducing peptide (AIP) (Wang, Zhao et al. 2015). The first modification is mediated by the transmembrane AgrB and involves cleavage of the C-terminal recognition sequence and formation of the Thiolactone ring. This step is energetically unfavourable, and the reaction is driven by rapid degradation of the cleaved C-terminal fragment (Wang, Zhao et al. 2015). The thiolactone intermediate is then exported out of the cell and further cleaved at the N-terminus to give the mature AIP molecule. When the extracellular concentration of AIP reaches a threshold, it is detected by the AgrC receptor-histidine kinase (RHK) which results in self-phosphorylation of AgrC and subsequent transfer of the phosphate group to the AgrA response regulator (RR) (Wang and Muir 2016).

Phosphorylated AgrA then binds, with differential affinity, to the P2 and P3 promoters to initiate transcription (Koenig, Ray et al. 2004). Transcription from the P3 promoter produces the RNAIII regulatory mRNA which has global effects on gene expression (Bronesky, Wu et al. 2016), and also encodes the δ haemolysin gene, while transcription from the P2 promoter creates a positive feedback loop. The RNAIII regulatory mRNA promotes, both directly and indirectly, the expression of several exotoxins and suppresses that of colonising factors, thus enabling S. aureus to switch to a pathogenic lifestyle (Bronesky, Wu et al. 2016). Perhaps one of the more intriguing aspects of the Agr system is its polymorphism within the *S. aureus* species. Four discrete Agr alleles have been isolated, with the variable region of the locus being the majority of *agrB*, the entirety of *agrD*, and the sensor domain of *agrC*. Each of the Agr groups produces its own type-specific AIP and with the exception of type I and type IV the effect of each AIP type on non-cognate Agr systems is inhibitory (Wang and Muir 2016).

It should be noted that the switch from an Agr "OFF" to "ON" state is not immediate and it has been shown that a heterogeneous population will transiently exist during the gradual activation of the system, which may play a role in the infection process (Garcia-Betancur, Goni-Moreno et al. 2017). Furthermore, this bifurcation is sensitive to environmental cues and is influenced by extracellular Mg^{2+} concentration which increases SigB mediated Agr repression (Garcia-Betancur, Goni-Moreno et al. 2017), constricting the activation threshold of the Agr system and increasing the potential for bifurcation into Agr ON and OFF subpopulations. In

addition, Agr activation is not strictly bound to the principles of a quorum sensing system. Indeed, the dynamics of the Agr system are more accurately described by the concept of 'diffusion sensing' proposed by Redfield in 2002 (Redfield 2002). Although Redfield's theory has come under scrutiny upon examination of more recent data (West, Winzer et al. 2012), the concept of diffusion sensing still stands, though it no longer seems to be mutually exclusive to quorum sensing as proposed by Redfield. The model of diffusion sensing posits that an autoinducer molecule functions as a sensor of diffusion. Under high-diffusion conditions, the autoinducer molecule is rapidly lost from the cell's local environment and this prevents wasteful production of secreted exoenzymes etc that would equally diffuse away. However, if there is a physical constraint in the local microenvironment of the cells, the autoinducer produced does not diffuse away and can accumulate to a sufficient concentration as to activate production of other exoproteins (Fig. 5).

The importance of the Agr system as a global virulence regulator in *S. aureus* has historically been robustly demonstrated through numerous infection models (Abdelnour, Arvidson et al. 1993, Cheung, Eberhardt et al. 1994, Gillaspy, Hickmon et al. 1995, Wright, Jin et al. 2005) and yet Agr negative isolates are commonly isolated (Somerville, Beres et al. 2002, Fowler, Sakoulas et al. 2004, Shopsin, Drlica-Wagner et al. 2008, Shopsin, Eaton et al. 2010), with evidence from as early as 2008 suggesting that a substantial proportion (~22%) of clinical isolates are completely Agr negative (Traber, Lee et al. 2008). Carriage of Agr deficient commensal strains is partly responsible for this (Shopsin, Drlica-Wagner et al. 2008, Smyth, Kafer et al. 2012) but

evidence suggests that Agr shutdown mutations also occur during the infection process (Traber, Lee et al. 2008). This propensity for Agr shutdown strongly implies that, under specific conditions, there is an advantage(s) conferred to Agr deficient strains (and by extension a disadvantage(s) conferred to Agr expressing strains). Indeed, evidence shows that Agr-deficient mutants are selected for in wound and abscess infections models and are better adapted for long-term chronic infections (Schwan, Langhorne et al. 2003, Suligoy, Lattar et al. 2018) as well as being associated with more severe infection outcomes (such as longer duration of bacteraemia (Fowler, Sakoulas et al. 2004)) and higher mortality (Schweizer, Furuno et al. 2011).

These phenomena could be explained in part by the greater biofilm forming ability of Agr deficient strains (Vuong, Saenz et al. 2000, Vuong, Kocianova et al. 2004, Kong, Vuong et al. 2006). Biofilms are known to protect *S. aureus* from host immune clearance (Paharik and Horswill 2016) by not only providing a physical barrier between immune cells and the bacteria, but also by influencing the host immune response. Bhattacharya et. al. show that *S. aureus* biofilms skew the Neutrophil response to the formation of Neutrophil Extracellular Traps (NETs), which are largely ineffective against biofilms, (Bhattacharya, Berends et al. 2018) while Hanke et. al. show that *S. aureus* biofilms cause accumulation of alternatively activated M2 macrophages, which are characterised by anti-inflammatory properties and abrogated degradation of pathogens (Hanke and Kielian 2012). Taken together, biofilms allow for *S. aureus* to escape immune-mediated clearance, which could

contribute to the poorer outcomes associated with infection by Agr deficient strains. Additionally, Agr deficient strains enjoy a lower energy burden relative to Agr expressing strains. *RNAIII* causes, both directly and indirectly, the expression of a large number of secreted toxins and other virulence factors (Bronesky, Wu et al. 2016). The production of these proteins is a costly endeavour for the bacteria, and Paulander et. al. suggest that this energy burden is exacerbated upon exposure to sub-lethal concentrations of antibiotics, imposing a fitness cost (Paulander, Nissen Varming et al. 2012). Furthermore, the human bloodstream is full of host immune factors and cells and creates a tight bottleneck for infecting pathogens: Laabei et. al. demonstrate that when grown *in vitro* in the presence of human serum there is a significant difference in relative fitness between high- and low-toxicity isolates of *S. aureus* (Laabei, Uhlemann et al. 2015). The advantage conferred by an inactive Agr system would mean magnitudes for the bacterial population.

However, the Agr system is not redundant. Indeed, some of the benefits enjoyed by Agr deficient strains seem to be created by an initially active Agr system. For example, an active Agr system is necessary for the initial establishment of osteomyelitis (Gillaspy, Hickmon et al. 1995) and endocarditis (Cheung, Eberhardt et al. 1994), both biofilm-associated infections. Furthermore, specific local production of the Agr-controlled surfactant-like Phenol Soluble Modulins (PSMs) is needed for proper structuring of biofilms and dissemination of cells from medical-device-associated biofilms (Periasamy, Joo et al. 2012). Additionally, Agr deficient strains have seemingly impaired inter-host transmissibility, and the current dogma is that

infections arising from Agr deficient strains are a ‘dead end’ as the infecting strain lineage should go extinct owing to the inability to spread between hosts (Shopsin, Eaton et al. 2010, Laabei, Uhlemann et al. 2015). According to the ‘evolutionary trade-offs’ concept proposed by Laabei et al, highly toxic *S. aureus* isolates that tend to be associated with superficial skin and soft-tissue infections have high inter-host fitness and are thus maintained at the epidemiological level, while low toxicity isolates that have propensity to cause invasive infections (bacteraemia) are impaired in inter-host spread (Laabei, Uhlemann et al. 2015).

Objective

The aim of this work is to investigate methods of adaptation beyond the stress response in the clinically important pathogen *Staphylococcus aureus*. Specifically, this thesis focuses on heterogeneity in the Agr system of *S. aureus*, an occurrence commonly observed in clinical *S. aureus* isolates. Mutations responsible for Agr-negative phenotypes usually locate in the *agrA* and *agrC* hot-spots with known Agr shut-down mutations including frame-shift insertion/deletions, non-synonymous SNPs, and poly(A) tract alterations (Somerville, Beres et al. 2002, Traber and Novick 2006, Adhikari, Arvidson et al. 2007, Shopsin, Eaton et al. 2010). The agr mutants isolated from patients usually have single mutations, suggesting that they are short lived (Shopsin, Eaton et al. 2010, Laabei, Uhlemann et al. 2015), and the current understanding is that these mutants are evolutionary irreversible derivatives which become extinct. Interestingly however, it had been described that Agr dysfunction in the common laboratory strain RN4200 is due to a change in the number of

successive adenines at the 3' end of the *agrA* coding region, being reminiscent of poly(A) tract phase variation (Traber and Novick 2006), but to date the reversibility had not been confirmed. Taken into consideration alongside the ubiquity of Agr dysfunction amongst clinical isolates, this study hypothesised that the phenomenon of Agr dysfunction is a result of reversible phase variation and constitutes a mechanism of heterogeneity resulting in adaptation of S*. aureus* beyond the stress response

In this study, I showed that a fraction of Agr-negative mutants are phase variants and retain the ability to revert their Agr activity. I first generated *in-vitro* Agr-negative variants using the *S. aureus* strains MW2 and s0437 and then subjected the variants to successive subcultures before screening for reversion of Agr activity. I isolated Agr positive colonies from the Agr-negative variants of both strains in this manner. Sequencing the Agr locus of the phenotypically reversible strains identified a genetic mechanism underlying the observed reversibility. In addition, I identified one clinical MRSA isolate, designated 66r, that gave rise to Agr revertant colonies. Although we could not obtain Agr revertant strains from a series of Agr negative primary patient isolates, I did identified colonies that reverted haemolysis, though no mechanism was identified. I demonstrated that the Agr revertant cells reactivate their Agr system when grown on solid media, possibly due to the reduced diffusion of the AIP pheromone from the local microenvironment. Finally, we showed that the Agr revertant cells also activate their Agr system upon phagocytosis by macrophages

and proposed a model whereby the Agr phase variants may play a cryptic role in adaptation to host-immune stress.

CHAPTER 2: METHODS

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1 . Strains were routinely grown in Tryptic Soy Broth (TSB) at 37^{o}C with shaking at 180 rpm. Twelve and a half μg/mL of Chloramphenicol (Cm12.5) was added when necessary.

Strain MW2 and s0437 were used to generate *in vitro* Agr negative mutants and to test their reversibility. Strain N315 was used in some fluorescent experiments.

A total of 173 clinical *S. aureus* samples, comprising 74 MRSA and 99 MSSA isolates, were collected from the Kanto region of Japan. Cells were picked from pure cultures on Müller-Hinton agar plates and grown in Tryptic Soy Broth (TSB) before making glycerol stocks. A further 39 primary patient samples (cotton swabs of infection sites) were obtained from the same source. Swabs were spread on SBA and non-haemolytic colonies were isolated for glycerol stocks.

Construction of agr deletion mutant

An *agr* deletion mutant was constructed by double-crossover homologous recombination using the pMAD-tet vector as described previously (Arnaud, Chastanet et al. 2004, Morikawa, Takemura et al. 2012). Briefly, the upstream and downstream regions flanking the entire *agr* locus were amplified using primer pairs: agr-up-f (BamHI) & agr-up-r (BamHI) and agr-down-f (ecoR1) & agr-down-r (bgl2) (Table 2), and then cloned into pMAD-tet to generate pMAD-tet-Δ*agr*. This plasmid

was introduced into strain MW2 by phage transduction after passaging through strain RN4220 and the *agr* mutant was constructed as previously described (Arnaud, Chastanet et al. 2004). The absence of the *agr* locus was confirmed by lack of haemolysis.

Variant generation

Agr-negative variants were generated from two *S. aureus* strains: MW2 and s0437. Independent cultures of MW2 and s0437 were grown overnight in TSB to stationary phase and then sub-cultured at a 1000-fold dilution into fresh TSB, TSB with 5% human blood, or TSB with 5% serum. Blood was mixed in a 3:2 ratio with filter-sterilised anticoagulation solution. The anticoagulation solution consisted of 0.32% (w/v) citric acid monohydrate, 0.88% (w/v) trisodium citrate dihydrate, and 0.88% (w/v) glucose prepared in Millipore water. Sub-cultures were repeated up to 3 ~ 7 times and samples were plated onto Sheep Blood Agar (SBA) at each stage to screen for non-haemolytic colonies.

Revertant strain generation

Agr-negative variant strains derived from MW2 and s0437 were grown overnight in TSB in 3 independent cultures to stationary phase. Samples were sub-cultured at a 1000-fold dilution into fresh TSB and grown to stationary phase. Subcultures were repeated up to 4 times and serial dilutions of the samples were plated onto SBA at each point to screen for haemolytic colonies.

Sixty-one of the 173 clinical isolates were characterised as being Agr-negative by the CAMP test. All 61 Agr-negative strains were tested in an initial revertant strain generation screening. A single culture of each strain was grown overnight in TSB to stationary phase. Samples were sub-cultured at a 1000-fold dilution into fresh TSB and grown to stationary phase. Subcultures were repeated up to 4 times and serial dilutions of the samples were plated onto SBA at each point to screen for haemolytic colonies.

Thirty of the 39 primary samples were characterised as being Agr negative by the CAMP test. These 30 strains were tested for Agr reversion as described above.

Assessment of Agr activity on sheep-blood agar plates

Traber et. al. demonstrated that a variation of the Christie-Atkins-Munch-Peterson (CAMP) test can be used to assess Agr activity of *S. aureus* by streaking test samples perpendicularly to a *S. aureus* strain that only produces β haemolysin and analysing the resulting patterns of haemolysis for the distinct pattern of Agr-controlled δ haemolysin (Christie, Atkins et al. 1944, Traber, Lee et al. 2008). Thus, the CAMP test was used to assess Agr activity of the isolated variant and revertant strains. An overnight culture of strain RN4220 (only produces β haemolysin) grown in TSB was streaked down the centre of a SBA plate using a cotton swab and the plate was incubated at 37^{o}C for 4-6 hours. Following this, test samples and controls (strain MW2 and its isogenic *Δagr* mutant) were streaked perpendicularly to RN4200, approaching the streak but not coming into direct contact with it. Plates were

incubated at 37^{o}C for 12-16 hours until the haemolysis patterns of the controls became clearly visible. Further incubation at 4^{o}C was carried out, if necessary, to enhance haemolysis.

agr locus sequencing

Genomic DNA was purified using standard procedures. The whole *agr* locus encompassing *hld* (NCBI Gene ID: 1004072, *Staphylococcus aureus subsp. aureus MW2*) through *agrA* (NCBI Gene ID: 1004076 *Staphylococcus aureus subsp. aureus MW2*) was amplified by PCR using the primers agr front and agr back (Table 2), and submitted to direct sequencing using the agr4, agr5, and agr back primers (Table 2) (Fasmac, Japan). The sequence data were analysed using the DNASTAR sequence analysis suite.

Plasmid Construction

The pRIT P3$_{(RNAIII)}$-*venus* Agr-reporter plasmid was constructed by Life Technologies, Japan. This plasmid has the P3-promoter region of RNAIII (-181 to +12 from transcription initiation site), the *venus* fluorescent protein coding region, and the terminator region from *clpB* cloned into the pRIT5H backbone (Inose, Takeshita et al. 2006).

Measurement of reversion frequency

Glycerol stocks of MW2, RVA_{MW2}, MW2 Δ*agr*, and 66r were inoculated in fresh TSB in 10 separate test tubes. Test tube cultures were grown to stationary phase and then sub-cultured at a 1000-fold dilution into fresh TSB. Sub-cultures were successively repeated 4 times in total. Serial dilutions from the final stationary phase sub-culture were plated on SBA and plates were incubated for 24 h at 37 °C after which the reversion frequency was scored as the percentage of haemolytic colonies on the final SBA plates.

Semi-quantitative RT-PCR

Cultures were inoculated in TSB media from glycerol stocks at time T=0. Cells were collected by centrifugation (10 000rpm, 4^{o}C, 5 min) from 2 mL aliquots at times T= 2, 4, 6, 8, 10 hours. Cell pellets were resuspended in 500 µL Tris-EDTA (TE) buffer and were transferred to Watson® tubes containing acid-washed glass beads and 500 µL of Phenol:Chloroform:Isoamyl Alcohol (25:24:1). Cells were lysed for 2 pulses of 60 seconds (with a 60 second interval) at a speed of 6.0 m/s in a Fast-Prep® machine. Samples were centrifuged (15 000rpm, 4^{o}C, 5 min) and aqueous phase lysates were transferred to tubes containing 1 mL of Trizol® reagent. Samples were mixed by inversion and incubated at room temperature for 2 minutes, followed by addition of 200 µL of chloroform. Tubes were centrifuged (15 000rpm, 4^{o}C, 5 min) and the aqueous phase was transferred to fresh tubes in 600 µL aliquots. Equal volumes of Isopropanol were added, and samples were mixed and placed at -20^{o}C overnight.

RNA was precipitated by centrifugation (15 000rpm, 4°C, 30 min) and was washed once with 1mL of 70% ethanol (15 000rpm, 4°C, 30 min).

DNase treatment was carried out using the TurboDNase® kit. Briefly, reaction mixtures of 50 µL were made in MilliQ to contain ~10 µg of RNA and 1x DNase buffer. Zero-point-five microliters of TurboDNase® was added and samples were incubated at 37°C for 30 minutes followed by addition of another 0.5 µL of TurboDnase® and a further 30-minute incubation. Enzyme was inactivated using the kit inactivation reagent (incubation for 2 min at rtp with occasional mixing) followed by centrifugation (11 000 g, 4°C, 2 min) and careful collection of DNA-free RNA. Approximately one microgram of DNase treated RNA was used to generate cDNA in a reverse transcriptase reaction using the SuperscriptIII® reverse transcriptase. A DNA contamination control (lacking reverse transcriptase) was made for each sample. Zero-point-five microliters of the generated cDNA was used for PCR using the QuickTaq® polymerase and the RNAIII and gyrA F/R primers.

Monitoring the population wide Agr activity

S. aureus strains MW2 P3*venus,* MW2*Δagr* P3*venus*, $AV1_{MW2}$ P3*venus,* RAV_{MW2} P3*venus,* N315 pRIT*asp-gfp*, and N315*ΔsigB* pRIT*asp-gfp* were grown overnight in TSB medium with Cm12.5. The *asp-gfp* reporter was used to detect σ^B activity. The number of cells was adjusted to 1×10^9 CFU/ml by referencing the OD_{600}. Each WT was mixed with its respective mutant to obtain a series of samples with final WT

percentage being 0%, 0.1%, 1%, 10%, and 100%. The samples were incubated at 37 °C with shaking at 180 rpm. At 0, 2, 4 and 8 hours, cells were observed by fluorescence microscope (FSX100, Olympus, Center Valley, PA) and the frequency of fluorescent cells was determined. To confirm that Agr-positive WT cells were not lost during the experiment, samples from the 8-hour time point were plated on SBA and the frequency of haemolytic colonies was determined. Additionally, 5 µl of the mixed samples were spotted onto TSA Cm12.5 and were incubated overnight at 37°C. $AV1_{MW2}$ P3*venus* and RAV_{MW2} P3*venus* samples were also spotted onto TSA Cm12.5 in a similar manner. Following incubation, colonies were viewed under a stereomicroscope to check for fluorescence.

Phagocytosis assay

The mouse macrophage cell line, RAW 264.7 (MΦ RAW 264.7; American Type Culture Collection) was maintained in suspension culture in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), L-glutamine (2 mM), and 10% (v/v) heat-inactivated foetal bovine serum (FBS; Equitech-Bio) at 37°C with 5% CO_2.

MΦ RAW 264.7 cells were seeded in a 16-well plate (0.5×10^4 cells/well, 200µl) and were grown over night until reaching a density of 1×10^4 cells per well. The cell culture medium was replaced with drug-free RPMI supplemented with glutamine and 5% FBS 1-hour before the infection.

MW2 P3*venus*, RAV_{MW2} P3*venus*, and MW2 Δ*agr* P3*venus* were grown overnight in TSB with Cm12.5. Cells were harvested by centrifugation (6000 rpm, 2 min, 4 °C) from overnight culture and re-suspended in RPMI. Cell density was adjusted to 1×10^9 cfu/ml by referencing the OD_{600} value. MΦ RAW 264.7 cells were infected with 10 µL of the bacterial cells for a multiplicity of infection (MOI) of ~10. At 1-hour post-infection, the culture medium was replaced with RPMI supplemented with glutamine, 5% FBS, and gentamycin 50 µg/mL and the samples were incubated for 1-hour to kill non-phagocytosed bacteria. Following this, the medium was once again replaced with drug-free RPMI supplemented with glutamine and 5 % FBS. Samples were prepared for microscopy at 1-, 5-, and 9-hours post infection by removing the supernatant and fixing the cells with 200 µL methanol. Images were captured using a light microscope (FSX100, Olympus, Center Valley, PA) and viewed with FSX-BSW software (Olympus, Center Valley, PA). To check whether the presence of MΦ RAW 264.7 affected Agr reversion frequency, bacteria were collected from a separate set of samples after the final time point by lysing MΦ cells with 200 µL of cold PBS supplemented with 0.02% triton X100 and mixing with a pipette. These samples were then plated on SBA and the frequency of reversion was assessed by the frequency of haemolytic colonies and compared to that of the pre-infection samples.

CHAPTER 3: RESULTS

Serial passaging of S. aureus generates Agr-negative variants

By their nature, phase variants emerge spontaneously over time with successive generations of growth. To isolate *in vitro* Agr-negative *S. aureus*, I serially passaged strains MW2 and s0437 and screened for non-haemolytic colonies on SBA. Haemolysis on SBA was used as the first screen as it provided a quick method to distinguish potential Agr negative colonies from a large number of samples. MW2 and s0437 were passaged in TSB, TSB containing 5% human blood, or TSB containing 5% human serum. Non-haemolytic colonies did not appear in some experiments even after several passages, but in others a large number were detected. Namely, the emergence of non-haemolytic colonies fluctuated greatly due to their spontaneous nature. All growth conditions allowed for generation of non-haemolytic variants. In total, 209 out of 14,948 MW2 colonies, and 4 out of the 308 s0437 colonies were non-haemolytic.

Agr-negative variants can phenotypically and genotypically revert to the wild type

I then tested if the isolated Agr-negative variants could revert their Agr activity over multiple generations. I carried out successive passages of 30 isolated Agr-negative variants and plated serial dilutions of the cultures onto SBA to check for the generation of any haemolytic colonies. Two *agr*-negative variants (termed RAV_{MW2}: Reversible Agr Variant derived from MW2, and RAV_{s0437} from s0437) were able to

reproducibly give rise to haemolytic colonies. To verify that these colonies were Agr-revertant colonies, I carried out CAMP tests and confirmed the presence of the characteristic δ-haemolysin arrowhead in the revertant colonies that was absent in the Agr-negative variants (Fig. 6). The average frequency of reversion after 4 successive cultures (i.e. the number of haemolytic colonies compared to non-haemolytic colonies on SBA plates) was ~2% in RAV_{MW2} and ~0.8% in RAV_{s0437} (Table 3). I also noted that RAV_{MW2} generated 4-fold higher number of rifampicin resistant colonies (median = 2.3×10^{-8}, mean =2.7×10^{-8}, n=10) after one overnight culture compared with MW2 (median = 5.4×10^{-9}, mean =6.8×10^{-9}, n=8) ($p<0.01$, in a two-tailed t-test), although this study did not further address if the higher mutation rate is responsible for the reversion rate of RAV_{MW2}. The background mutation rates (measured by rifampicin resistance) in the s0437 WT strain (median = 1.4×10^{-7}, mean = 1.4×10^{-7}, n=10) and RAV_{s0437} (median = 1.0×10^{-7}, mean = 9.7×10^{-8}, n=10) were also determined and no significant difference was observed. Interestingly, I observed a differential effect of 5% Human serum on the reversion frequency of RAV_{MW2} (0.45 ± 0.48, n=10) and RAV_{s0437} (3.27 ± 1.92, n=9) when compared to the frequencies in TSB. This could be due to the different mechanisms of phase variation observed in the two strains, suggesting the existence some sort of mechanistic pathway, but this was not explored further in this work.

Sequencing of the *agr* locus of the variant (both the reversible isolates and some of the irreversible isolates) and revertant strains uncovered underlying genetic mechanisms for Agr shutdown (Fig. 7). RAV_{MW2} had a duplication and inversion in

agrC. The *agr* locus in the RAV_{MW2} revertant strain was identical to the WT. RAV_{s0437} had an alteration in a poly(A) tract within *agrA*, which reverted to the WT sequence in the RAV_{s0437} revertant strain. Most of the sequenced irreversible Agr-negative MW2 isolates had insertions and/or point mutations across the *agr* locus in line with previous studies (Somerville, Beres et al. 2002, Shopsin, Drlica-Wagner et al. 2008, Traber, Lee et al. 2008). Although one irreversible isolate ($AV3_{MW2}$) had a duplication within *agrA*, I was unable to generate any revertant strains. Taken together, these results show that a fraction of the Agr-negative strains isolated *in vitro* can phenotypically revert their Agr activity and that there are multiple underlying mutational events behind this reversion.

I also carried out the revertant strain generation procedure on 61 clinically isolated Agr negative strains. Although some strains showed phenotypic Agr reversion in some experiments, only one strain, an MRSA isolate designated RAV(66r), was reproducibly reversible (Table 3). However, the detection limit of the initial screening was ~ 3.0×10^{-3}, and thus the possibility remains that more reproducibly reversible strains could be identified using a higher detection limit. The mean frequency of reversion in RAV(66r) was 0.12% (Table 3). Upon sequencing of the *agrA* and *C* locus of RAV(66r) and the RAV(66r) revertant strain, a reversible poly(A) tract alteration was found in *agrA* (Fig. 7). The average background mutation rate for RAV(66r) was 7.0×10^{-9} (median = 5.4×10^{-9}, n=10).

A summary of all the Agr variant (AV) and revertant strains generated or collected in this study, along with the growth conditions used for their generation as well as the origin strain, can be found in Table 3 in Appendix 1.

RAV strains have different Agr shutdown characteristics

A study by Gomes-Fernandes et. al. identified that there can be a discordance between CAMP assay results and actual RNAIII expression (Gomes-Fernandes, Laabei et al. 2017). Therefore, I followed-up the CAMP results for the variant and revertant strains with semi-quantitative RT-PCR using primers for *RNAIII* and the housekeeping *gyrA* gene. Intriguingly, although I observed a clear abolishment and subsequent recovery of *RNAIII* expression in the MW2 and 66r variant and revertant strains respectively (Fig 8A), RAV_{s0437} shows a time-dependent impairment of *RNAIII* expression with *RNAIII* levels impaired at 4 hours of growth but recovered by 6 hours (Fig 8B). This could suggest a differential Agr-shutdown mechanism (a delayed activation, for example) dependent on the underlying genetic mutation creating the variant, though this possibility remains unexplored.

A minor population of agr-intact cells does not activate its own Agr system in an AIP non-producing population but can through local segregation on solid media.

Due to its quorum sensing nature and the low reversion frequencies, it is expected that the minor population of revertant cells emerging from the Agr-negative population in a liquid culture would sustain the Agr OFF state. Revertant

cells may be able to activate their Agr system on solid structured media as it allows for segregation of the revertant cells into a discrete locality.

To test this, Agr activity was monitored using a *venus* reporter system under the control of the *agr* P3 promoter in populations of an isogenic *Δagr* strain unable to produce any AIP (MW2 *Δagr* P3*venus*) with various percentages of *agr*-intact cells (MW2 P3*venus*) mixed in, as well as in populations of the reversible RAV_{MW2}. Venus fluorescent protein is stable enough to detect even transient activation of a promoter at the end point of the experiment. When spotted onto TSA Cm12.5, no fluorescence was observed in the spotted areas of samples with an MW2 P3*venus* content less than 10% (Fig. 9A). However, I was able to visualise streaks of localised fluorescence on the outer edges of spotted areas in samples with an MW2 P3*venus* content as low as 0.1% (Fig. 9A). This suggests that a minor population of *agr*-intact cells can activate their Agr system upon propagation on solid media, likely because the AIP concentration in the locality can exceed the threshold. A similar pattern of fluorescence could be detected in RAV_{MW2} but not in the irreversible strain $AV1_{MW2}$ (Fig. 9B). RAV_{MW2}, which carries a mutation in *agrC,* would still be able to produce basal levels of AIP, but the local signals in the RAV_{MW2} colony suggests the presence of a minor population of *agr*-intact revertant cells that were generated through colony expansion.

Fig. 9C shows a control reporter system expressing *gfp* under the control of the SigB dependent *asp* promoter in N315 and its isogenic *ΔsigB* strain. GFP-positive cells remained detectable within the entire spotted area of samples with low N315

content (Fig. 9C), confirming that a quorum-independent reporter system can be detected in the same experimental settings.

When the proportion of Agr-positive cells in liquid culture corresponded to the observed reversion frequency, i.e. <10%, reporter activity was undetectable during the time course tested (Fig. 10A). In contrast, when the proportion of WT cells was increased to 10%, reporter expression was observed, suggesting that this is the threshold that is necessary for Agr activation in planktonic conditions. The frequency of SigB active cells remained constant and detectable for all samples over the time course tested (Fig. 10B). To eliminate the possibility that the *agr*-intact cells had been lost during the time course tested, we plated the samples on SBA after the experiment and confirmed that the frequency of haemolytic cells had not changed from the start of the experiment (Fig. 10C).

Taken together, these data indicate that a minor population of Agr revertant cells does not activate its Agr system when growing in planktonic conditions.

Phagocytosis triggers expression of the Agr system in revertant cells

The results demonstrate that *agr*-intact cells sustain the Agr OFF state when they are a minority (<10%) in an Agr-negative population. It is known that a single *agr*-intact cell can activate its own Agr system when it is packed into a narrow matrix mimicking the phagosome environment (Carnes, Lopez et al. 2010) in a phenomenon termed 'diffusion sensing' (Redfield 2002, West, Winzer et al. 2012) and that Agr

activation is induced upon phagocytosis (Tranchemontagne, Camire et al. 2015, Münzenmayer, Geiger et al. 2016). Therefore, the phagosome environment must allow *agr* revertant cells to switch the Agr system ON.

To test this, a phagocytosis assay on RAV_{MW2} P3*venus* was carried out. RAV_{MW2} overnight culture contains minor revertant cells (see Fig. 11C, RAV_{MW2}, Before infection). These minor revertant cells are negative in GFP expression in liquid culture without macrophages (Fig 11A, - MΦ). As expected, fluorescent cells were detected within macrophages at 4 and 8 hours post infection (Fig. 11A, B). To test whether the presence of macrophages affected the *agr* reversion frequency, serial dilutions of RAV_{MW2}P3*venus* were plated on SBA before and after infection. The frequency of *agr* revertant haemolytic colonies was not significantly different between the two time points, indicating that macrophages do not affect *agr* reversion (Fig. 11C). Taken together, these results suggest that revertant cells activate their *agr* system when in the confined environment of the phagosome and suggests a possible function of revertant cells.

Prevalence of haemolytic revertant strains amongst clinical isolates

The previous collection of clinical samples had undergone multiple clonal expansions before I received them. Thus, it is possible that any highly reversible Agr variants would have already reverted their Agr activity before we received the samples and would end up being characterised as Agr positive. Therefore, in order to assess the significance of our findings in the clinical field, I collected a series of primary patient samples from Kotobiken laboratories. These samples had not

undergone clonal expansion since their isolation from the patient and thus if any hyper reversible Agr variants were present in the clinical setting, they would be identified using these samples. I identified one strain out of the thirty Agr negative primary patient samples that phenotypically reverted its Agr activity (Fig. 12 B). However, upon sequencing its Agr locus, I could only identify a single SNP within the *agrC* ORF that did not correspond to any known phase variation mechanisms. Interestingly however, I identified two strains that reverted haemolysis without reverting to an Agr positive phenotype (Fig. 12C). Furthermore, I identified a single strain that reverted to a CAMP phenotype that is characteristic of Alpha haemolysin (*hla*) production (Fig 12D). However, I could not identify any mutations in either the *agr* locus (direct controller of *hla*) or in the *hla* locus; thus the mechanism of Hla reversion remains elusive.

CHAPTER 4: DISCUSSION

In this study I show that some Agr-negative isolates generated *in vitro*, as well as those isolated from patients, can phenotypically revert their Agr activity with underlying genetic mechanisms reminiscent of phase variation. In addition, while revertant cells are generated at a low frequency, allowing them to sustain an Agr OFF state in planktonic culture, they can activate their Agr system upon phagocytosis. This suggests a cryptic strategy for the bacterial population to survive phagocytic stress and allow for continued infection (Fig 13).

It is clear that the role of the Agr system and its activation during the infection process is an extraordinarily complex and detailed operation. There are demonstrated advantages to having an inactive Agr system and yet evolutionarily it seems implausible that such an important gene regulatory system would be mutationally inactivated in a 'dead end' manner. Conceptually, the evidence suggests that the unattainable ideal would be an Agr system whose function could be switched on and off at will. My data demonstrating the emergence of reversible Agr phase variants could in part address this complicated phenomenon. I show that a small fraction of Agr deficient strains can genotypically and phenotypically revert to an intact Agr system (Fig. 6). Considering this, it is feasible to suppose that upon successfully establishing infection, *S. aureus* could inactivate its Agr system, leaving behind subpopulations that can revert to a genetically intact Agr locus. Importantly, while these subpopulations do not express Agr when present as a minority in planktonic conditions (Fig. 10A), I show that they can cause local Agr activation when

grown on solid structured media (Fig. 9A), possibly due to the local extracellular AIP concentration exceeding the required threshold for activation. These Agr active subpopulations could, for example, produce the low-level of PSMs needed for proper biofilm structuring, as well as allow subpopulations of cells to carry out an exodus from the mature biofilm, while enabling the population as a whole to enjoy the Agr-deficient benefits of biofilm-mediated immune evasion and a low energy burden.

Regarding impaired inter-host transmission of Agr deficient strains, Shopsin et. al have shown that family members have been colonised by identical Agr-deficient strains, suggesting that these strains are in some way transmissible (Shopsin, Drlica-Wagner et al. 2008). Interestingly, Kernbauer et. al. have demonstrated that intravenous injection of *S. aureus* in a murine model, even at non-lethal doses, results in dissemination of the bacteria from the blood to the gastrointestinal tract (presumable via the gall bladder) and subsequent faecal shedding and transmission to naïve mice housed in the same cage (Kernbauer, Maurer et al. 2015). Importantly, an isogenic Agr deletion strain did not exhibit any impairment in dissemination or transmission, strongly suggesting that Agr deficient strains could attenuate their poor inter-host fitness via gastrointestinal dissemination.

A common mechanism of phase variation is frame-shifts within short sequence repeat (SSM) regions, such as ploy(A) tracts, resulting from slipped-strand mispairing (SSM) events which causes changes in transcription or translation (van Belkum, Scherer et al. 1998, Buckling, Neilson et al. 2005). In our study, I describe

two strains which exhibit this pattern of phase variation mutation (Fig. 7). Although one of these strains, s0437, is a lab strain the other , 66r, was obtained from a collection of clinical isolates gathered from the Kanto region of Japan. However, only one of the isolates within that collection demonstrated Agr reversion, thus statistical descriptions of the prevalence of Agr reversion could not be made. Interestingly though, a study investigating the link between epidermal *S. aureus* colonisation in children and the development of Atopic Dermatitis (AD) observed that virulence was correlated with the development of AD. They noticed that continuous *S. aureus* epidermal colonisers of children who did not present with AD were associated with mutations within the Agr locus with the mutation rate of the locus being in the order of 10^{-5}, which is within the lower threshold of phase variation. Importantly, although their sample size was small (4 mutated clones detected from a sample of 24), two of the frame-shift mutations they observed were poly(A) alterations within the coding sequences of *agrA* and *agrC*, respectively (Nakamura, Takahashi et al. 2020). The nature of the mutations, coupled with the mutation rate, strongly suggests that these Agr deficient strains could be phase variants.

Unlike our other identified variants, RAV_{s0347} showed an interesting pattern of delayed *RNAIII* production rather than outright abolishment, with expression returning to the WT pattern in the s0437 revertant strain (Fig. 8B). This is not the first such report of delayed Agr activation conferring an Agr negative phenotype. Traber et. al. solved the longstanding mystery of *S. aureus* strain RN4220's Agr negative phenotype, and its parent's RN450's mixed haemolysis, by discovering that there was

a poly(A) tract alteration in the coding sequence of *agrA* that resulted in delayed Agr activity (Traber and Novick 2006). Indeed, the s0437 variant has a poly(A) tract alteration in its *agrA* sequence. Interestingly, a study by Sloan et. al. showed that natural mutations in *agrC* have an effect on the temporal activation of Agr, attenuating virulence (Sloan, Murray et al. 2019). In an elaborate study Wang et. al. have demonstrated that there is a contiguous helix between the two domains of AgrC (the Dimerisation and Histidine Phosphotransfer domain, which includes the AIP binding site, and the Catalytic and ATP-binding domain) which rotates upon AIP binding and transmits the conformational change that allows for activation of AgrA (Wang, Zhao et al. 2017). Xie et. al. have identified a non-covalent interaction of amino acids in AgrC that functions as a 'latch' to stabilise the histidine kinase in its inactive form that must be 'opened' by the helical twisting induced by AIP binding before activation of AgrA can occur (Xie, Zhao et al. 2019). Sloan et. al. suggest that some of the naturally occurring AgrC mutations they observed indirectly strengthen the hydrogen bond interaction of the abovementioned latch requiring a greater twisting torque input from AIP binding before AgrA can be activated (Sloan, Murray et al. 2019). Furthermore, studies by Geisinger et. al. and Wang et. al. have shown that there is a striking difference in temporal activation of Agr between the four Agr allelic types and that this results from differential AgrC autokinase activity in the cognate AIP bound state, proposed to be due to differential intensities of interactions of the cognate AIP with the AgrC sensor domain (Geisinger, Chen et al. 2012, Wang, Zhao et al. 2017). There is a clearly documented association between

infection niches and Agr types, with clinical isolates from discrete infection sites rarely exhibiting Agr type variation (Traber, Lee et al. 2008, Wang and Muir 2016). Could the temporal differences in Agr activation amongst the allelic types have evolved to adapt each type their different preferred infectious niches? Taking all the evidence into account, it may not be too far-fetched a suggestion that phase variation within the Agr locus that results in changes in the temporal activity of Agr, due either to mutations in *agrA* or in *agrC*, could be a strategy that allows a single *S. aureus* clone access to different infectious niches.

Although the Agr revertant strains did not activate their Agr system when growing in planktonic conditions, the single revertant cells were able to activate their Agr system when they were phagocytosed (Fig. 11). While *S. aureus* is not a classical intracellular pathogen, it has long been known that it can survive phagocytosis (Rogers and Tompsett 1952). Tranchemontagne et. al. show that not only does the highly virulent USA300 clone of *S. aureus* survive macrophage phagocytosis, it can actually comfortably replicate within the phagosome (Tranchemontagne, Camire et al. 2015). Importantly, they showed that acidification of the phagosome was vital for this survival and replication ability, and that this was due to the low pH activating the Agr system. Very interestingly, in accordance with a study from Kubica et. al. (Kubica, Guzik et al. 2008), they show that unlike in non-phagocytic cells, *S. aureus* does not immediately escape to the cytoplasm upon phagocytosis by macrophages. Instead, the majority of S*. aureus* cells remain enclosed within the phagosome for a number of days, without compromising Macrophage viability or function, before finally

escaping the macrophage by causing cell lysis (Kubica, Guzik et al. 2008, Tranchemontagne, Camire et al. 2015). Critically, survival within, and eventual escape from, the phagosome has been clearly demonstrated to be dependent on the Agr system (Kubica, Guzik et al. 2008, O'Keeffe, Wilk et al. 2015, Tranchemontagne, Camire et al. 2015, Münzenmayer, Geiger et al. 2016). The evidence of *S. aureus'* survival within the phagosome vacuole and its delayed escape from it has led a number of reports to suggest that phagocytic immune cells could function as a 'Trojan Horse' for disseminating *S. aureus* throughout the body (Edwards and Massey 2011, Thwaites and Gant 2011, Horn, Stelzner et al. 2018). The potential benefits of this strategy are notable. Enclosed within phagocytic cells, *S. aureus* would not only be protected from the worst of the immune complement attack but would also be sheltered from many antibiotics. Furthermore, cells like macrophages are relatively free-moving within the host vasculature and tissue and could thus be an efficient way for *S. aureus* to travel to far reaching sites to establish secondary infections. Indeed, there are studies that support this suggestion. Gresham et. al. have shown that polymorphonuclear Neutrophils isolated from sites of *S. aureus* infection contain viable bacterial cells, and that these Neutrophils are sufficient to establish infection in naïve mice (Gresham, Lowrance et al. 2000). This finding was confirmed more recently by Lehar et. al. with the additional observation that intracellular *S. aureus* reservoirs were protected against Vancomycin (Lehar, Pillow et al. 2015). Taken together, there is the strong possibility that the Agr phase variants act as a cryptic strategy against host immunity, remaining hidden within the Agr negative population

but becoming active upon phagocytosis whereby they subvert the host immune cells and utilise them to escape to new sites of the body.

While I was unable to determine the prevalence of Agr reversion in the clinical setting, I noticed reversion of haemolysis amongst a number of primary clinical isolates (Fig. 12C). Altman and colleagues have described the tendency of Agr defective *S. aureus* to accumulate mutations during infection (Altman, Sullivan et al. 2018). They observed that one of their samples had acquired mutations that increased its virulence in a murine bloodstream infection model while still having a defective *agr* locus. Thus, it is possible that compensatory mutations that revert aspects of virulence while bypassing the Agr system can be selected for during the infection process. Very interestingly, I also identified a single strain that reverted to an Alpha haemolysin-positive phenotype (Fig. 12D). Although I could not identify any genetic changes within the *agr* or *hla* ORFs, it is possible that there is a reversible mutation in a higher regulatory pathway that results in this phenotypic switching.

Heterogeneity within bacteria is not an uncommon phenomenon, and recent studies have uncovered several mechanisms that bacteria employ to generate this variation within their populations.

Many transcriptional regulators are embedded with a positive-feedback response and are thus primed to take advantage of biological noise to generate heterogenous subpopulations and phenotypic variation. A good example of this is competence development versus sporulation in *Bacillus subtilis* (Grimbergen, Siebring et al. 2015). Endospore formation is an effective strategy at surviving

starvation and under nutrient depletion most cells in a colony activate the sporulation programme driven by the sporulation regulator Spo0A (Veening, Smits et al. 2008). However, a small number of cells can instead activate a state of natural competence (allowing for the uptake of extracellular DNA), and this is driven by the competence regulator ComK (Martins and Locke 2015). Sporulation and natural competence are mutually exclusive processes, however the pathways can progress independently of each other within a cell until a certain point is reached, after which the cell must choose its fate based on the relative levels of ComK and Spo0A (Martins and Locke 2015). ComK has a positive feedback circuit which generates a bistable expression system, resulting in cells highly expressing ComK to proceed down the natural competence pathway while those that do not continue to form endospores (Veening, Smits et al. 2008).

Bacteria can also incorporate extrinsic noise (fluctuations in the microenvironment potentially preceding a drastic global environmental shift) to regulate intrinsic biological noise and generate phenotypic variation for bet-hedging. An example of this is elegantly described by Carey et. al. in *Escherichia coli* anaerobic respiration (Carey, Mettert et al. 2018). In the absence of oxygen, *E. coli* can use trimethylamine oxide (TMAO) as a terminal electron acceptor in the respiratory pathway. This requires expression of the *torCAD* operon which is controlled by the TorTSR two-component system. The TorTS complex is activated by TMAO in the bacterial periplasm and phosphorylates the response regulator TorR. Carey et. al. show that in the presence of oxygen, there is high variability in *torCAD* expression,

and only cells expressing a high amount of *torCAD* can survive upon rapid oxygen depletion from the environment since the adaptation time-lag is too long for the maladapted cells. However, the variability in *torCAD* expression is not due to noise in *torCAD* expression but instead due to noise in *torTS*. Sensing of periplasmic TMAO is dependent on TorT while phosphorylation of the response regulator is carried out by TorS. Therefore, stochasticity in the relative ratios of TorT and TorS will strongly affect the levels of phosphorylated TorR, directly affecting *torCAD* expression. Furthermore, Carey et. al. demonstrate that *torTS* transcription itself is controlled by an oxygen-sensitive transcriptional regulator, IscR. Under aerobic conditions IscR represses *torTS* transcription which drastically reduces the number of TorT and TorS molecules in the cell, increasing the propensity for stochastic fluctuations in their relative ratios and thus ultimately driving variability in *torCAD* expression and the generation of subpopulations that are pre-adapted to rapid oxygen depletion. This is an elegant example of how environmental cues can be incorporated into stochastic phenotypic variability.

Phenotypic heterogeneity can often be observed in nutrient metabolism/uptake, and an example is described by Solopova et. al. (Solopova, van Gestel et al. 2014). They demonstrate that, contrary to the traditional model of bacterial diauxie, the lag period of growth exhibited by *Lactococcus lactis* during a shift from glucose to cellobiose metabolism is a result of a bifurcation of the population into two stable cell states: a cellobiose metabolising and non-metabolising population. The non-metabolising population remains viable but does not grow in the presence of

cellobiose. The group shows that the decision for the split seems to be dependent upon two factors: the timing of the initiation of the stringent response after Carbon Catabolite Repression (CCR) is lifted and the history of the metabolic pathway of the cell in question. *L. lactis* can activate two metabolic routes: homolactic and heterolactic fermentation. While both routes can be used for glucose fermentation the latter is the only route used for cellobiose fermentation, and cells that have activated this route when using glucose are those that go on to metabolise cellobiose after the nutrient source switch. Initially, it seems like the cellobiose non-metabolisers are a failed dead-end. However, the group identified that this subpopulation is able to grow more rapidly if there is a further change of carbon source from cellobiose to galactose. Computer modelling predicted that a population that is heterogenetic for cellobiose metabolism would have the greatest geometric fitness in an environment with unpredictably changing carbon sources, characteristic of a bet-hedging strategy.

Very interestingly, phenotypic variance in a population can be achieved epigenetically through the phenomenon of hysteresis, as alluded to by the results of Solopova et. al. when they demonstrated that *L. lactis* metabolic history plays a role in the bifurcation of the population into cellobiose and non-cellobiose fermenters (Grimbergen, Siebring et al. 2015). Hysteresis can be defined as a situation where a physical property lasts longer than the effect that caused it (Veening, Smits et al. 2008). In the biological sense, this could be a protein that remains stable and existent in the cell long after the stimulus that caused its expression is over (e.g. one involved

in nutrient uptake). If this active protein is then unevenly passed on to daughter generations of cells it can result in epigenetically inherited bistability. A particularly good example of this is the well-studied *lac* operon of *E. coli*. In perhaps one of the earliest examples of bistability, Novick et. al. noticed that at threshold concentrations of inducer *E. coli* cells bifurcated into two subpopulations, *lac* expressing and non-expressing, the split being influenced by the recent history of the cells (Novick and Weiner 1957, Cohn and Horibata 1959). It was later identified that this was due to the stability of the lactose permease protein. Cells in which the *lac* operon was previously induced contained stable active lactose permease within their membranes which could be distributed unevenly to daughter cells upon cell division. Daughter cells that received a relatively larger number of active lactose permease form the parent cell were able to efficiently take up the inducer molecule from the environment even at very low concentrations, initiating the positive feedback cycle of the operon and ensuring *lac* expression remained active, bifurcating the population into two subpopulations (Müller-Hill 1996, Veening, Smits et al. 2008). This phenomenon was termed “all-or-none” enzyme induction (Novick and Weiner 1957) and is a striking example of how epigenetic mechanisms, rather than biological noise, can create heterogeneity.

Perhaps the most clinically relevant application of phenotypic heterogeneity is the role it plays in bacterial persistence (Maisonneuve and Gerdes 2014). Persister cells comprise a small sub-population that is characterised by arrested or vastly attenuated cell growth resulting in transient tolerance to lethal doses of antibiotics.

Persister cells can revert back to a non-persister phenotype allowing for proliferation pf the bacterial population once antibiotic stress has been lifted. Thus, bacterial persistence has been held accountable for recurrent outbreaks of bacterial infection once antibiotic therapy has been ended, and its importance in chronic infections and the subsequent healthcare burden should not be ignored. Bacterial persistence is generally induced by toxin-antitoxin (TA) modules (Maisonneuve and Gerdes 2014). TA modules consist of a stable toxin molecule, which targets and inhibits an essential cellular process leading to growth arrest, and an unstable antitoxin molecule which inhibits its cognate toxin. Activity of the toxin molecule has been convincingly shown to be responsible for a switch into a persister phenotype, with *E. coli hipAB* being the first TA module identified as a persister gene (Moyed and Bertrand 1983). Earlier work demonstrated that persister cells are a pre-existing subpopulation rather than an inducible one (Balaban, Merrin et al. 2004), suggesting a stochastically generated bet hedging mechanism. Interestingly, TA modules have a complex self-regulatory mechanism, termed conditional co-operativity, whereby the toxin acts as both a co-repressor and de-repressor of its own expression (Maisonneuve and Gerdes 2014). The antitoxin component neutralises the toxin but also represses transcription of the TA operon, in both its free and toxin-bound state. At low levels, the toxin is completely bound to the antitoxin and the TA complex binds strongly to the operon's promoter region, repressing transcription. In contrast, transcription is de-repressed when there are high levels of free toxin, which promotes operon transcription. Furthermore, cellular proteases (e.g. Lon or Clp proteases) degrade the antitoxin

component (Maisonneuve and Gerdes 2014), adding an additional layer of regulation. Lastly, it has been demonstrated that a threshold level of the toxin is necessary to induce dormancy, and the degree by which this threshold is surpassed dictates the time of toxin-induced dormancy (Maisonneuve and Gerdes 2014). Taken together, TA modules are bistable systems, primed to exploit cellular noise and create a bifurcated population. Maisonneuve et. al. and Germain et. al. (Germain, Castro-Roa et al. 2013, Maisonneuve and Gerdes 2014) elegantly construct a system wherein the bifurcation of a population into persister and non-persister phenotypes is dependent on cellular concentrations of the alarmone (p)ppGpp. The model relies on stochastic activation of RelA and/or SpoT, by an as-yet unknown mechanism, to create a subpopulation with high alarmone levels. This inhibits the Exopolyphosphatase (PPX) enzyme that is responsible for the degradation of inorganic phosphate (PolyP), resulting in an accumulation of PolyP which stimulates Lon proteases to degrade the antitoxin component of TA modules, increasing free toxin molecules leading to a persister phenotype. Alarmone molecules positively stimulate RelA, allowing for amplification of the initial noise (Shyp, Tankov et al. 2012). Furthermore, the HipA toxin can inactivate glutamyl tRNA-synthatase (GltX) by phosphorylation (Germain, Castro-Roa et al. 2013, Kaspy, Rotem et al. 2013) which leads to accumulation of uncharged $tRNA^{Glu}$ which is speculated as a second positive feedback loop in the above model (Maisonneuve and Gerdes 2014) leading to even greater single-cell accumulation of (p)ppGpp. A different study by Verstraeten et. al. (Verstraeten, Knapen et al. 2015) links single-cell variability in

levels of *E. coli* Obg (a conserved GTPase that is at the nexus of DNA and protein synthesis) and (p)ppGpp to persister cell formation through a different TA module. They show that subpopulations of cells expressing high levels of Obg switch to a persister phenotype by the Obg -dependent expression of the HokB toxin, which reduces membrane potential and arrests respiration. This role of Obg is not related to its GTP metabolism or ribosome association and requires (p)ppGpp. These findings robustly demonstrate the stochastic nature of the underlying mechanisms of the persister phenotype.

Although fewer in number, there are reports that have uncovered heterogeneity in *S. aureus*. A report by Buckling and colleagues (Buckling, Neilson et al. 2005) demonstrated phase variation in the *mapW* gene that was the result of an SSM event. The *S. aureus* Map protein is a multifunctional protein that is important in pathogenesis, possessing immunomodulatory activities that affect human T-cells (Lee, Miyamoto et al. 2002, Harraghy, Hussain et al. 2003). There is also robust evidence of variation in the biofilm phenotype of *S. aureus*, and interestingly there seem to be numerous mechanisms of phase variation that are responsible for this. Tormo et. al (Tormo, Úbeda et al. 2007) show that phase-variable expression of the Biofilm-Associated Protein (Bap) can create biofilm-negative colonies for a WT strain. Kiem et. al. show that *icaC* of the Intracellular Adhesin (ica) operon can be inactivated by the insertion sequence IS256, while Valle et. al. (Valle, Vergara-Irigaray et al. 2007) provide evidence of an extra layer of control to this by showing that Sigma B regulates IS256. Finally, Brooks and colleagues (Brooks and Jefferson 2014)

show that phase variation in *icaC* can also arise through an SSM event. Taken together, these evidences lead to the curious observation that all documented instances of phase variation in *S. aureus* occur in systems that are crucial for pathogenesis: either in immune-modulatory or virulence proteins. Does phase variation not exist within other, more benign gene systems in *S. aureus*? If so, what does that mean for the pathogenic lifestyle of this organism? The existence of reversible shut-down mechanisms within virulence factors strongly suggests that these genes are only useful during host pathogenesis. The development of such an elaborate control mechanism could have important implications for the evolutionary history of this organism. Did this dangerous pathogen make a shift to commensalism through its history, evolving mechanisms to shut down virulence to allow this? If so, this would indicate that commensalism is the preferred lifestyle of *S. aureus*. Could we hasten this evolutionary drive and thus push *S. aureus* firmly into the niche of a commensal, removing its threat as a pathogen? While these questions may not provide any immediately tangible benefit to clinical treatment plans, they may be valuable avenues of research to better understand, and ultimately control, this ubiquitous bacterium.

CHAPTER 5: CONCLUDING REMARKS

In conclusion, this study shows that Agr defective strains can be phase variants that arise by reversible genetic mutations within the *agr* locus. These revertant cells cannot activate their Agr system when growing as a minority in planktonic populations but can activate it on solid structured media or, importantly, in the confines of a phagosome. This ability of the revertant cells to remain hidden when they are a minority but to awaken upon host immune attack and unleash their arsenal of weapons could have developed as an adaptive strategy beyond a simple response to immune stress (Fig. 13). These subpopulations are not Agr-active as a minority and thus do not disrupt the Agr negative phenotype of the whole population. This allows them to enjoy the benefits of a sessile lifestyle that can expertly evade host detection and killing. However, in the chance that the host is able to mount a successful immune response, these populations are not only able to survive phagocytic attack, but may also be able to use the host phagocytes as trojan horses and migrate to greener pastures within the body. These findings add a new layer of complexity to the phenomenon of Agr dysfunction in *S. aureus* and take us a step further in understanding the multi-faceted lifestyle of this important pathogen.

APPENDIX 1: FIGURES AND TABLES

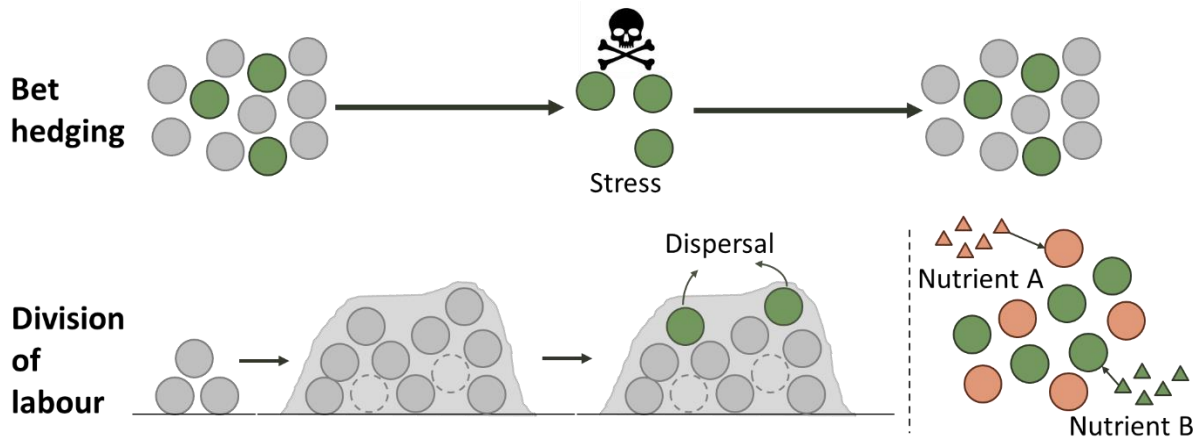

Fig. 1 Heterogeneity in Bacterial Populations Allows for Population-Level Strategies

Heterogeneity creates subgroups of specialised cells within the larger population. This allows the bacterial population to have a diverse mix of phenotypes that can, amongst other things, either aim to predict future stress conditions to improve the population's geometric mean fitness or can allow the population to carry out division of labour. Division of labour can either be in the form of groups of cells carrying out individual tasks to create a complex phenotype, such as biofilms, or groups of cells expressing different gene systems enabling selective consumption from a diverse nutrient pool, increasing efficiency of resource management.

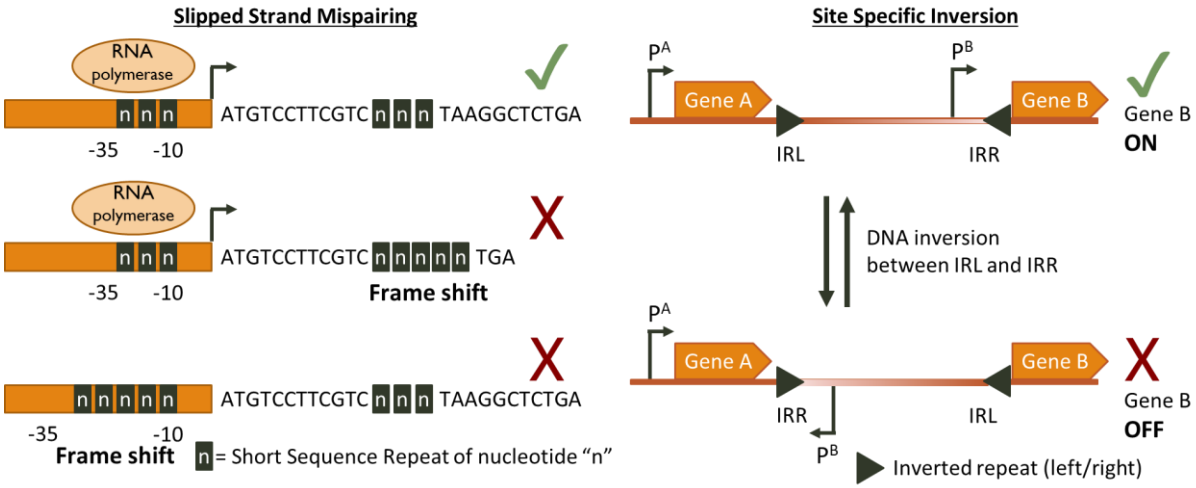

Fig. 2 Genetic mechanisms of Phase Variation

Left: A Short Sequence Repeat (SSR) is a run of identical nucleotides in the DNA sequence, and commonly occurs with Adenine (A)/ Thymine (T). A Slipped-Strand Mispairing (SSM) can alter the length of these A/T tracts and cause a frame shift. Depending on the location of the SSR, this can either result premature termination in translation or can alter the promoter region and prevent polymerase binding and subsequent transcription.

Right: Site-specific inversion occurs between two inverted repeat sequences. If the promoter region of a gene (depicted in cartoon) or the gene's ORF lies within the region that is spanned by the inverted repeats, a site-specific inversion event will temporarily shut-down gene transcription.

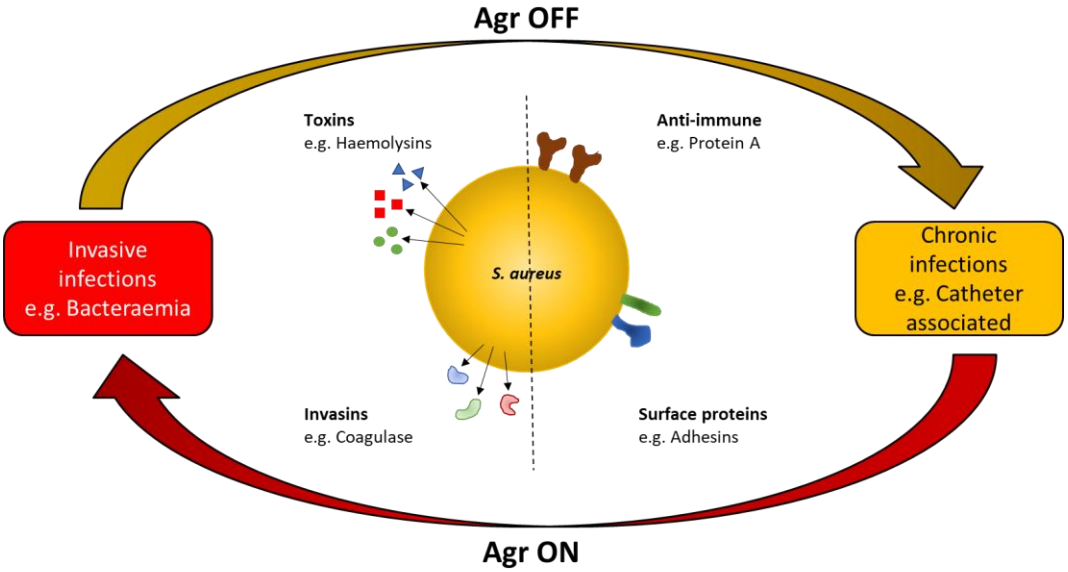

Fig. 3 Agr mediated control of *Staphylococcus aureus'* lifestyle

S. aureus has a manifold arsenal of virulence factors that allow it to inhabit a diverse array of host niches. Some of these factors, such as Protein A and cell-surface adhesins, are tailored to enable a more sessile lifestyle which is characterised by immune evasion and chronic infections. Others, such as the haemolysins and invasins, are geared for a more aggressive lifestyle and often cause a lot of damage to host tissues. The balance between the two approaches must be delicately maintained, and this is achieved by the Agr system. Upon Agr activation, the expression of virulence factors promoting invasive infections is upregulated while those enabling chronic infections are suppressed and *vice-versa*. This global control allows *S. aureus* to switch its virulence expression profile to best meet its needs in its current environment.

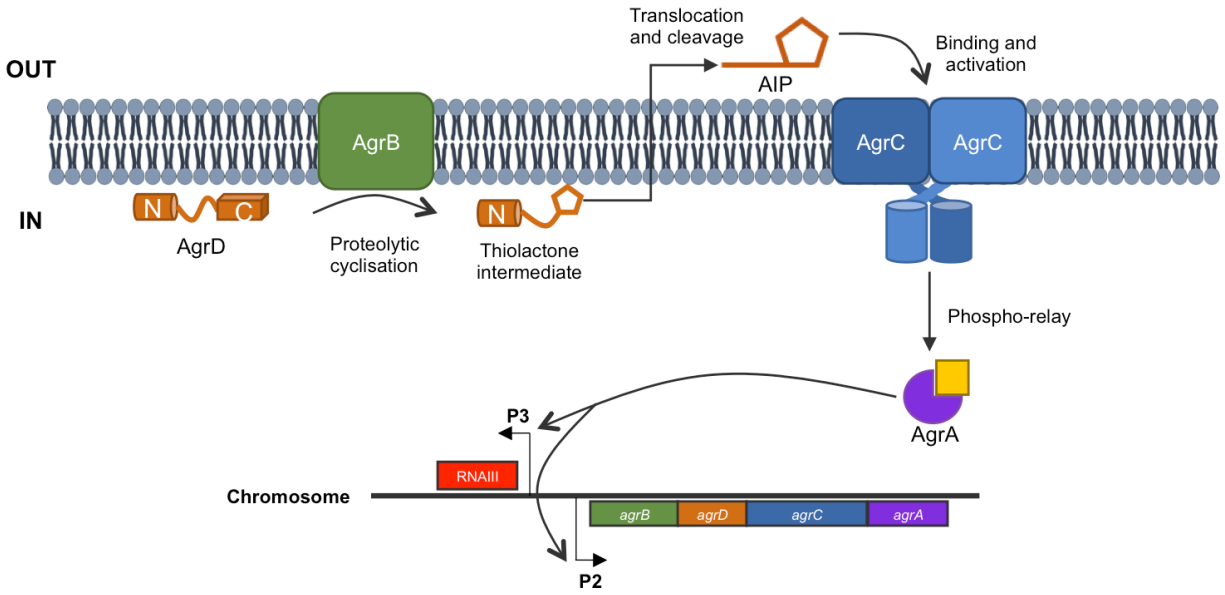

Fig. 4 Basic architecture of the Agr quorum sensing system

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(*Contd*) Fig. 4 Basic architecture of the Agr quorum sensing system

The *agr* locus is composed of two divergently transcribed units: the *agrBDCA* operon under the control of the P2 promoter and the *RNAIII/hld* gene under the control of the P3 promoter. The AgrD pre-cursor peptide undergoes two modifications to convert it into mature autoinducing peptide (AIP). The first modification is mediated by the transmembrane AgrB and involves cleavage of the C-terminal recognition sequence and formation of the Thiolactone ring. This step is energetically unfavourable, and the reaction is driven by rapid degradation of the cleaved C-terminal fragment. The thiolactone intermediate is then exported out of the cell and further cleaved at the N-terminus to give the mature AIP molecule. When the extracellular concentration of AIP reaches a threshold, it is detected by the AgrC receptor-histidine kinase (RHK) which results in self-phosphorylation of AgrC and subsequent transfer of the phosphate group to the AgrA response regulator (RR). Phosphorylated AgrA then binds, with differential affinity, to the P2 and P3 promoters to initiate transcription. Transcription from the P3 promoter produces the RNAIII regulatory mRNA, which has global effects on gene expression and also encodes the δ haemolysin gene, while transcription from the P2 promoter creates a positive feedback loop.

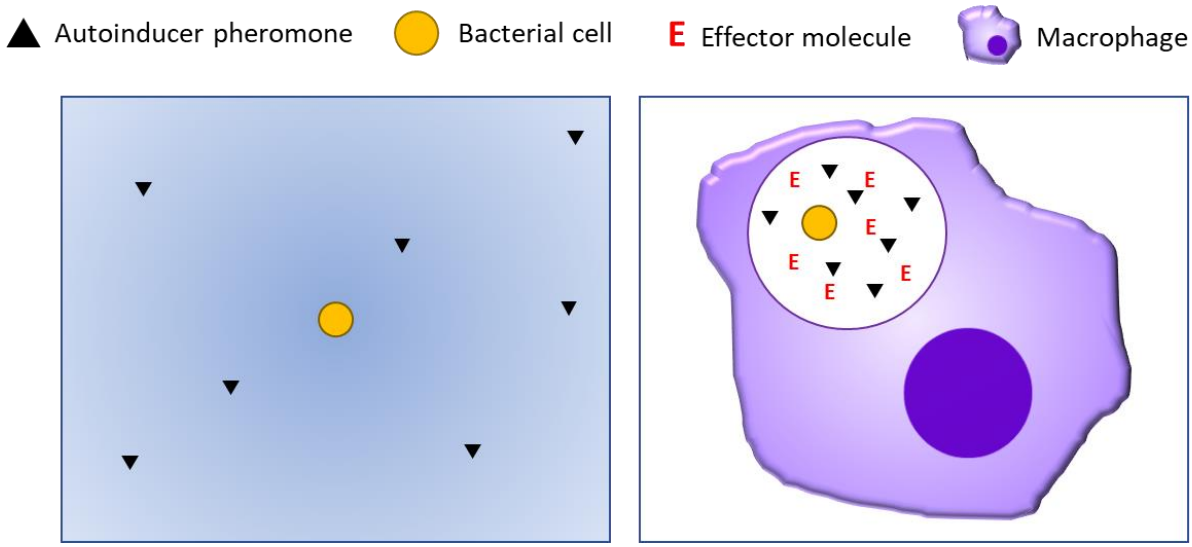

Fig. 5 Cartoon Illustrating the Concept of Diffusion Sensing

When produced under high diffusion conditions, a secreted autoinducer produced by a single cell (or small group of cells) is rapidly lost to the greater environment and prevents the production of exoproteins that would similarly be lost and thus wasted **(left)**. However, when there are constraints on the microenvironment of the cell, such as between soil particles or in phagosomes, the local concentration of the autoinducer can be sufficient to overcome the threshold of activation as there is limited diffusion to the greater environment resulting in the producer of effector exoproteins **(right)**.

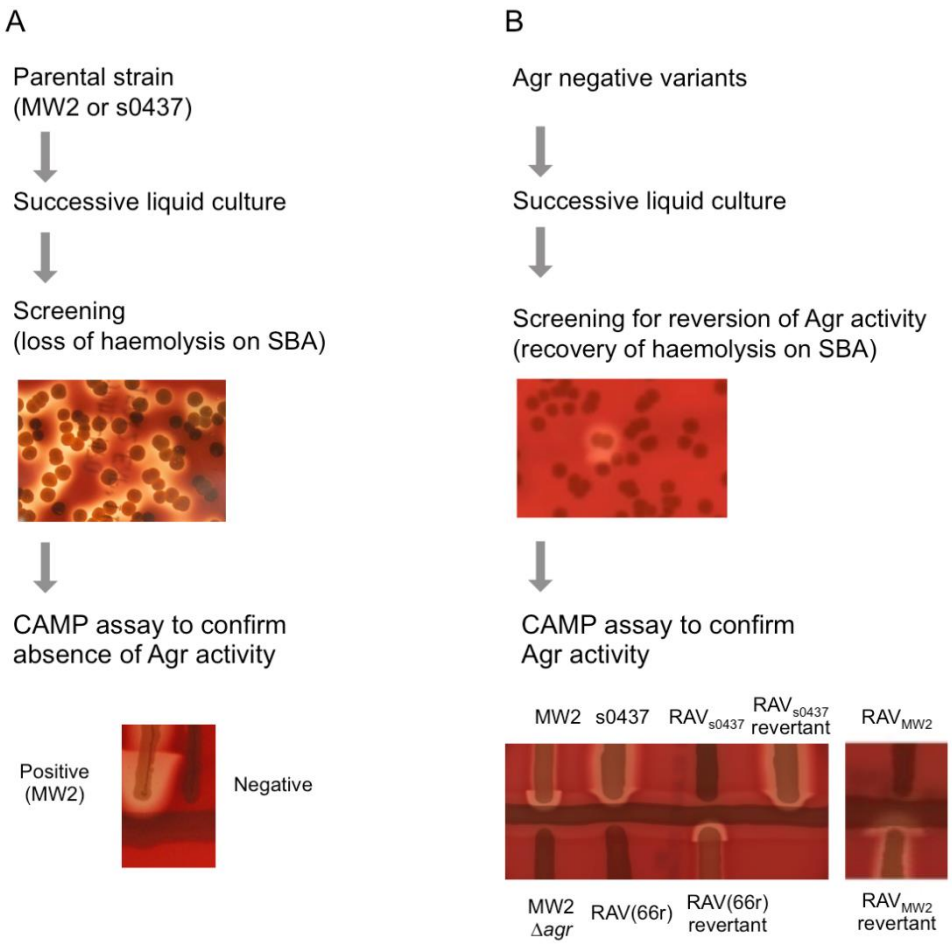

Fig. 6 Serial passaging of Agr-negative strains can give rise to colonies with reverted Agr activity.

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(*Contd*) Fig. 6 Serial passaging of Agr-negative strains can give rise to colonies with reverted Agr activity.

Flowchart describing the screening procedure used to isolate strains in this study. **(A)** Parental strains MW2 and s0437 were passaged in successive liquid cultures followed by screening for non-haemolytic colonies. CAMP tests were carried out on the non-haemolytic colonies alongside the parent strains to confirm loss of Agr activity. The CAMP insert depicts a non-haemolytic isolate generated from MW2. **(B)** Agr negative variants were passaged in successive liquid cultures followed by screening for haemolytic colonies. CAMP tests were carried out on haemolytic colonies alongside the reversible Agr-variant (RAV) strains and the original parental strains. The CAMP inserts depict RAV_{MW2} and RAV_{s0437} alongside their respective revertant and WT stains. The Agr-negative MRSA clinical isolate RAV(66r) and the RAV(66r) revertant strains are also shown. MW2 Δ*agr* was used as a negative control.

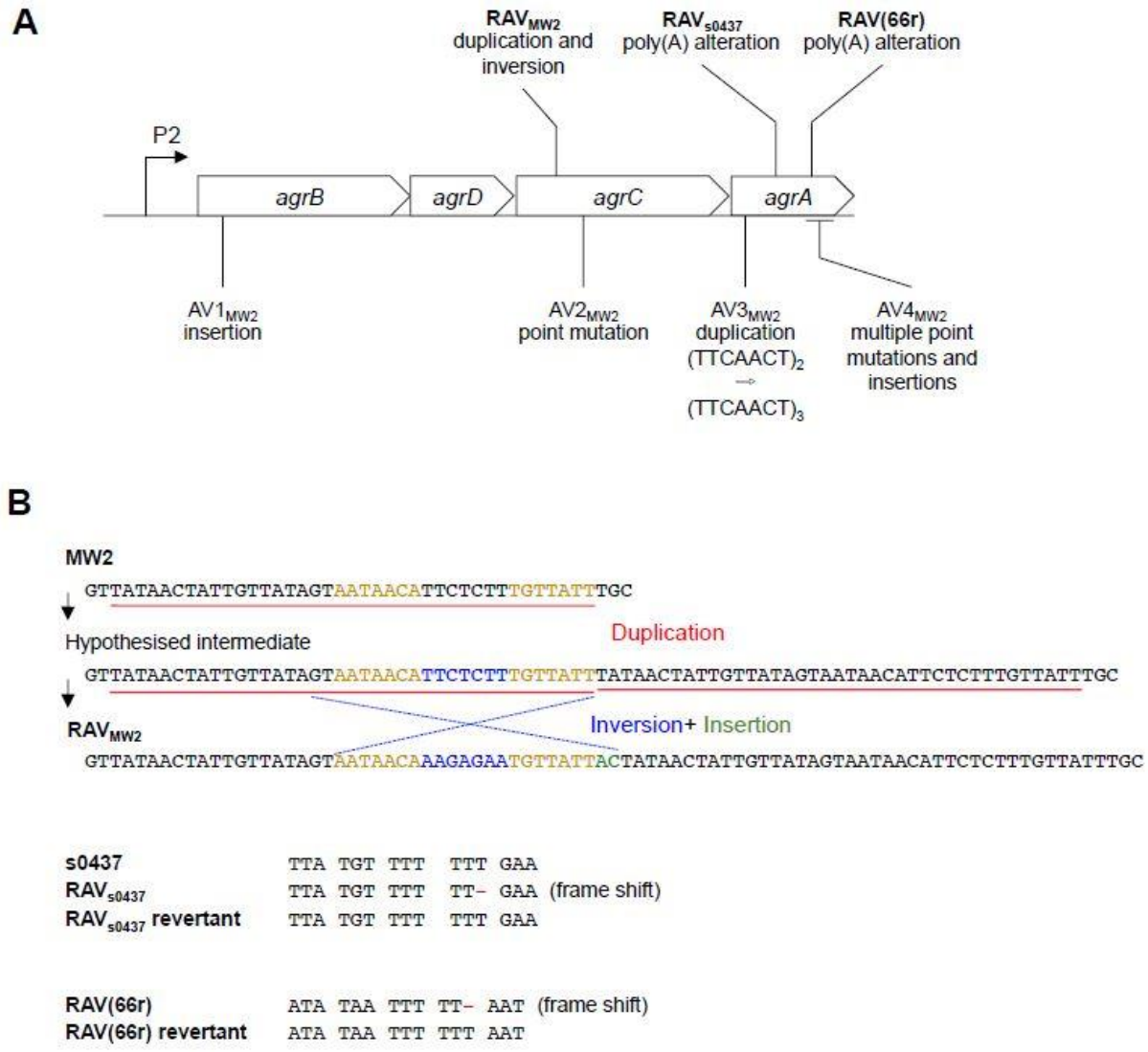

Fig. 7 Sequence diagram of MW2 variant and AT2 revertant strains

(A) A map of the *agr* P2 locus showing the location of the changes identified in the reversible and irreversible Agr mutants generated or collected in this study. The duplication event identified in AV3$_{MW2}$ is also depicted. **(B)** A cartoon depicting the inversion and duplication event inferred in RAV$_{MW2}$ as well as the affected sequences in RAV$_{s0437}$ and the RAV(66r) revertant strain.

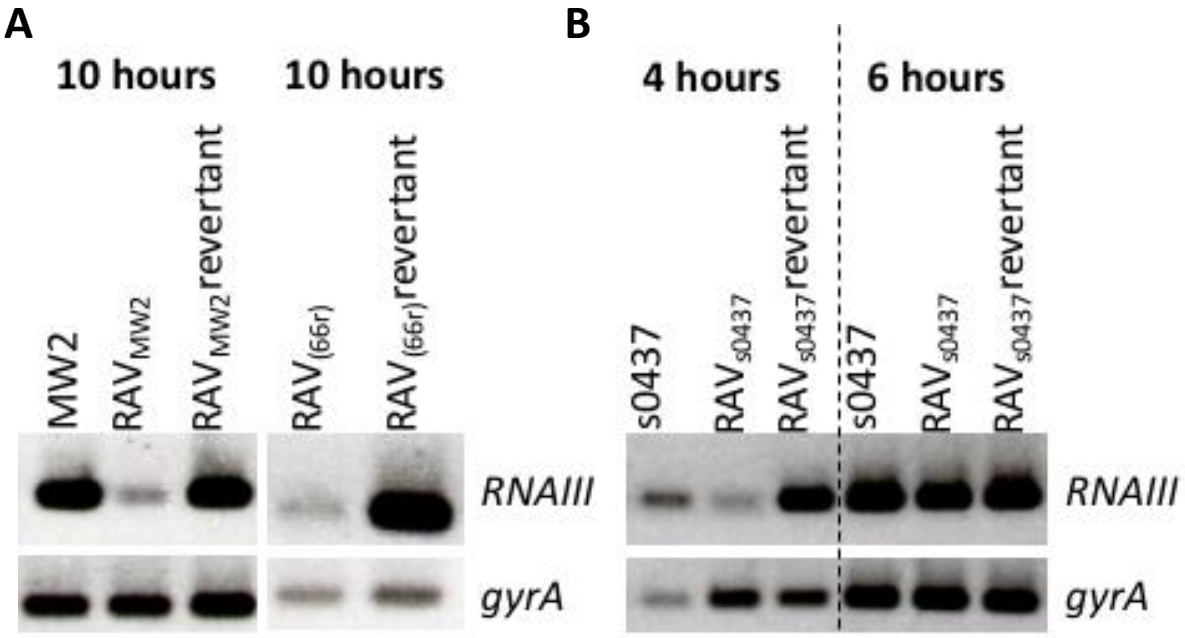

Fig. 8 Variable Agr shutdown dynamics between the RAV strains

Gel images of semi-quantitative RT-PCR carried out on RNA extracted from RAV strains and their respective revertant strains in a time course manner. **(A)** RAV_{MW2} and $RAV_{(66r)}$ have completely abolished *RNAIII* production. **(B)** Compared to the wild type and the revertant strain, RAV_{s0437} has abolished *RNAIII* production at 4 hours post inoculation but recovers production by 6 hours of growth. *gyrA* primers used as a housekeeping gene control.

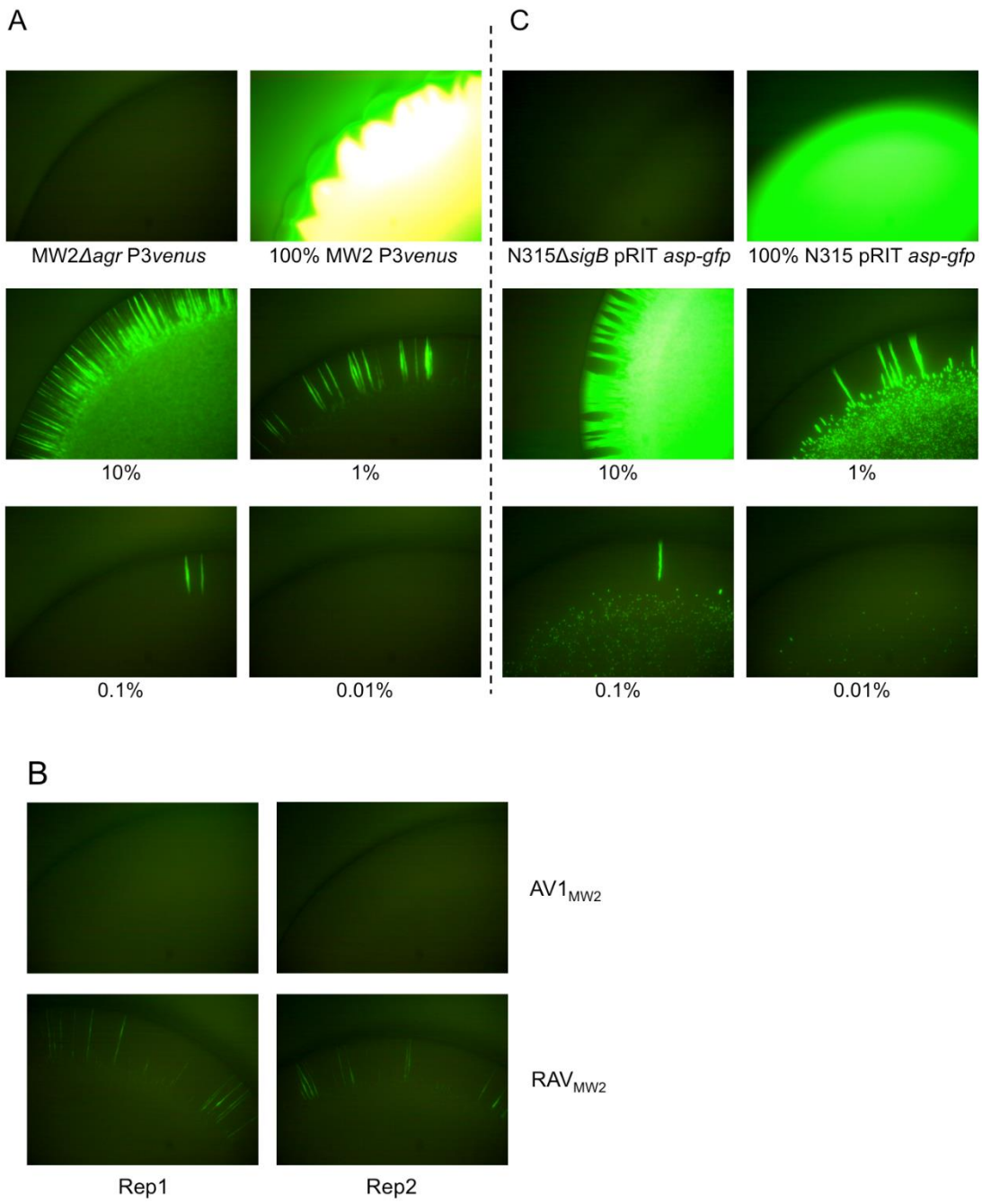

Fig. 9 Agr intact cells do not activate their Agr system as a minority unless they undergo local segregation

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(*Contd*) Fig. 9 Agr intact cells do not activate their Agr system as a minority unless they undergo local segregation

Representative fluorescence images of colonies of MW2 P3*venus* and mixtures of 10%, 1%, 0.1%, and 0.01% MW2 P3*venus* in MW2 Δ*agr* P3*venus* **(A)** or N315 pRIT *asp-gfp* and mixtures of 10%, 1%, 0.1%, and 0.01% N315 pRIT *asp-gfp* in N315*ΔsigB* pRIT *asp-gfp* **(C)**. Mixtures were spotted onto TSA and grown overnight before fluorescence was viewed under a stereomicroscope. **(B)** Representative fluorescence images of colonies of the irreversible $AV1_{MW2}$ and reversible RAV_{MW2} Agr variants on TSA. Two independent observations are shown (rep1 and rep2).

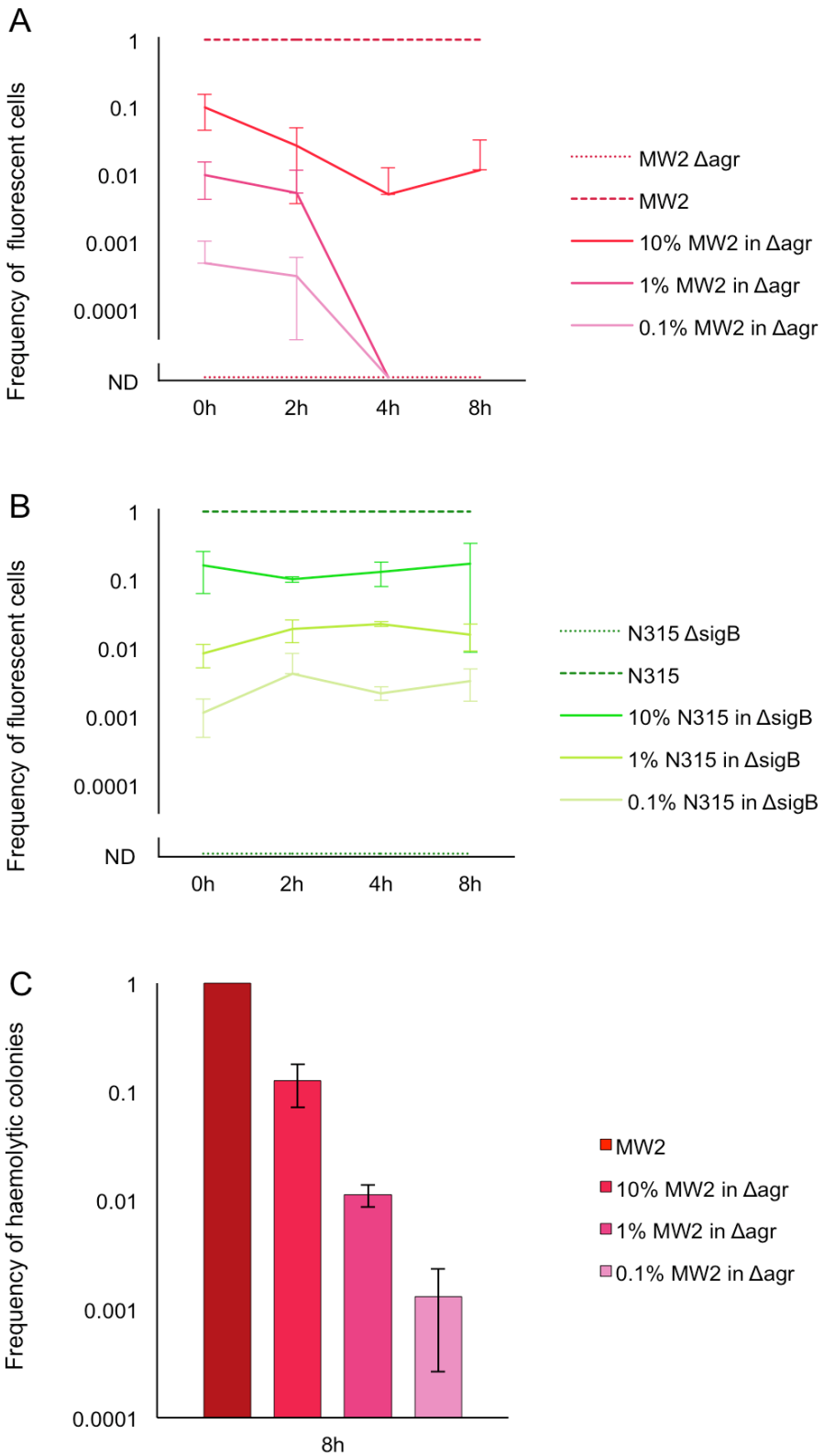

Fig. 10 Agr intact cells do not activate their Agr system when they are a minority in an Agr-negative population growing in liquid culture

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(*Contd*) Fig. 10 Agr intact cells do not activate their Agr system when they are a minority in an Agr-negative population growing in liquid culture.

(A) Frequency of fluorescent cells in liquid cultures of MW2 P3*venus*, MW2 Δ*agr* P3*venus*, or mixtures of 10%, 1%, and 0.1% MW2 P3*venus* in MW2 Δ*agr* P3*venus.* Aliquots were taken at 0, 2, 4, and 8 hours for microscopic analysis. **(B)** Frequency of fluorescent cells in liquid cultures of N315 pRIT *asp-gfp*, N315*ΔsigB* pRIT *asp-gfp*, or mixtures of 10%, 1%, and 0.1% N315 pRIT *asp-gfp* in N315*ΔsigB* pRIT *asp-gfp.* Aliquots were taken at 0, 2, 4, and 8 hours for microscopic analysis. **(C)** Frequency of haemolytic colonies on SBA. Samples from **(A)** were taken at the 8-hour time point and spread on SBA to confirm the continued presence of Agr-positive cells.

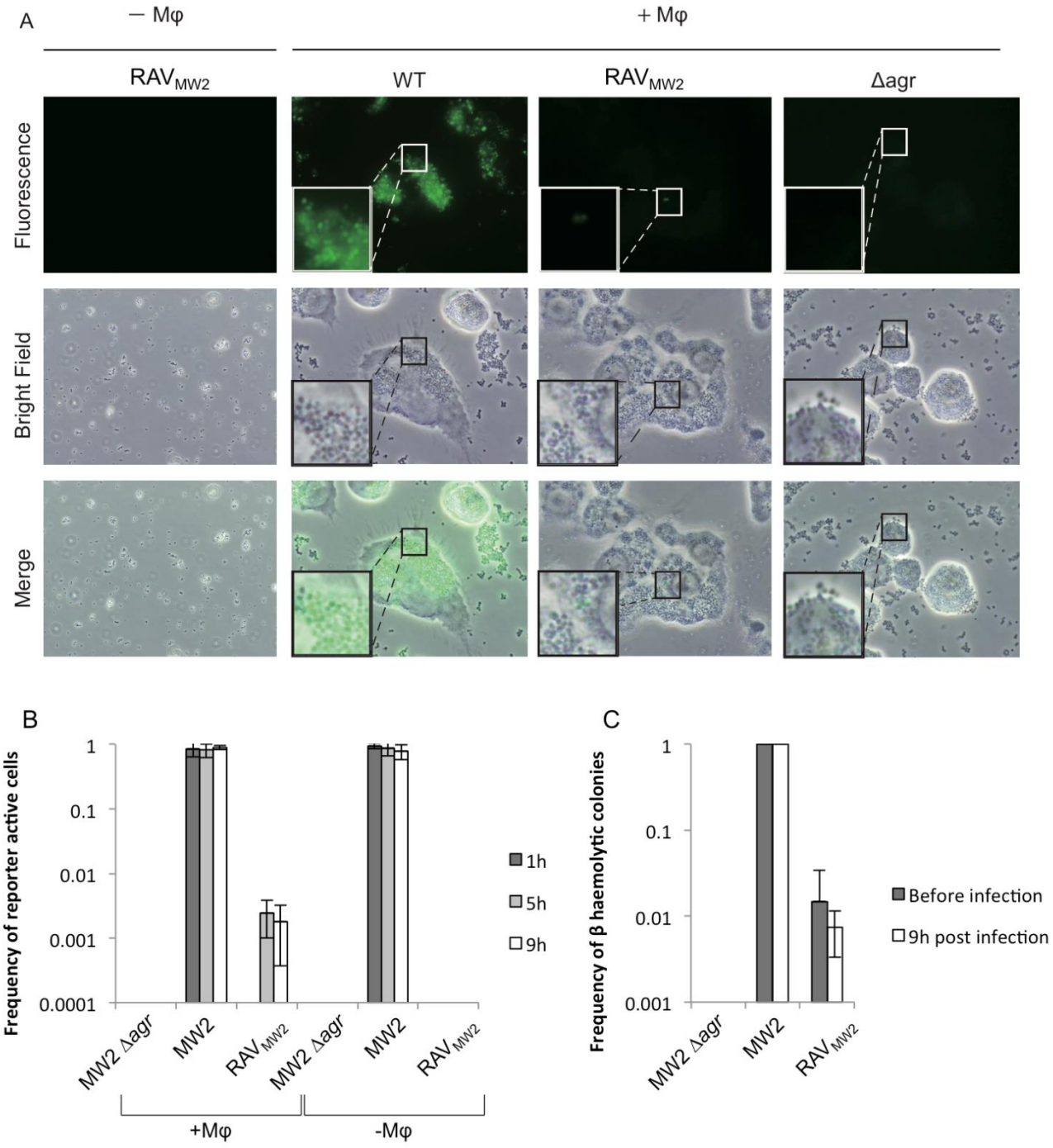

Fig. 11 Agr revertant cells can activate their Agr system in macrophages

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(*Contd*) Fig. 11 Agr revertant cells can activate their Agr system in macrophages

(A) Representative fluorescence microscopy images of MW2 P3*venus*, RAV_{MW2} P3*venus*, and the MW2 Δagr P3*venus* in the presence and absence of macrophages (Mϕ) at 9 hours post infection. **(B)** Average frequencies of reporter active cells from 3 independent Mϕ experiments. **(C)** Frequency of haemolytic colonies on SBA from samples spread before and after exposure to macrophages.

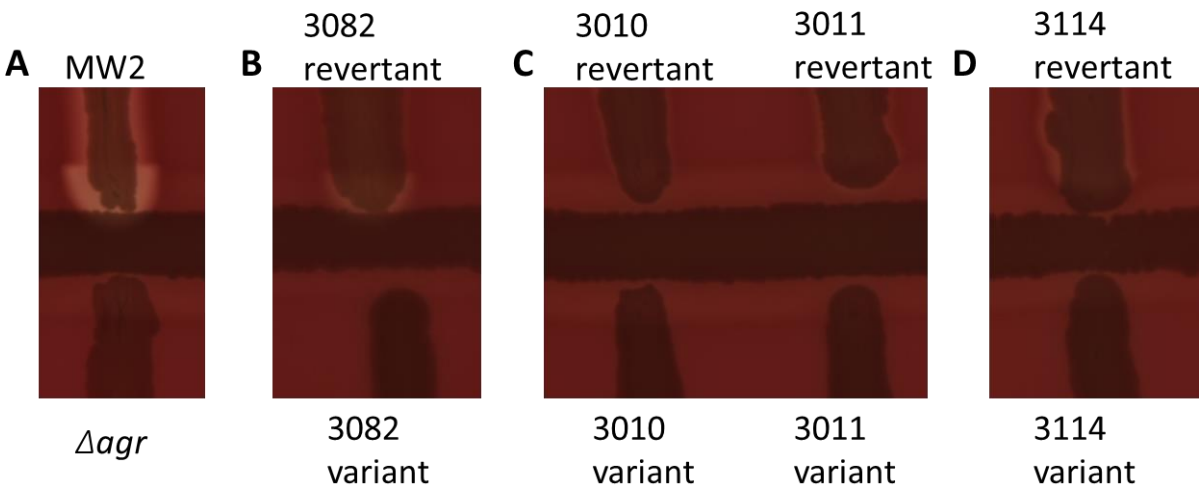

Fig 12 Reversion amongst primary patient isolates

Sections of CAMP plates showing a series of primary clinical isolates and their revertant strains. **(A)** MW2 and MW2Δagr as positive and negative controls, respectively. **(B)** Strain 3082 and its Agr revertant strain. Only a single SNP in the *agr* locus was detected between the variant and revertant strains. **(C)** Haemolytic revertants of strains 3010 and 3011. Note how the tell-tale arrowhead for δ haemolysin is absent in both variant and revertant strains. **(D)** 3114 and its Hla revertant strain. Note the inhibition of Hla by Hlb close to where the sample meets the streak of the Hlb producer.

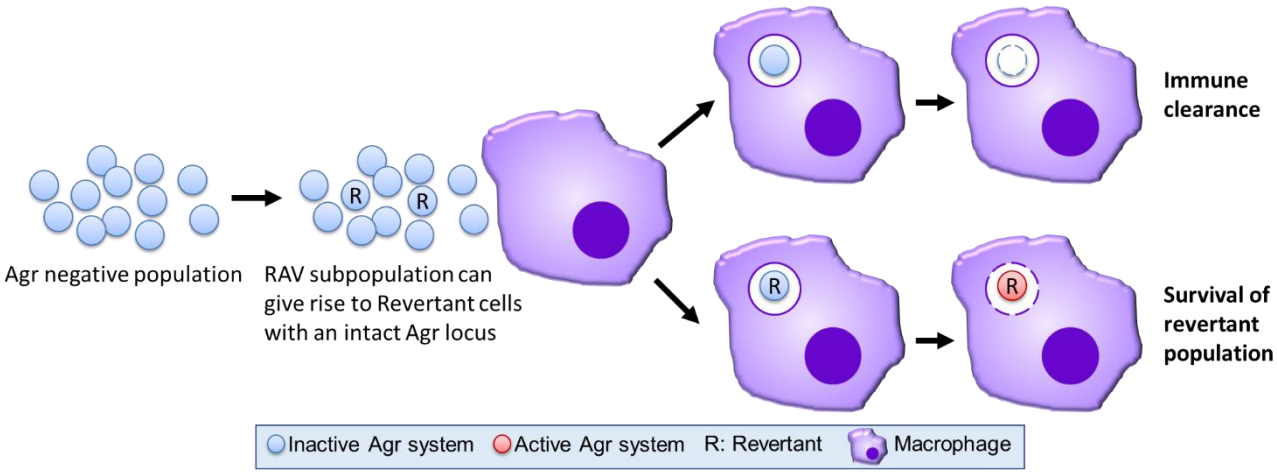

Fig. 13 Proposed model of Agr Phase Variation as a Predictive Adaptation Mechanism

An Agr deficient population can enjoy the benefits of immune evasion and enhanced biofilm formation conferred by an Agr OFF phenotype, allowing the bacteria to chronically persist in the host system. Reversible Agr Variant (RAV) subpopulations can give rise to revertant cells (designated R) that have an intact Agr locus but are phenotypically indistinguishable from their Agr deficient counterparts. This allows them to continue sharing the common benefits of the Agr OFF phenotype. However, upon successful detection by the host and resultant phagocytic attack, one of two outcomes are possible. The majority of the Agr deficient population will be destroyed upon phagocytosis. However, the revertant cells will be activate their Agr system within the phagosome and survive the phagocytic attack. They can then possibly remain dormant in the Phagocytes until they disseminate from the original site of phagocytosis after which they burst out from the immune cells and establish a new site of infection.

TABLE 1 . ***STAPHYLOCOCCUS AUREUS*** **STRAINS AND PLASMIDS USED IN THIS STUDY**

Strains	Relevant characteristics	Source / reference
RN4220	Derivative of 8325-4, restriction minus, modification plus	(Kreiswirth,
MW2	Community acquired MRSA	(CDC 1999)
MW2 *ΔAGR*	Isogenic MW2 mutant lacking the whole *agr* locus	This study
RAV_{MW2}	Reversible Agr variant derived from MW2	This study
$AV1_{MW2}$	Irreversible Agr mutant derived from MW2	This study
$AV2_{MW2}$	Irreversible Agr mutant derived from MW2	This study
$AV3_{MW2}$	Irreversible Agr mutant derived from MW2	This study
$AV4_{MW2}$	Irreversible Agr mutant derived from MW2	This study
S0437	Clinical isolate, MSSA	(Inose,
RAV_{S0437}	Reversible Agr variant derived from s0437	This study
RAV(66R)	Clinical MRSA isolate, Reversible Agr mutant	This study
3010	Primary patient isolate; Haemolysis reversible	This study
3011	Primary patient isolate; Haemolysis reversible	This study
3082	Primary patient isolate	This study
3114	Primary patient isolate; Alpha haemolysin rversible	This study
MW2 P3*venus*	MW2 carrying the $pRIT_{RNAIII}$*venus*	This study
MW2 *Δagr* P3*venus*	MW2 *Δagr* carrying the $pRIT_{RNAIII}$*venus* Agr reporter plasmid	This study
RAV_{MW2} P3*VENUS*	RAV_{MW2} carrying the $pRIT_{RNAIII}$*venus* Agr reporter plasmid	This study
PLASMIDS		
N315 PRIT*asp-gfp*	N315 carrying the $pRIT_{asp}$*gfp* SigB reporter plasmid	(Morikawa,
N315 *ΔSIGB* PRIT*asp-gfp*	N315 *sigB* deletion strain carrying the $pRIT_{asp}$*gfp* SigB	This study
PRIT5H	shuttle vector, used to make pRIT derivatives, Cm^R	(Inose,
$PRIT_{RNAIII}$*venus*	Agr P3 promoter-*gfp* transcriptional fusion	This study
PRIT*asp-gfp*	P*asp23-gfp* transcriptional fusion, SigB reporter	(Morikawa,

TABLE 2 **PRIMERS USED IN THIS STUDY**

Primers	5’- 3’
AGR FRONT(RNA3)	AGTTGGGATGGCTTAATAAC
AGR BACK	CAGCTATACAGTGCATTTGC
AGR4	CCGGTCTTCGAGACTATTTC
AGR5	AAGCCTATGGAAATTGCCCT
AGR-UP-F (BAMH I)	TATGAGGATCCAAATTTATCAATTACCGA
AGR-UP-R (BAMH I)	TTAAGGGATCCCAACTTAATAACCATGTA
AGR-DOWN-F(ECOR1)	GGCGAATTCAATTGTAAATCTTGTTGG
AGR-DOWN-R(BGL2)	TCAGATCTTTACGAAGCAAATTGGTGGC
RNAIII F	CGATGTTGTTTACGATAGCTT
RNAIII R	CCATCCCAACTTAATAACCA
GYRA F	AAGGTGTTCGCTTAATTCGC
GYRA R	ATTGCATTTCCTGGTGTTTC

TABLE 3 . **SUMMARY OF AGR VARIANT AND REVERTANT STRAINS GENERATED AND COLLECTED IN THIS STUDY**

Strain Name	Phenotype	Original Strain	Culture condition	Revertant Frequency Mean ± SD (%) (n = number of experiments)
$AV1_{MW2}$	Non-haemolytic	MW2	TSB	
$AV2_{MW2}$	Non-haemolytic	MW2	TSB	
$AV3_{MW2}$	Non-haemolytic	MW2	TSB	
$AV4_{MW2}$	Non-haemolytic	MW2	TSB	
RAV_{MW2}	Non-haemolytic	MW2	TSB	2.07% ± 0.68 (n=10)
RAV_{MW2}REVERTANT	Haemolytic	RAV_{MW2}	TSB	
RAV_{S0473}	Non-haemolytic	s0473	TSB + 5% Blood	0.82% ± 0.22 (n=10)
RAV_{S0473}REVERTANT	Haemolytic	RAV_{s0473}	TSB	
RAV(66R)	Non-haemolytic			0.12% ± 0.08 (n=6)
RAV(66R)REVERTANT	Haemolytic	RAV(66r)	TSB	

APPENDIX 2: REFERENCES

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